Temporal and Spatial Trends in the Abundance of Functional Denitrification Genes and Observed Soil Moisture and Potential Denitrification Rates.

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1.1 Nitrogen Cycle Overview

Nitrogen (N) is a major constituent of the atmosphere, making up roughly 78% in the form of inert dinitrogen gas, N_2 (Rennenberg and Dannenmann 2015). Nitrogen gas is biologically unavailable for use by plants and microbes despite its ample abundance. For use, N must be made available in the reduced form of ammonia, NH_4^+ or NH_3, through the process of nitrogen fixation. Through its subsequent uptake by plants and microbes, it cycles through a range of oxidation states, ranging from -3 to +5 (Zehr and Kudela 2011). It is first oxidized through nitrifying processes, then reduced through denitrifying processes with include both assimilatory and dissimilatory mechanisms. Assimilatory processes lead to the incorporation of nitrogen compounds into biomass, while dissimilatory reactions result in the formation of gaseous nitrogen compounds.

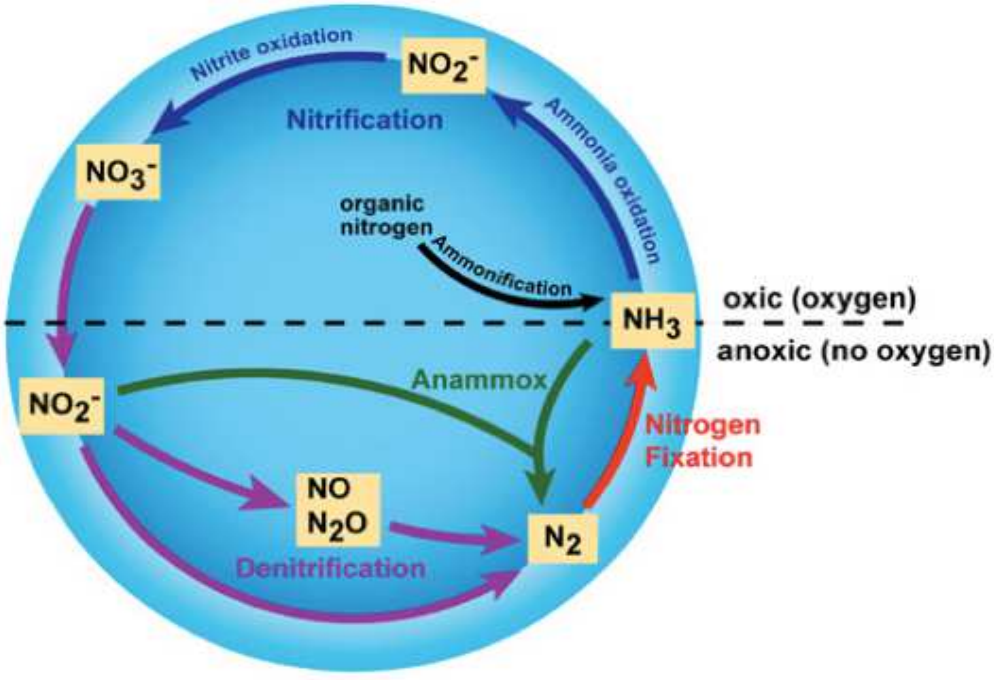


Figure 1.1. Basic Nitrogen Cycle.[1]

[1] Credit to Jake Bailey for creation of the figure.

Assimilatory nitrate reduction is carried out by many bacteria, algae, and fungi and the reduction of nitrate/nitrite, leads to its incorporation into larger organic molecules, such as amino acids. Nitrate anions are imported into the cell via transporters, where it is subsequently incorporated into biomass primarily by the glutamine synthase (GS) – glutamate synthase (GOGAT) cycle (Imamura et al. 2010). Not all microbes are capable of carrying out this reaction.

There are two main pathways of dissimilatory nitrate reduction that have been identified: denitrification, or the sequential reduction of nitrate to dinitrogen gas, and dissimilatory nitrate reduction to ammonia, referred to as DNRA (Brunet and Garcia-Gil 1996). Denitrification can occur as complete, or true, denitrification, or as incomplete denitrification. True denitrification leads to the production of inert N_2 gas, thereby removing nitrogen from terrestrial ecosystems. Incomplete denitrification fails to fully reduce nitrate to dinitrogen gas, and is thereby not considered true or full denitrification. DNRA can occur under the same anaerobic conditions as those required by denitrification, though the end product is biologically available ammonia as opposed to N_2 (Fazzolari, Mariotti, and Germon 1990). Though both DNRA and denitrification pathways are dissimilatory, the end product of DNRA does not lead to nitrogen removal from the system as it remains as ammonia.

1.2 Denitrification

Due to the continually increasing demand for N-based fertilizers for agricultural use they are often applied in excess, frequently leaching from soils into streams and groundwater (Stark and Richards 2008). Once in the waterway, biologically usable nitrogen sources can, and do, often lead to eutrophication processes. Moreover, conversion of NO can lead to the production of the potent greenhouse gas nitrous oxide, N_2O (Giles et al. 2012). Eutrophication itself has further implications, leading to hypoxia and dead zones which can be either temporary or permanent and on massive scale, such as what is seen in the Gulf of Mexico (Diaz and Rosenberg 2008; Ligi et al. 2013). Additionally, human health can suffer adverse effects from nitrogen stimulated algal

blooms known as “red tides” (Oehler, Bordenave, and Durand 2007), or the accumulation of NO_3^- in ground water leading to “blue baby syndrome”.

The largest source of emissions of N_2O has been demonstrated to be from agricultural soils (Uchida et al. 2014). The losses due to leaching, as well as the fact that N_2O is emitted in significant amounts from agricultural systems, serves as further evidence that nitrogen is often applied in excess relative to the ability of the plants to take up, and the ability of the soil to retain, the fertilizers. Denitrification is the primary process by which N_2O gas is produced (Guo et al. 2014).

Denitrification is carried out by many microbes under anoxic conditions (Philippot and Hallin 2005). In addition, when oxygen becomes limiting as an electron acceptor for respiration, the less energetically favorable nitrate can be used as an alternate electron acceptor (Correa-Galeote, Tortosa, and Bedmar 2013). It is generally recognized that the basic requirements for denitrification to occur are: 1) anoxia, 2) NO_3^- availability as an electron acceptor, and 3) an organic carbon source to be used as an electron donor, though other donors have been identified (Orr et al. 2007).

The sequence of reducing transformations is as follows: nitrate (NO_3^-) → nitrite (NO_2^-), NO_2^- → nitric oxide (NO), NO → nitrous oxide (N_2O), and finally, N_2O → dinitrogen gas (N_2) (Zumft 1997) (Figures 1.1, 1.2). Denitrification functional genes have been identified that are responsible for each of the transformative steps and are, in stepwise order, *narG* or *napA*, which encode slightly different, but equivalent, nitrate reductase enzymes, *nirK* or *nirS*, which encode nitrite reductase, *cnorB* encoding nitric oxide reductase, and *nosZ* which encodes nitrous oxide reductase (Barrett et al. 2013; Correa-Galeote, Tortosa, and Bedmar 2013). Each of the enzymes encoded by the above mentioned genes are inhibited by the presence of O_2 and require anoxic conditions to carry out their respective reactions (Guo et al. 2014). It is important to note that often incomplete denitrification also exists so that nitrogen is not fully reduced to dinitrogen gas but to other oxidized forms of N (Braker and Conrad 2011).

While some microbes are true denitrifiers and can produce N_2 gas as the end product of the pathway, denitrification can also be considered a community process in that not all microbes should be assumed to possess all genes necessary for each step of

denitrification. In this case, different microbes facilitate different steps in the pathway and the encoded non-redundant enzymes, or carry out functions discrete from one another (Braker and Conrad 2011; Catherine E. Dandie et al. 2008). For example, it is has been reported that approximately one third of all denitrifying microbes lack the *nosZ* type gene, and therefore cannot reduce nitrous oxide to dinitrogen gas (Isobe and Ohte 2014).

Denitrifiers are ubiquitous due to their metabolic flexibility – and the fact that they are facultative anaerobes means that they can exist in a variety of environments and be successful as opposed to obligate microbes, whose distribution is highly restricted (Peralta, Matthews, and Kent 2014). Despite resilient metabolic capabilities, however, denitrifiers as a group only constitute ~5% of the soil microbial community (Correa-Galeote, Tortosa, and Bedmar 2013). Southern hybridization analyses support the contention that the greatest number of denitrifiers are thought to exist within the top 2 cm layer of soil (Mergel et al. 2001; Philippot and Hallin 2005). However, recent studies suggest that even at depths of 20-40cm below the topsoil there are significant rates of denitrification which suggests some level of thus far poorly characterized microbial activity (Oehler, Bordenave, and Durand 2007).

Extreme diversity exists in terms of the role and function of denitrifying microbes, which is evidenced by their representation within numerous phylogenetic groups (Isobe and Ohte 2014; Levy-Booth, Prescott, and Grayston 2014). For example, more than 60 genera of bacteria (see Philippot, Hallin, and Schloter 2007 for a comprehensive list of genera) have been identified as denitrifiers (Braker and Conrad 2011), and there most certainly exist others that have not been characterized due to limitations in isolation and identification. Additionally, diversity exists within the genes themselves, and sequences that encode the same functional process can vary between different groups of bacteria. For example, while diversity has been shown to exist in the sequence of *nirS*, this often correlates to spatial distribution of microbes sequenced (Braker et al. 2000).

Current efforts to quantify or characterize denitrification processes have focused on either genetic or chemical methods that measure the gaseous intermediates produced by the individual steps that comprise the pathway. Due to the nature of denitrification, it

being a functional process as opposed to a species-specific one, standard 16S rRNA methods are insufficient (Bellini et al. 2013). The range of phylogenies represented within the denitrifier community would make identifying, and/or targeting them, with 16S rRNA genes impossible - those capable of denitrification would be inseparable from those incapable within the same phylogenetic grouping (Braker and Conrad 2011).

Measurement of the gases produced during denitrification is often done by employing the acetylene block method, which inhibits the production of N_2O as acetylene is preferentially reduced to ethylene (Yoshinari and Knowles 1976). This is much easier than trying measure N_2 gas itself. With this measurement, often the goal is to find a relationship in order to correlate microbial community roles with denitrification potential (Isobe and Ohte 2014).

Although the environmental and genetic factors that are thought to control denitrification are generally agreed upon (there are examples of contradictory conclusions in the literature), it remains at best difficult, and at worst impossible, to predict rates of denitrification on any scale. In fact, the scale and scope of a study are important as the factors thought to control denitrification are impacted at all levels: from ecosystem, to regional, landscape and even down to micro levels (Kulkarni et al. 2014). For example, oxygen availability in the soil can be impacted by drought or increased rainfall, soil texture, and the amount of microscale aggregation. In the case of this example, it is important to understand the scale of the study in order to correctly determine how oxygen availability is being impacted.

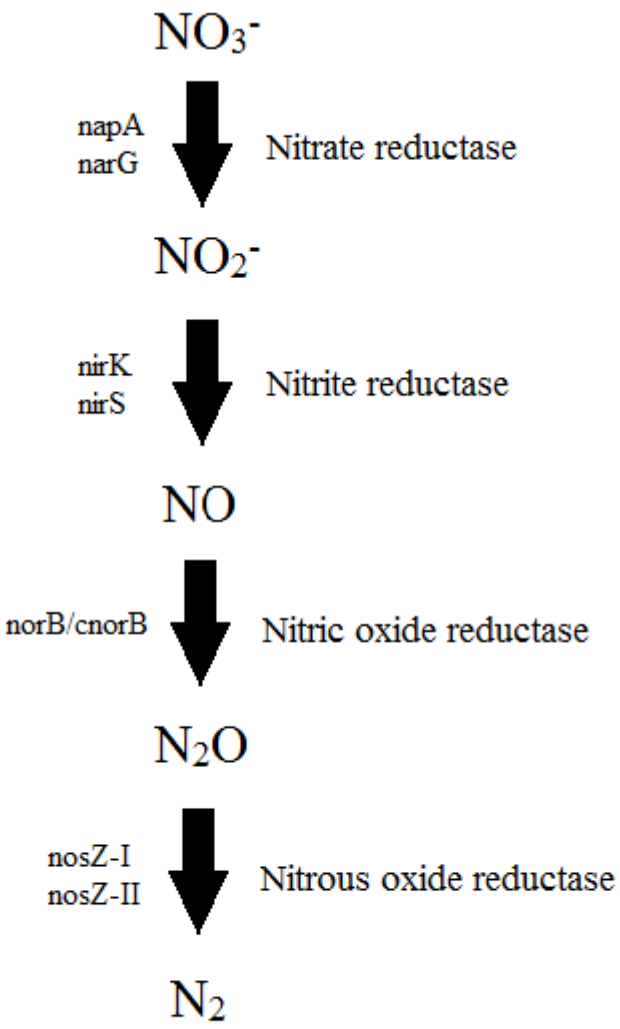


Figure 1.2. Flow chart depicting each of the steps in the complete denitrification pathway, ordered in sequence. Corresponding functional genes (right of arrow) and enzyme responsible for respective transformation (left of arrow) are also indicated.

1.3 Denitrification Hotspots

The concepts of denitrification hot spots and hot moments have drawn a lot of attention as potentially and likely significant sources of denitrification activity despite remaining largely elusive. A hot spot is a location at which, for some reason, assumed to be due to environmental parameters that vary at the specific site, there are significantly increased rates of denitrification. Similarly, hot moments are ephemeral periods that result in increased denitrification rates, attributed to the same variables as that of hot spots. The main drivers of both hot spots and hot moments are thought to be soil O_2 concentrations, moisture levels, and availability of both nitrate and an organic carbon source (L. E. McPhillips et al. 2015). Development of predictive models that enable the determination when and where these locations and moments are likely to occur are of utmost importance and much effort has been spent trying to understand these processes.

Riparian ecosystems, zones in which water and land directly interface, such as the banks flanking the channel of a small stream, have been implicated in increased rates of nitrate removal and denitrification (Cooper 1990; L. E. McPhillips et al. 2015). This has largely been the focus of research that aims to more fully understand and characterize denitrification rates. Riparian areas cannot be considered hot spots, in a general sense, as they are quite heterogeneous (Burgin, Groffman, and Lewis 2010), but this variability is also likely what attributes to the increased incidence of observed hot spots/moments. One study attributed > 99% of the observed denitrification to what are known as riparian hollows, which are dips or depressions in the topography, despite comprising only 0.5 to 1% of total riparian area (Duncan, Groffman, and Band 2013). The authors attributed the hot spot status to these topographical depressions, although further study is required to validate this observation and subsequent conclusion.

Ultimately, however, more questions remain than answers with regard to denitrification. Generally speaking, an understanding of how the environment shapes and controls denitrification and the denitrifier community is lacking (Braker and Conrad 2011). This lack of understanding is demonstrated repeatedly as various publications report relationships that contradict one another, leading to inconsistencies in claims amongst studies (Orr et al. 2007). The reasons for such disparities are not known, but two possible suggested explanations include differences in methodologies and scale of study,

as well as a lack of characterization of the denitrifier community (such as available libraries of data and sequencing data). What can be concluded with confidence, however, is that understanding why such disparities are observed could be, and likely is, a key to understanding controls over the denitrification pathway itself.

1.4 Controls over Denitrification

The central question to be asked when discussing denitrification rates and activities is that of what conditions or parameters affect it and, ultimately, what controls it. Results of numerous studies, however, have been contradictive to one another. For example, throughout the literature the most common environmental controls over denitrification are suggested to be O_2, NO_3^-, and organic carbon and it is thought that these variables are regulated at the ecosystem level, or perhaps even at the regional level (Kulkarni et al. 2014). Others deny such claims, and argue that the effect of spatial location as it relates to temporal scales is not significant (Catherine E. Dandie et al. 2008).

1.4.1 O_2 and Denitrification

As denitrification is an anaerobic process and is often carried out by facultative organisms that would preferentially use oxygen, it requires that the environment be anoxic in order to proceed. Oxygen availability is determined by a complex network of variables that are both biotic and abiotic in nature. Biological activity can impact the concentration of O_2 in a system through processes such as photosynthesis via microbes, or on the macro scale, such as with plants, and by decomposition (Giles et al. 2012). Additionally, soil texture and moisture levels can both impact O_2 levels as water can act as a barrier, thereby decreasing diffusion into the soils; diffusion is also more problematic with finer, more tightly condensed soils (Opdyke and David 2007). Microbial respiration also tends to consume O_2 and produce CO_2 as a byproduct, together reducing the partial pressure of O_2.

Oxygen availability impacts denitrification not only by directly inhibiting the process, but by impacting the environment as well, which has indirect effects for denitrification rates and on denitrifier community structure. As with all organisms, the

microbes responsible for denitrification have nutritional and energetic requirements. The presence of oxygen impacts the redox conditions of the system (Pett-Ridge 2005), which in turn influences the community structure of microorganisms, but also nutrient availability (Burgin, Groffman, and Lewis 2010). The form in which various compounds can exist is largely determined by both redox condition as well as pH of the system, and can therefore render previously bioavailable forms unavailable and vice versa. Additionally, on a larger scale, topography of a system can have more influence over oxygen availability than rainfall, despite moisture often having great impact (Duncan, Groffman, and Band 2013).

1.4.2 Organic Carbon and Nitrate

Organic carbon and nitrate are both thought to be key controls over the denitrification pathway, likely due to being the electron donor and acceptor, respectively. Under the assumption that organic carbon is the primary electron donor for denitrification, the claim that organic carbon availability is a major influencing factor over denitrification (Hopfensperger, Schwarz, and Kirtman 2014) is logical and is likely important. Despite this, however, no study has been able to definitively show that there is a relationship between organic carbon and denitrification rates (L. E. McPhillips et al. 2015). Nitrate, similarly, is thought to hold some level of control over denitrification, with some evidence to support this claim coming from stream environments (Manis et al. 2014). In some cases, however, there has been no meaningful correlation of nitrate concentration with denitrification rates (Orr et al. 2007). That said, the assertion that nitrate stimulates the denitrification pathway when O_2 is limiting (Giles et al. 2012), or under anoxic conditions, seems to be widely accepted.

1.4.3 Moisture

Moisture content of soil can be linked to several other parameters such as oxygen levels, microbial community structure, soil texture, seasonality, and hydrology, and is therefore likely to have some control over denitrification either directly or indirectly. Water filled pore space (WFPS) is thought to exhibit some control over denitrification, though this may be an indirect effect as there is a relationship between water content and oxygen availability (Oehler, Bordenave, and Durand 2007). When comparing the

moisture content of soils, wet soils tend to have a higher rate of denitrification or denitrification enzyme activity (DEA) than their more dry counterparts (Ducey et al. 2015). Measurements taken of enzyme levels have also demonstrated an increase in denitrification enzymes, two-fold increase in denitrification enzymes in wet soils compared to dry ones, further evidence of higher rates of activity (Burgin, Groffman, and Lewis 2010). This has been reasoned by the fact that dry conditions in soils presents a lack of available water, required by all microorganisms for basic function, as well as decreased substrate availability as required nutrients are not as readily accessible (Guo et al. 2014). Also, in dry soils there is likely increased oxygen diffusion, which itself inhibits denitrification.

Hydrology of the landscape, flooding, and water residence time both appear as if they may influence spatial and temporal differences in denitrification rates. With more flooding, or longer lasting floods, such that the soil becomes inundated thereby reaching saturation, it has been noted that denitrification rates are potentially affected as rises in rate have been observed (Ligi et al. 2013). It seems reasonable that with longer lasting flooding events, or residence times, denitrification rates would increase due to a lack of oxygen being able to readily diffuse (L. E. McPhillips et al. 2015). One factor that seems to effect flooding is impervious surface, which is a direct result of the types of soil, plants, and ground cover (Hopfensperger, Schwarz, and Kirtman 2014).

1.4.4 Temperature and pH

Temperature and pH are additional parameters which exist in a network of factors that potentially affect denitrification and are intertwined with several of the others previously discussed, such as oxygen availability and soil type. It is unclear if pH is an important factor in denitrification directly, as it has been documented to occur over a range of pH values – from 4 to 8, with no known optimum (Peralta, Matthews, and Kent 2014). The impacts of pH are likely due to altering the bioavailability of some chemical compounds (such as NH_3 and NH_4^+) and elements needed for enzyme activity (Giles et al. 2012). Enzymes are almost universally sensitive to parameters such as pH and temperature and such changes in either would likely impact denitrification rates.

1.4.5 Land Properties and Management

The properties of the land, such as the topography and ground cover, as well as soil type and texture, have implications for denitrification because they lead to changes in O_2, moisture content, and organic carbon concentrations. Soil texture/type is important because it relates directly to both organic carbon content and moisture content (Opdyke and David 2007). With more fine grained soils, such as silts and clays, there tend to be higher levels of moisture as well as available organic carbon, therefore denitrification rates could present differently than in sandy soils. Additionally, the implications of O_2 are that in more inundated soils, diffusion is more difficult and there are lower concentrations. The presence of soil aggregates, which can create anoxic micro environments, may allow denitrification to occur in otherwise oxygenated environments, and is influenced by soil type (Højberg, Revsbech, and Tiedje 1994).

Land management practices that have impact on denitrification are a contentious topic and conclusions are often varied. Some believe that land management has absolutely no effect on denitrification rates and that they remain independent of changes in the land (Barrett et al. 2013) while others suggest that it is of utmost importance, particularly in areas of farming and cultivation (Bannert et al. 2011). For example, land management can lead to significant changes in denitrification rates in dairy farms and pastures (Chroňáková et al. 2009). Urea and feces both provide compounds which can be utilized directly or indirectly by denitrifiers, while grazing leads to diminished ground over as well as compacted soils from the physical movement over the land. It would seem obvious, therefore, that this use of land could and should have implications for denitrifying processes nearby, yet some still disagree that land management plays a role. It is of utmost importance to consider the system under study when drawing conclusions, as land management may have negligible influence over a less disturbed system as opposed to that of a dairy farm.

1.5 Genetics of Denitrification

The most commonly used functional gene markers for the study of denitrification are *nirK*/*nirS* and *nosZ*, which encode nitrite reductase (Sonia Henry et al. 2004) and nitrous oxide reductase (S. Henry et al. 2006) respectively. Across a range of known

denitrifying species, the *nirS* encoded nitrate reductase gene is much more common than others, with *nirK* being present in roughly 30% of microorganisms or environments (Chon et al. 2011). In some cases, *nirK* dominates over *nirS*. Additionally, the simultaneous presence of both *nirK* and *nirS* in a single bacterium has not yet been observed (Correa-Galeote, Tortosa, and Bedmar 2013), thereby giving credence to the treatment of these two groups as discretely separate entities. To further support the distinction, studies have demonstrated that *nirK* and *nirS*-type denitrifiers respond differently to environmental conditions (Levy-Booth, Prescott, and Grayston 2014).

Nitrous oxide reductase, encoded by *nosZ*, has perhaps received much more attention due to its pivotal role in removal of the greenhouse gas N_2O via reduction to inert N_2 gas, thereby completing the full denitrification pathway. It has been characterized as the most oxygen sensitive enzyme of any involved in the pathway (Giles et al. 2012). Interestingly, and potentially of great significance, is the fact that no known Archaea or anaerobic ammonia oxidizers (ANAMMOX) are known to possess the *nosZ* gene (Philippot et al. 2011), nor are fungi (Kim et al. 2009), which could potentially be useful for distinguishing bacterial from non-bacterial denitrifiers. Conversely, however, it has also been demonstrated that not all bacteria that possess *nosZ*-type genes reduce N_2O (Sanford et al. 2012). Recently, it has been reported that two distinct microbial clades possess the *nosZ* gene, referred to as clades I and II (Isobe and Ohte 2014). It has been revealed that, while *nosZ-II* lacks adequate study due to its relatively recent discovery, it is at equal abundance as *nosZ-I* (Jones et al. 2013), leading to a large gap in knowledge and therefore understanding of the final step in the denitrification pathway.

Understanding denitrification activity through DNA-based parameters alone can be difficult and sometimes misleading, which also seems a plausible explanation for the wide variance in conclusions drawn throughout the literature. RNA based techniques, however, which focus on isolating mRNA, are more likely to give an accurate understanding due to only being produced when the cell is active. There is, however, no significant relationship between genetic abundance of DNA and mRNA levels (Chen et al. 2015), which implies that no activity level assumptions can be made based on the presence of DNA alone. It has also been demonstrated that *nirS*/*nirK* and *nosZ* mRNA

levels show no correlation to nitrous oxide emissions while DNA levels showed no relationship (Uchida et al. 2014). While DNA analysis can be useful it cannot always tell the full story of what is happening and for a complete snapshot, mRNA levels should be considered additionally.

Thus far, however, understanding denitrification from a genetic point of view remains highly incomplete and restricted. No study has been able to definitely establish a relationship or correlation that holds true in all systems, as evidenced by the contradictions and inconsistencies between studies. Countless studies have concluded that no seasonal trends can be established between denitrifier community or activity/abundance and the denitrification pathway used (Chon et al. 2011), the same true for other temporal and spatial scales.

Current opinion is that there is no clear pattern between denitrification and microbial community structure (Manis et al. 2014) or community composition (Levy-Booth, Prescott, and Grayston 2014). This is likely due to the functional redundancy in encoding several of the enzymes involved, such as *nirK*/*nirS* encoding nitrite reductase, from an ecological perspective. Changes in the species that make up the community, such as a decrease in number of *nirS* nitrite reducers, would have little impact if the difference is then made up with a simultaneous increase in *nirK* nitrate reducers. To fit this fact, it may be useful to cease looking at denitrification as a pathway in its entirety; given that denitrification is a community process, no single species carries out the entire pathway, it may be most useful consider each step independently in analysis. It has been demonstrated that different environmental variables impact specific genes in dissimilar ways (Ligi et al. 2013), highlighting the need for a more exacting and discrete approach in attempting to characterize findings.

1.6 Difficulties and Potential Future Considerations

1.6.1 Assumptions

Quite often, studies involving denitrification involve the measurement of genetic information, most commonly DNA. Though relatively easy to extract from the environment and subsequently process, an assumption often made is that the presence of a specific gene, for example *nirS*, can be used as a proxy for measurements of activity (Chon et al. 2011) when in fact it cannot. The presence of a gene alone says nothing specifically about functionality because it is possible for a microbe to be capable of expressing a protein, but it is disfavored due to environmental conditions and is therefore not actively transcribed (Philippot and Hallin 2005). DNA is also known to, at times, persist in the environment in an extracellular form on a timescale of months (Recorbet et al. 1993). In the case where DNA based functional genes are used as a measure of community structure, difficulty can arise in the assumption that one copy of a gene corresponds to one microbe. For example, *narG* is known to be present in multiple copies within one organism. Additionally, the *nosZ* gene has also been found in non-denitrifying microbes (Giles et al. 2012). Thus, these functional genes cannot always correctly reveal the community structure in an ecosystem with respect to function.

Other assumptions that have long been made in countless studies are starting to come under scrutiny as evidence to suggest that other considerations should be made come to light. For example, it has long been accepted that denitrifier abundance is quite large, and perhaps the largest, in the uppermost layers of soil. However, at least one study as suggested that depths 20-40cm below the surface can contribute significantly to overall denitrification in a system (Oehler, Bordenave, and Durand 2007).

The importance of various compounds is frequently discussed, such as organic carbon being a necessity for denitrification to occur. However, other studies have shown that other compounds can be used as electron donors for this pathway (Hedin et al. 2015). As a result there are likely incorrect assessments made with respect to organic carbon availability and resulting denitrification patterns observed, which may lead to incorrect conclusions drawn, such as a relationship existing where a focus on organic carbon alone provides no such indication.

The most obvious problem of all, however, is assuming that bulk soil conditions are representative of the entire sampling site (Burgin, Groffman, and Lewis 2010). Microbial abundance and community composition is known to change over very small scales, and has a patchy distribution, likely affecting hot spots and hot moments.

1.6.2 Limitations

Currently, research is only as useful as the means of measurement are effective. Primers, used for identifying sequences of DNA that correspond to specific genes, are one tool of measurement which is currently lacking in adequate coverage of denitrifying genes in many species (Green et al. 2010). With primers unable to capture full diversity of microbes and different genes which encode the same enzymes (Levy-Booth, Prescott, and Grayston 2014), there is undoubtedly underestimation of denitrifier abundances leading to inaccurate correlations with environmental parameters. Metagenomic advances are expected to reduce these issues as more sequences are revealed and primers subsequently designed to cover more diversity (Correa-Galeote, Tortosa, and Bedmar 2013).

Other issues affecting interpretation of denitrification data include the lack of ability to extrapolate findings over larger scales due to the possible fluctuation in and lack of predictability with which microbial communities and species abundances shift over small spatial scales (Giles et al. 2012). While it is generally agreed upon that hot spots and hot moments account for a large percentage of total denitrification, they remain difficult to study due to a lack of predictive power in determining where and when they will occur (Groffman 2012).

1.6.3 Future Considerations

Denitrification is not the only pathway that uses nitrate as a substrate. Dissimilatory nitrate reduction to ammonia (DNRA) is an alternative process that uses NO_3^- to produce NH_4^+, thereby disallowing removal to N_2 gas (Hardison et al. 2015; C. J. Smith et al. 2015). Generally speaking, DNRA as an alternative fate of nitrate is overlooking in studies despite for having studies that indicate that, in some ecosystems, up to 30% of nitrate is removed in this way (Giblin et al. 2013). This is largely possible

for bacteria with nitrogen fixation genes. Competition for nitrate is likely to have an impact on denitrification rates (Rütting et al. 2011) and is therefore important to consider when attempting to explain observed denitrification rates in an ecosystem. The *nrfA* gene, for example, could easily be analyzed against denitrification genes for a qualitative measure of relative abundance to that of denitrifiers (Giles et al. 2012; C. J. Smith et al. 2007).

As discussed, soil microbial properties such as community structure and relative abundances of species within that structure can vary significantly over short distances and over time, thereby conferring further complexity to the topic of study. Future studies should take into consideration the importance of soil aggregates which allow for microenvironments that differ from bulk conditions. Some work has been done in the past on this topic, with the use of micro sensors within soils, and the results suggested that O_2 concentration ranges can vary from 0 to 20%, over spans on the order of millimeters, which is significant with respect to denitrification (Højberg, Revsbech, and Tiedje 1994). While the soil texture and type is often mentioned and discussed with respect to moisture content and organic carbon concentrations, it may be of use to try to characterize the tendency of the system to form these aggregates.

Fungi are also known to carry out parts of the denitrification pathway and despite this are almost universally disregarded with study of questions relating to denitrification. This neglects a potentially huge source of nitrate reduction and subsequent nitrogen removal from the ecosystem (Braker and Conrad 2011). In fact, though it varies from system to system, it is not uncommon for fungal biomass to be many times greater than that of bacterial biomass (Ruzicka et al. 2000).

Chapter 2. Spatial and Temporal Perspectives Regarding the Genetic Basis for Denitrification

ABSTRACT

In an attempt to identify a genetic basis for observed denitrification rates, denitrification functional genes *nirS*, *nirK*, *narG*, *cnorBB*, *nosZ1*, and *nosZ3* were quantified using qPCR of DNA extracted from soils and sediments taken from agricultural runoff ditches in the Seven Mile Creek watershed. Gene copy numbers were analyzed with respect to both spatial and temporal scales, as well as compared to soil moisture levels and potential denitrification as determined by the acetylene inhibition method. Results of this study suggest a link between soil moisture and potential denitrification, although more formal relationships could be determined. Discriminant analyses also show similarities in genetic composition of analyzed samples over both spatial and temporal scales.

2.1 Introduction

Agricultural demand for N-based fertilizers is continually increasing due to their need for application in the production food and biofuels. Often fertilizers are applied in amounts exceeding that which the plants are capable of utilizing, leading to subsequent leaching from the soils and ultimately finding their way into groundwater and streams (Stark and Richards 2008). Fertilizer based nitrogen loading, once in the waterways, often leads to eutrophication and subsequent hypoxia-induced dead zones, such as that which is seen in the Gulf of Mexico (Diaz and Rosenberg 2008; Ligi et al. 2013). Eutrophication and resultant algal blooms can prove problematic with respect to human health due to algal toxin production in "red tides" (Oehler, Bordenave, and Durand 2007). Additionally, given the correct conditions allowing for the necessary chemical transformations that make up the process of denitrification, the potent greenhouse gas nitrous oxide, N_2O, is produced (Giles et al. 2012).

Denitrification, or the pathway through which nitrate is reduced in several intermediate producing steps, ultimately results in the production of N_2 gas (Chon et al. 2011). This is of importance for nitrogen removal from an ecosystem, and leads to the

production of the greenhouse gas nitrous oxide, N_2O (H.-W. Hu, Chen, and He 2015). The process of denitrification is the primary pathway implicated in N_2O production (Guo et al. 2014). The pathway is also, however, the only known biological sink for nitrous oxide (J. Hu et al. 2015), in which N_2O is converted to N_2.

Denitrification is often carried out by facultative anaerobes, or those that are capable of oxygenic (aerobic) respiration in addition to anaerobic respiration. As oxygenic respiration is more energetically favorable, true denitrification will not occur unless oxygen is absent, and nitrate is then used as an electron acceptor by denitrifying microorganisms (Correa-Galeote and Tortosa 2013; Philippot and Hallin 2005). Due to the metabolic flexibility of being able to use oxygen and other compounds as electron acceptors, denitrifiers are quite ubiquitous, are able to exist and persist over a range of environments or microhabitats (Peralta, Matthews, and Kent 2014).

The ubiquity of denitrifiers is further demonstrated as they are represented across a large number of phylogenetic groups, which also demonstrates the diversity of the group in terms of role and function, aside from simply existing over a range of habitats (Isobe and Ohte 2014; Levy-Booth, Prescott, and Grayston 2014). The pathway is often considered a community process, completion of which requires representation across these different phylogenies as discrete groups mediate steps in the process separate from one another. (Braker and Conrad 2011; Catherine E. Dandie et al. 2008). It should be noted, however, that there are many examples known of single microorganisms with the ability to completely denitrify.

Denitrifier groups are necessarily defined by the specific steps in the pathway which they mediate, distinguished by bearing functional genes responsible for encoding enzymes necessary to catalyze each of the steps. The sequence of reductions begins with nitrate to nitrite, then with nitrite to nitric oxide followed by the reduction of nitric oxide to nitrous oxide, then concluding with nitrous oxide to inert dinitrogen gas (Zumft 1997). Each transformation step has an enzyme associated with it, encoded by a specific gene: *narG* or *napA* encoded nitrate reductase, *nirK* or *nirS* encoded nitrite reductase, *cnorB* encoded nitric oxide reductase, and *nosZ* encoded nitrous oxide reductase, respectively

(Correa-Galeote and Tortosa 2013), (Barrett et al. 2013). Currently, there is no species known to bear redundant genes *nirK* and *nirS*, or *narG* and *napA*.

Hot spots, or hot moments, are places or times of increased rates of denitrification (Palta, Ehrenfeld, and Groffman 2014) and they contribute a disproportionately high fraction to the overall amount of denitrification that occurs. These processes remain somewhat elusive and unpredictable by current standards and methods. The ephemeral increases in denitrification are thought to be driven by factors such as O_2 content, moisture levels, available organic carbon, as well as the topography and hydrology (Duncan, Groffman, and Band 2013; Hopfensperger, Schwarz, and Kirtman 2014; L. E. McPhillips et al. 2015).

Riparian zones are heterogeneous areas of interface between an aquatic system such as a river and the land and have been identified as key zones for denitrification (Burgin, Groffman, and Lewis 2010), often containing hot spots and hot moments. Some hypothesize that riparian zones themselves should be considered hot spots of denitrification, due to the frequency with which the process is observed at increased rates.

The Seven Mile Creek Watershed is located south of St. Peter, Minnesota and contains the 6.1 mile long Seven Mile Creek (SMC). The watershed is located in Nicollet Country and is roughly 95.3 km^2, 86% of which is made up of agricultural land. The majority of runoff that enters the creek comes from constructed ditches and tile drains that are located throughout this farmland. The site was selected for multiple reasons: 1) the area is largely agricultural; 2) nitrate concentrations have been found to be elevated at times, up to 25mg/L; and 3) previous research has been done at the site, including members of the current project, so that there is ample background information. The SMC system also allows for the study of moisture levels due to intermittent flow and drying within the ditches, which leads to a dampened contribution of water to the creek within the Park, and geographic isolation of bacteria within the creek system in individual pools.

Of the three sites in the study, two (SM1 and SM2) fall into the category of containing agricultural runoff ditches, which are steep riparian slopes that are intermittently flooded by the streams that they flank. The third site is located in Seven Mile Creek Park and sampling was done within the stream channel itself. The SM1, SM2,

and Park sites were selected from the variety of potential site locations of study after considering known history from previous studies as well as similarities and differences between them. Sites SM1 and SM2 were selected due to having different plant cover (crops planted), with SM1 having soybean and corn, and SM2 corn. Preliminary survey also distinguished the two from one another, with SM2 having a severe snail infestation problem and a finer stream bottom, while SM1 did not, and had a stream alternating between sandy and muddy. Additionally, SM1 is situated below and next to State Highway 99, a main thoroughfare, and SM2 is only near an unpaved dirt road. The Park site was included due to its drastically different location, as well as being the location into which each of the other sites drain.

The goal of the study was to identify denitrification hot spots and to attempt to determine how the types and presence of specific functional genes relate to enhanced denitrification. To do this, I examined the genetic basis for denitrification, expressed as copy number of a functional gene, and how it relates to both denitrification rates, measured as denitrification potential, and moisture on both temporal and spatial scales. Selection of genes was based on the attempt to obtain the most complete picture of the denitrification process as possible. As such, at least one gene per pathway step was selected. In the case of nitrite reductase, both *nirK* and *nirS* were included as they represent two different groups responsible for the same step. Also, as has been recently discovered, *nosZ*-bearing denitrifiers exist in two clades, designated *nosZ-I* and *nosZ-II* (Isobe and Ohte 2014). In an effort to better encompass the entire step, two primer sets were selected to represent these different clades: *nosZ1* corresponding to *nosZ* clade I and *nosZ3* representing subsets of both clades, targeting overlap between them. The *nosZ2* gene, which represents clade II, was omitted as it did not reliable results.

The denitrification functional genes *narG*, *nirK*, *nirS*, *cnorBB*, *nosZ1*, and *nosZ3* were considered in two ways. First, with relation to total 16S rDNA to give a relative abundance of genes in the samples with respect to overall microbial abundance, and second, as gene copy number normalized to dry weight soil/sediment, to give an idea of absolute abundance in the bacterial community as well as in soil/sediment. Both

measures were compared against moisture content and potential denitrification over time and across sampling locations independently.

Temporally, it was hypothesized that there should be some fluctuation over the course of a year, possibly due to seasonal shifts as well as differences in weather patterns, and that these fluctuations would correlate with soil moisture content as well as measured potential denitrification values. With respect to spatial scales, both inter site and intra site, the hypothesis is that there would be differences between locations for each of the functional genes, and these differences would correspond to differences in soil moisture content and potential denitrification. Implications of these hypotheses are of great import in understanding microbial denitrification and its role as a potential sink of nitrogen from inundated systems.

2.2 Materials and Methods[2]

2.2.1 Field Site Locations

Three sites, Seven Mile Creek site 1 (SM1), Seven Mile Creek site 2 (SM2), and Seven Mile Creek Park, were sampled several times throughout each of the two years. In 2014 each site was sampled on June 12, August 20, and October 20 for a total of three sample sets. In 2015 each site was sampled on May 13, June 15, July 27, August 17, and October 26 for a total of five sample sets.

At both SM1 and SM2 the samples were taken in triplicate in the channel and in two places on the bank, designated the flooding and non-flooding zones. The flooding zone is low enough on the bank that it is periodically submerged when the channel flooding increases sufficiently. The non-flooding zone is always above the submersion zone. Channel samples are designated ‘1’, flooding zone samples are designated ‘2’, and non-flooding zone samples are designated ‘3’ with triplicates A-C for each site. For example, SM2-3B refers to replicate B in the non-flooding zone at Seven Mile Creek site 2.

[2] Methods done in collaboration with Abby Tomasek

The sites were located by GPS coordinates (Table 2.1). Within each site, the exact sampling location was found by measuring from a set location on the landscape. At SM1, a stake was placed 0.45 meters up the bank as a reference for future sampling. Into the center of the channel from the stake, the samples for the 'in channel' group were taken. At the location of the stake in the bank, the flooding zone was sampled, and 2.3 meters up the bank and away from the channel the non-flooding zone samples were obtained. The SM2 locations were determined by measuring 12.4 meters upstream from a tree and from that point moving straight down into the channel's center. The flooding zone samples were taken 0.85 meters from the middle of the channel. The non-flooding zone samples were then taken by measuring 7.6 meters up the bank from the flooding zone. The park samples were taken 30 meters upstream of a tree, on the edge of the channel at the other side of the channel.

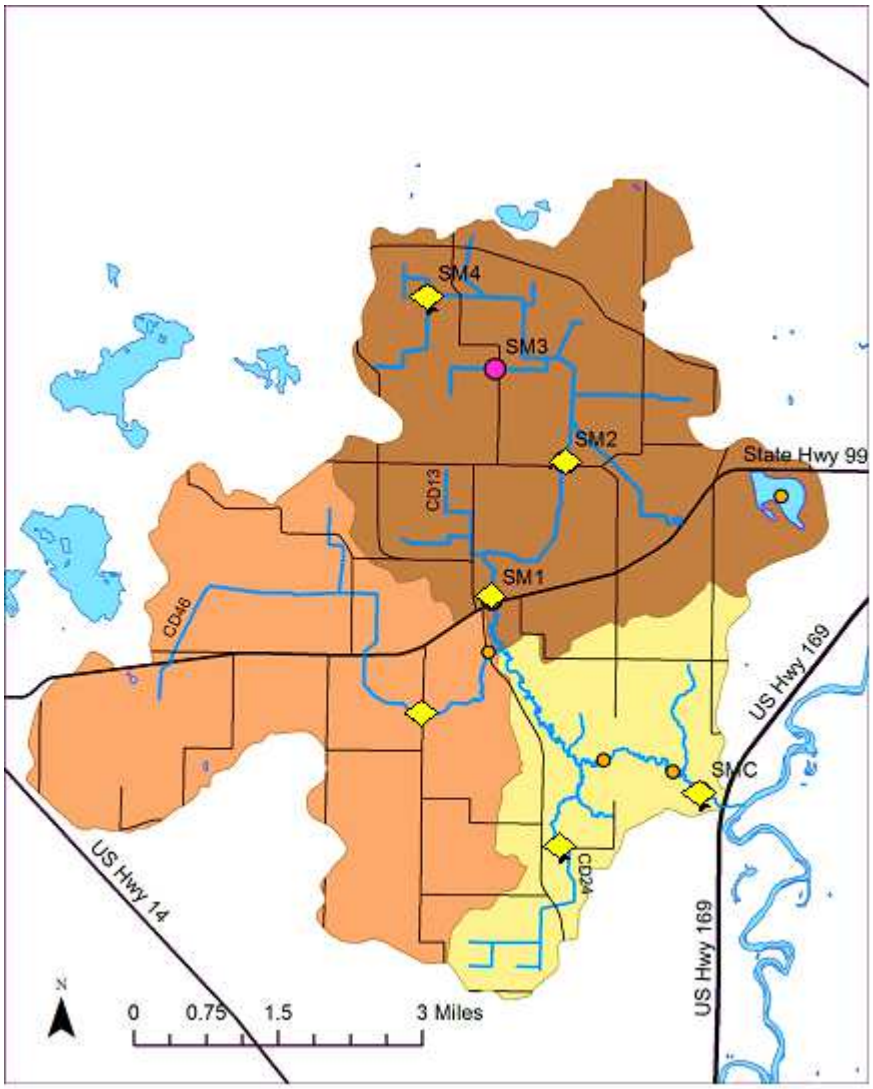


Figure 2.1. Map showing the locations of the three sampling sites, SM1, SM2 and Park (SMC on the map). Near St. Peter, MN. The other locations were not used in this study.

Table 2.1. GPS coordinates of sampling sites.

Site	Coordinates
SM1	44.2925, -94.0759
SM2	44.3117, -94.0614
Park	44.2634, -94.0319

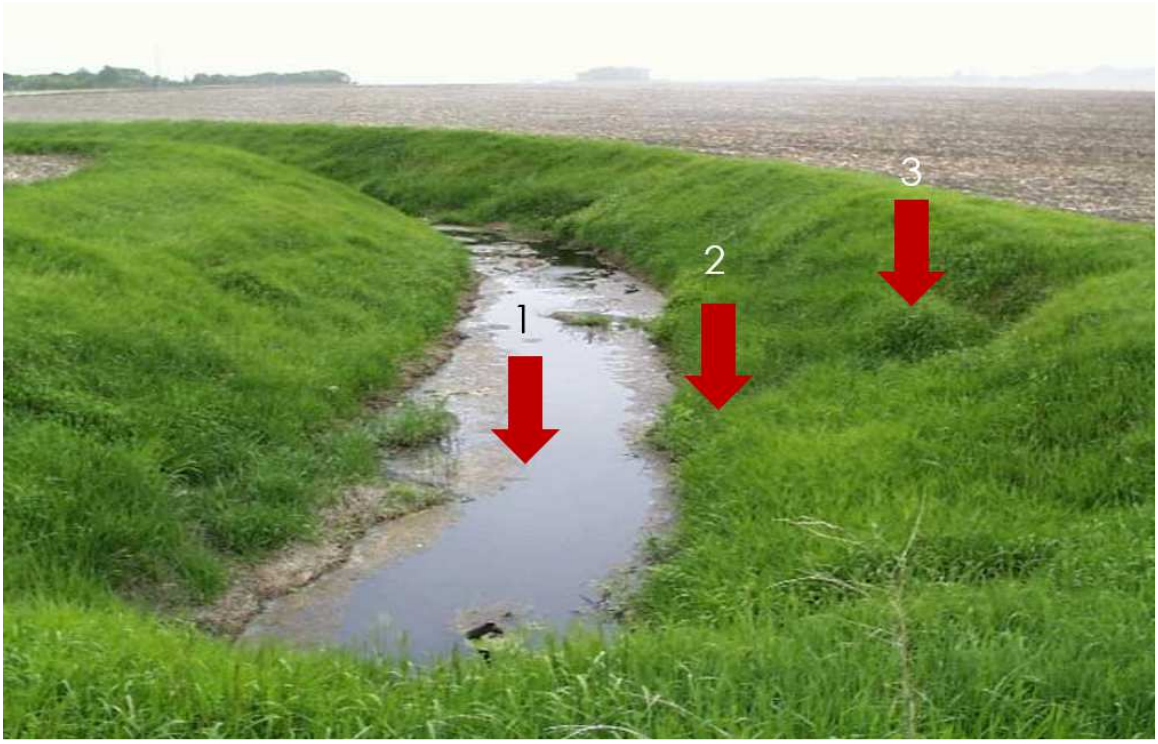


Figure 2.2. Bank sampling scheme. At sites SM1 and SM2 locations 1, 2, and 3 were sampled and represent in channel, flooding zone, and non-flooding zone, respectively. At the park site only location 2 was sampled. The park sampling location is technically in the channel but it acts as a flooding zone as it is not always submerged due to temporal fluctuations in water level throughout the year and was analyzed as such.

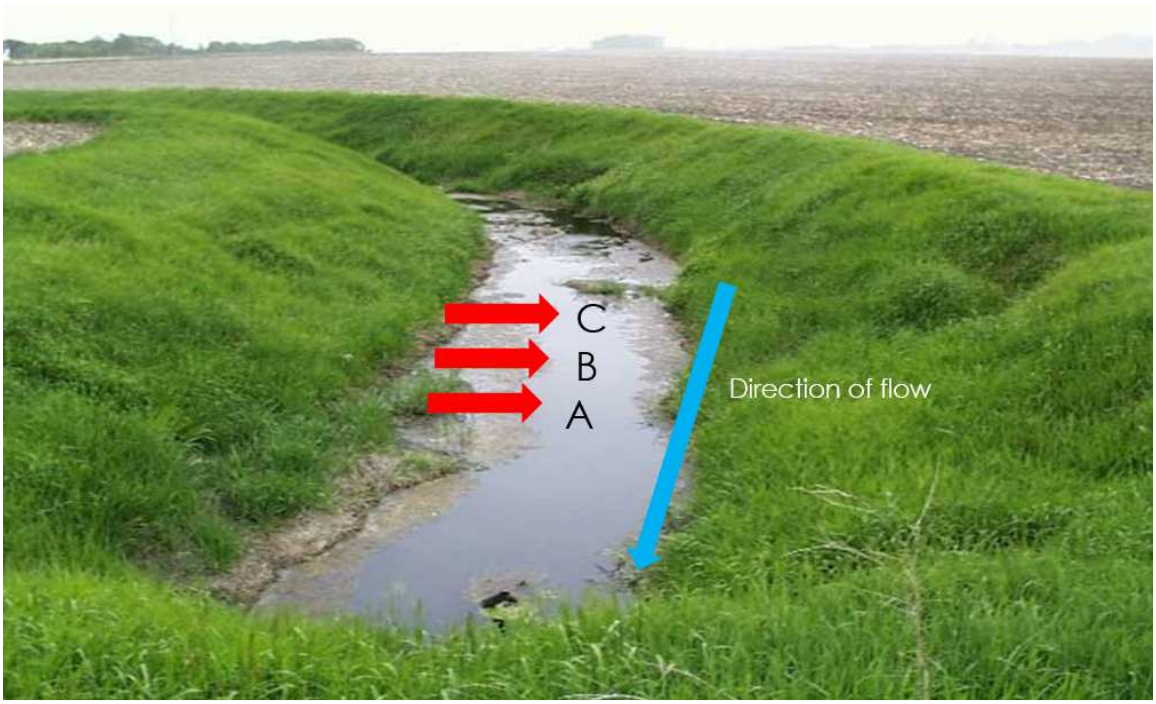


Figure 2.3. In channel replicate sampling scheme. The first triplicate, ‘A’, is the furthest downstream, with ‘B’ further upstream, and ‘C’ the furthers upstream.

2.2.2 Microbiological Sampling

Triplicate within channel samples were taken from the bottom sediments with a 60mL syringe by plunging it downward so that it includes depths below the oxygenated surface (including the first 5 cm). The sediment sample was transferred to a 15 mL Falcon tube and placed into a cooler for transport back to the lab where it was stored at -20°C until extraction. Samples were thereafter moved to -80°C for long term storage. Triplicate samples were taken next to one another, by moving progressively upstream so that replicate 'C' is the most upstream sample and 'A' is the furthest downstream. The bank replicates were taken to form a triangle with 'A' the bottom left and moving clockwise (Figure 2.3). The bank soil samples were collected in the same manner as the stream and stored at -80°C after processing.

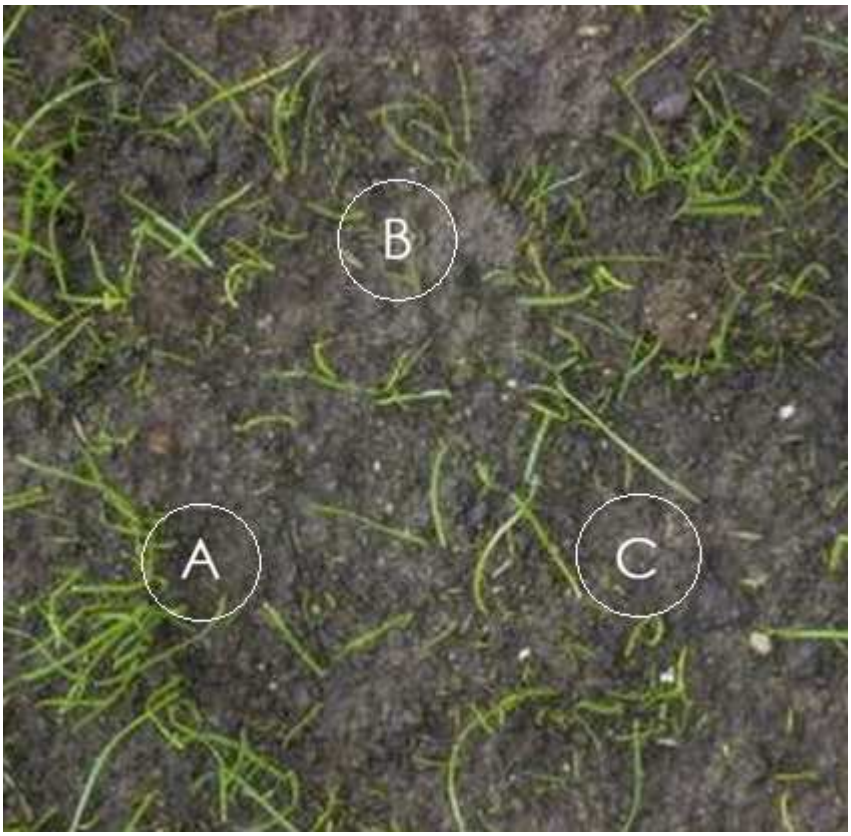


Figure 2.4. Microbiological flooding zone and non-flooding zone sampling scheme. Sample A is taken at the lower left and subsequent samples move clockwise, making a triangle.

2.2.3 DNA Extractions and Concentration Measurement

The sediment samples stored at -20°C were removed and allowed to thaw fully before DNA extraction. For each sample, 0.5g ± .01g wet weight sediment or soil was weighed, double the suggested weight as per the manufacturer's protocol. DNA was extracted from the samples using a PowerSoil DNA Isolation Kit (MoBio Laboratories, USA) following the kit's accompanying instructions (Costa et al. 2015; Stea et al. 2015) with minor changes. This included waiting 5 min. after the addition of solution C5 as well as repeating this step before proceeding with the published instructions. Each sample was done in duplicate and then the extracted DNA from duplicated pseudo-replicates was combined for a total volume of 200µl. This volume was then distributed into three tubes to avoid repeated freeze/thawing and stored at -20°C. The sediment samples were placed in the -80°C for long term storage.

DNA concentrations were measured using a Qubit (Invitrogen, Massachusetts, USA) as per the manufacturer protocol. A stock solution of 199µl buffer and 1µl dye per sample was made and then 198µl was used per tube (corresponding to each sample), combined with 2µl of the extracted DNA for measurement. Dilutions were done as necessary when readings were too high, and appropriate calculations to adjust for dilutions were made in determining final concentration.

2.2.4 Standard Dilutions

Stock dilutions were prepared for subsequent use in quantitative PCR analyses (qPCR) for determining standard curves using gBlock Gene Fragments synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) designed from the selected primers for denitrification functional genes. Using the information provided by the manufacturer regarding concentration and weight, dilution schemes were determined based on the required copy numbers for the standard curve containing 300,000, 30,000, 3,000, 300, and 30 copies. The fragments received were as follows: a fragment with *BAC515* (16S rDNA gene), *narG*, *nirS*, and *nirK*, a fragment with *cnorBB* and *nosZ1*, and a fragment bearing *nosZ3*. Plasmid standards were used for the *nirS* gene due to the

inability of the gBlock *nirS* to function reliably or efficiently with PCR or qPCR protocols.

2.2.5 PCR

To test the efficiency of the selected primers with the gBlocks and *nirS* plasmid standards, PCR and subsequent gel electrophoresis were done. Each of the dilutions were run as per the protocol in Table 2.3 depending on the gene, as well as with a random sample taken from the field and a known stock sample to be used as a positive control.

Each PCR reaction (one reaction per sample/standard dilution) included 23µl of master mix and 2µl of DNA template. If the DNA templates were of low or high concentration, these volumes were adjusted by altering water addition per sample in preparing the master mix. The master mix for a 23µl (1x) volume total was prepared as follows: 2.5µl 10x buffer solution, 1µl 10mM dNTPs (Denville Scientific, Holliston, MA, USA), 0.5µl 10mM of each primer (one forward, one reverse primer), 0.25µl Taq polymerase (Denville Scientific, Holliston, MA, USA), and the rest was water to a final total of 23µl. Each master mix was done separately for different genes due to primer differences.

The run cycle protocol for PCR varies from that of qPCR (Table 2.3) only in that there is an additional 95°C for 10 min. to start the reaction, as well as a 4°C hold at the conclusion of the cycle.

Table 2.2. Forward and reverse primers listed by corresponding gene

Gene Name		Primer Sequence	Ref.
***BAC515F* (16S rDNA)**	U515F: U806R:	GTGCCAGCMGCCGCGGTAA GGACTACHVGGGTWTCTAAT	
cnorBB	cnorB-BF: cnoB-BR:	AIGTGGTCGAGAAGTGGCTCTA TCTGIACGGTGAAGATCACC	(C. E. Dandie et al. 2007)
narG	narG_1960m2fE: narG_2050m2R:	TAYGTSGGGCAGGARAAACTG CGTAGAAGAAGCTGGTGCTGTT	(López-Gutiérrez et al. 2004), (Kandeler et al. 2006)
nirK	nirK876F: nirK1040R:	ATYGGCGGVCAYGGCGA GCCTCGATCAGRTTRTGGTT	(Bru et al. 2011; Petersen et al. 2012)
nirS	m-cd3AF: m-R3cd:	AACGYSAAGGARACSGG GASTTCGGRTGSGTCTTSAYGAA	(Hallin and Lingren 1999; Kandeler et al. 2009)
nosZ1	nosZ_F: nosZ_1622R:	CGYTGTTCMTCGACAGCCAG CGSACCTTSTTGCCSTYGCG	(Rosch, Mergel, and Bothe 2002)
nosZ3	nosZ2F nosZ2R	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA	(Bru et al. 2011; Petersen et al. 2012)

Table 2.3. Details regarding each of the parameters used listed per gene for master mix.

Gene Name	Primer Set	qPCR Specifications	PCR/qPCR protocol
***BAC515F* (16S rDNA)**	U515F U806R	.8µl each primer 10^{-4} dilutions	(95°C:30s, 50°C: 30s, 72°C:30s) x40
cnorBB	cnorB-BF cnorB-BR	.8µl each primer 50ng BSA	(95°C:30s, 60°C:30s, 72°C:30s) x46
narG	narG_1960m2fE narG_2050m2R	.5µl each primer	(95°C:15s, 58°C:30s, 72°C:30s) x46
nirK	nirK876F nirK1040R	.5µl each primer	(95°C:30s, 63°C →58°C [-1°C/cycle] :30s, 72°C:30s) x51
nirS	m-cd3AF m-R3cd	2µl each primer Plasmid standard	(95°C:30s, 63°Cà58°C [-1°C/cycle] :30s, 72°C:30s, 81°C:30s) x55
nosZ1	nosZ_F nosZ_1622R	1.2µl each primer 10^{-2} dilutions	(95°C:30s, 65°C:30s, 72°C:30s) x51
nosZ3	nosZ2F nosZ2R	1.2µl each primer 10^{-2} dilutions	(95°C:30s, 65°C→60°C [-1°C/cycle] :30s, 72°C:30s) x51

The column entitled specifications lists only the variables that change between setups, therefore excludes the amount of iTAQ SYBR Green used (10µl) and the volume of DDI (which is variable, but makes up the difference depending upon the amount of each primer used), the total master mix volume of each reaction to equal 15µl.

2.2.6 qPCR

Each quantitative PCR reaction was done using the SYBR Green kit and mostly done using a Roche Light Cycler 480 Real Time PCR System, but several were done using Applied Biosystems StepOne Real-Time PCR System, in 96 well plates. Each master mix setup was done at 110X concentration and followed the specifications as described in Table 2.3 according to the gene being analyzed. Each volume totaled 20µl and consisted of 15µl (as per Table 2.3) plus 5µl DNA template. Each sample was run in triplicate.

The template DNA was diluted 100-fold (10^{-2}) for all genes, except for that of 16S rDNA, which was diluted 10,000-fold (10^{-4}). Dilutions were necessary due to concentrations that were too high and exceeded the Ct value output by the machine for the highest copy number dilution (300,000), therefore requiring extrapolation. Instead, it was decided to dilute and back calculate to obtain actual values. Each standard curve dilution and sample was done in triplicate and values were averaged for analysis.

The standard curve was run and calculated each time a plate was run. Determination of the standard curve came from the average of the Ct values output by the machine plotted against the log of the copy number of the dilution (Table 2.4). The curve is created by plotting the average log of the copy number against the average Ct value, then displaying the trend line equation. Using this trend line equation, log of the copy numbers for each of the samples was obtained by inserting the generated Ct value in place of the y value and solving for x. Finding the true copy number requires then undoing the log function and then adjusting for dilutions. The R^2 should be as close to 1 as possible, with greater than or equal to .99 being the most desired result to give an accurate copy number (x variable) based on the Ct values (y variable) obtained. The exact equations used differed by gene but were generated so that the efficiency (slope) was between 80 to 110%, with a slope of -3.2 corresponding to 100% efficiency.

The melting curve shows the temperature at which the amplified fragment of DNA denatures. This is referred to as the Tm value and the more similar the sequences, the closer the Tm values are. One gene, *nosZ3*, had differing melting curves (Figure 2.6) for the samples and so they were sequenced and analyzed against a database to confirm that they were the correct gene, despite varying sequences.

Table 2.4. Calculation scheme for establishing the standard curve.

Standard Curve				
copy num.	**log(copy num.)**	**ct value**	**mean log(copy num.)**	**mean ct value**
300000	5.47	20.85	5.47	20.91
300000	5.47	20.98		
300000	5.47	20.91		
30000	4.47	24.42	4.47	24.41
30000	4.47	24.43		
30000	4.47	24.38		
3000	3.47	27.99	3.47	27.39
3000	3.47	27.67		
3000	3.47	26.52		
300	2.47	31.68	2.47	31.28
300	2.47	31.55		
300	2.47	30.62		
30	1.47	35.3	1.477	34.20
30	1.47	34.88		
30	1.47	32.42		

2015 15 June *narG* data. Copy number is known as per the dilution scheme and the Ct value is output by the qPCR machine.

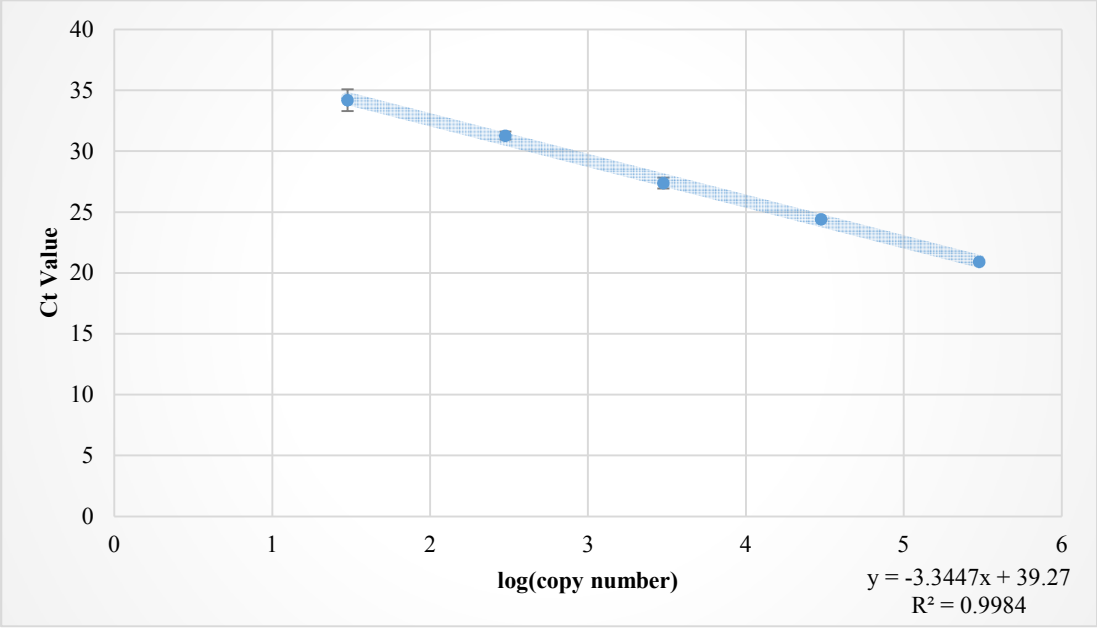


Figure 2.5. June 15, 2015 *narG* standard curve with standard error bars.

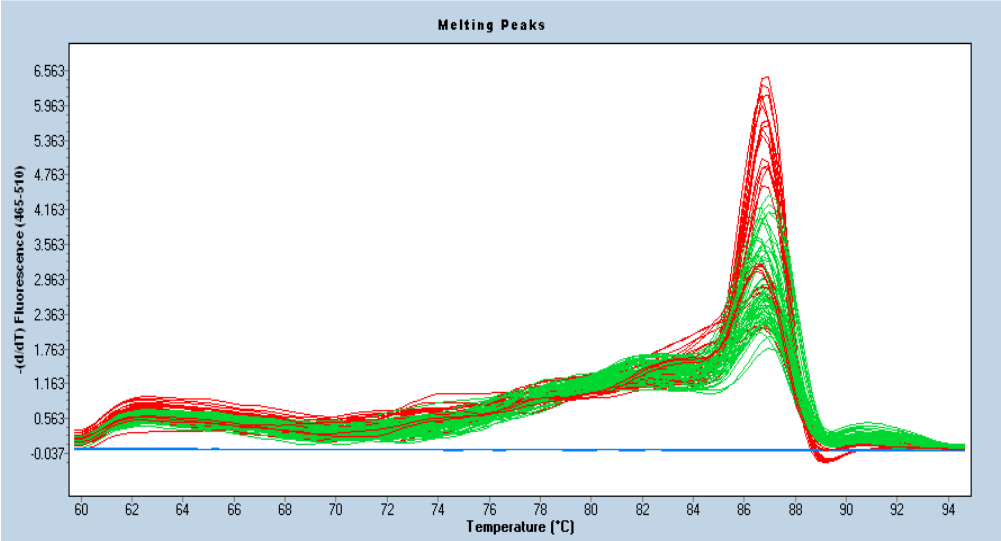


Figure 2.6. Melting curve (Roche Light Cycler 480) of *cnorBB* for sampling date May 26, 2015. Single peak which is expected with samples all of the same gene.

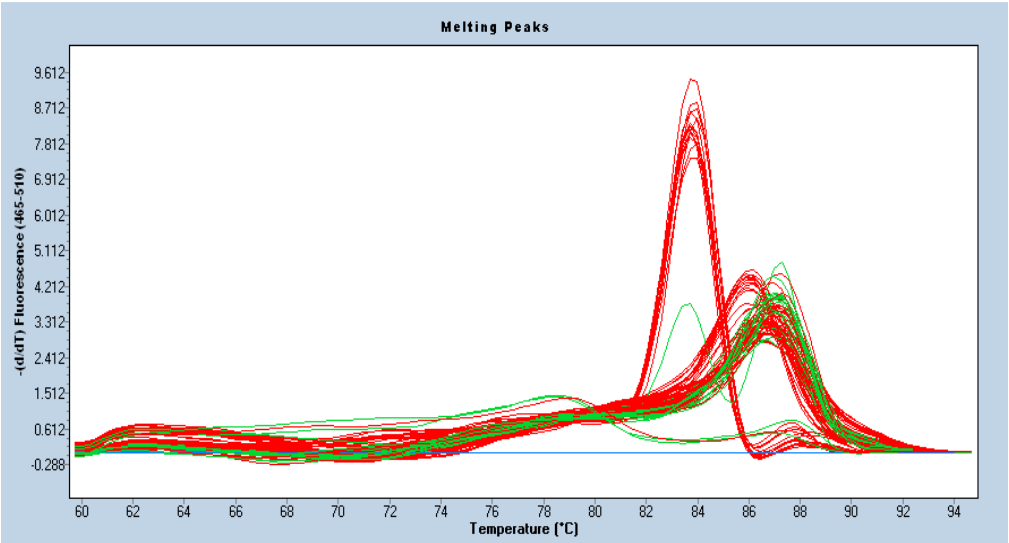


Figure 2.7. Melting curve (Roche Light Cycler 480) of *nosZ3* for sampling date October 20, 2014. Example of a poor melting curve. Samples of varying Tm values were sequenced and compared against a database to confirm that they were the correct gene despite differences.

2.2.7 Soil Moisture Content[3]

Soil cores were taken following the same pattern as, and corresponding to, the microbiological soil samples to be used for DNA extraction. For in channel locations at both SM1 and SM2, triplicate 'A' samples were taken so that they were most downstream of flow with respect to the other replicates. The 'B' replicates were taken just upstream of the location of replicates 'A', and 'C' were taken just upstream of replicate 'B'. Flooding zone and non-flooding zone (bank) triplicate samples were taken to correspond to the microbiological soil samples as well, taken in a triangle shaped pattern with 'A' at the bottom left point and moving clockwise to end with 'C'(Figure 2.3).

Soil cores were collected with a 35 mL syringe, sampling to include the top 5 cm of the soil layer as was done with the microbiological sampling, and then placed into small cups and stored on ice until transport to the laboratory for subsequent processing. The depth of sampling was recorded as measured by the syringe. Due to submersion in water as well as being unable to see the collection process, in channel sampling was done as carefully as possible with the intent of remaining consistent with the depth of flooding zone and non-flooding zone sampling.

To determine volumetric water content, the volumes and weights (wet weight) of the sample-containing cups were recorded before being placed in an oven and incubated at 110°C for 24 hours. The samples were then reweighed to determine dry weights. Volumetric water content was calculated by subtracted dry weight plus cup from the wet weight plus cup, then normalizing to the measured pre-drying volume and depth of sampling. It was assumed that the weight of the cup did not change over the 24 hour period of drying.

[3] Work done by Abby Tomasek

2.2.8 Potential Denitrification[4]

Sediment and soil samples were taken following the same scheme as detailed in both the Microbiological Sampling and Soil Moisture Content sections (2.2.2 and 2.2.7) with in channel sampling moving from downstream to upstream and flooding zone and non-flooding zone following the same triangular sampling pattern. As with the sampling for volumetric water content, a 60 mL syringe was used to collect the samples, including the top 5cm of sediment, which were then placed in bags and put on ice until transport back to the laboratory. Samples were processed within two days of retrieval and kept at -4°C until analyses were initiated.

The denitrification enzyme activity (DEA) assay was used to determine denitrification potential of the samples. This assay relies upon the use of acetylene to inhibit N_2O reduction to N_2, the final step in complete denitrification (M. S. Smith and Tiedje 1979). Blocking this final step leads to the accumulation of measurable N_2O, the rate of which is used as a proxy for denitrification rate. Samples were amended with carbon as glucose (40 mg C/L), phosphate as KH_2PO_4 (13.84 mg P/L), and nitrate as KNO_3 (100 mg N/L) previous to the assay being run to mimic conditions if no nutrients were limiting in the system, thereby giving the absolute potential of the communities to denitrify.

For each sample, 40 g of sediment/soil, 4 mL of water taken from corresponding site locations, and 10,000 μg/L chloramphenicol were added to a 125 mL Wheaton bottle. The bottles were closed with rubber septum caps to create anoxic conditions, and flushed with He for at least 5 min. To each bottle, 10 mL of pure acetylene (C_2H_2) was added using a needle and luer lock syringe and they were carefully shaken to create a slurry before being allowed to rest for 20 min for adequate diffusion of the C_2H_2. For each sample, initial N_2O readings were taken after this period of diffusion by removing 10 mL of gas with the same syringe, 5 mL of which were expelled into the air to clear the needle, and the remaining 5 mL were injected into a 10 mL helium flushed gas vial for gas chromatograph reading. The bottles were incubated for 4 hours on a roller table, after which time another sample of the same volume and protocol was taken and injected into

[4] Work done by Abby Tomasek

a second gas vial for a final N_2O concentration reading, once more by gas chromatography (GC).

A Hewlett-Packard 5890 Series II GC was used to analyze the initial and final nitrous oxide concentrations taken from each sample. The gas chromatograph was equipped with an electron capture detector and a headspace autosampler (Hewlett-Packard 7694). The temperatures were as follows: flame ionization (FID) and thermal conductivity (TCD) detectors 75°C-isothermal, the electron capture detector (ECD) was run at 35°C-isothermal and the autosampler oven temperature was 40°C. Helium was flushed through each of the detectors at the corresponding flow rates: TCD at 30 ml/min, FID at 12 ml/min, and ECD at 30 ml/min. In addition to the helium, the ECD was also flushed with 5% CH_4-Ar at 45 ml/min. Production of nitrous oxide was measured as accumulation N_2O over the 4h incubation period and was adjusted by using the Bunsen solubility coefficient (Breitbarth et al. 2004).

2.2.9 Other Methods

In channel water temperatures and dissolved oxygen measurements were obtained by using a Hydrolab DS5X multiprobe (Hach, Loveland, CO, USA). The probe was placed in the center of the channel upstream of where sediment sampling was done to ensure no particulate was loosened that could interfere with the probe's sensors. The submersible system was deployed for at least 1 hour, allowing values to stabilize, and recorded water temperature and dissolved oxygen values every minute. Readings obtained were averaged in laboratory for use in subsequent analyses.

Maximum air temperature values for each sampling date were obtained using past climate data from the National Weather Service Reporting Station in St. Peter, MN. Data was accessed using the Minnesota Department of Natural Resources website (http://www.dnr.state.mn.us/climate/historical/acis_stn_meta.html) and was converted from the reported temperature in Fahrenheit to Celsius.

2.3 Results of Temporal Studies

2.3.1 Genetic Analyses

Each site was considered individually over both the 2014 and 2015 field sampling years on the scale of months, and analyzed using ANOVA with a p-value < .05 to qualify for statistical significance. Analyses were done using normalization with respect to 16S rDNA as well as per gram dry weight sediment or soil, and converted to $\log_{10}$ values.

The relative functional gene copy numbers, normalized with respect to copy number of 16S rDNA (Table 3.1) over either year, were not statistically different across all months for the *cnorBB* gene (p=0.88 in 2014, p=0.56 in 2015). Each of the *nirS*, *nirK*, and *nosZ1* genes showed significant differences across months in 2015 (p-values $3.8\text{x}10^{-26}$, 0.03, $6.14\text{x}10^{-8}$ respectively), but not in 2014 (*nirS* p=0.21, *nirK* p=0.055, *nosZ1* p=0.73), while *nosZ3* was significantly different across months in 2014 (p=0.02), but not in 2015 (p=0.089). However, *narG* was significantly different (p=0.005 in 2014, p=0.014 in 2015) in both 2014 and 2015 across sampling months. Aside from *narG*, the copy number of the other tested genes were not significantly different (p>0.05) over both years on a monthly scale.

When absolute copy numbers were analyzed, as measured by copy number per gram dry weight soil/sediment there were statistically significant (p=0.03) differences between months in 2014 for only *nirK* (Table 3.2). In 2015, however, each functional gene studied showed significant variation over the months sampled with p-values provided in Table 3.2.

ANOVA analysis done between years excluded the months of May and July in 2015 as there was no direct comparison in 2014. Thus, only the months of June, August, and October were compared between 2014 and 2015. Normalization of copy number of each functional gene to 16S rDNA copy number, as well as to dry weight soil, were considered in analyses (Table 3.3). Copy numbers of *nirS*, *nirK*, and *nosZ1* were significantly different when normalized to 16S rDNA (p-values of 0.01, $1.7\text{x}10^{-13}$, and $5.1\text{x}10^{-8}$ respectively). In contrast, there were no significant differences *cnorBB*, *narG*, and *nosZ3* by years (p-values of 0.055, 0.099, and 0.53 respectively). With respect to soil dry weight normalization, all genes were significantly different across the scale of years (Table 3.3) except for *narG* (p=0.86).

Discriminant analyses[5] were done using the averaged triplicate values for gene copy number as normalized to 16S rDNA for each of the functional genes studied for 2014 data (Figure 3.1), 2015 data (Figure 3.2), and both years combined (Figure 3.3). Analyses shows that in 2014 the profiles of functional gene relative abundances were much more discrete than in 2015, where the samples by month tended to be more tightly clustered. While there is still separation similar to that of 2014 alone, both years combined were less variable than that of 2014.

Table 3.1. Details regarding ANOVA analysis done with the gene copy numbers normalized to 16S rDNA gene copy numbers between months listed, done for each year independently.

	Year							
	2014			**2015**				
	June	**Aug.**	**Oct.**	**May**	**June**	**July**	**Aug.**	**Oct.**
cnorBB		-				-		
narG		p = 0.005				p = 0.01		
nirS		-				$p = 3.8 \times 10^{-26}$		
nirK		-				p = 0.03		
nosZ1		-				$p = 6.14 \times 10^{-8}$		
nosZ3		p = 0.02				-		

Significant differences stated against a p-value of 0.05 with the exact p-values from the analysis given. Dashes represent results that were not statistically significant.

[5] Credit to Abby Tomasek for discriminant analyses and resultant figures

Table 3.2. Results of ANOVA analysis for gene copy number per gram dry weight soil/sediment between months listed, done for each year independently.

	Year							
	2014			**2015**				
	June	**Aug.**	**Oct.**	**May**	**June**	**July**	**Aug.**	**Oct.**
cnorBB		-			$F = 20.156, p = 9.6 \times 10^{-7}$			
narG		-			$F = 3.05, p = 0.03$			
nirS		-			$F = 28.29, p = 8.5 \times 10^{-10}$			
nirK	$F = 5.28, p = 0.03$				$F = 3.14, p = 0.02$			
nosZ1		-			$F = 2.76, p = 0.04$			
nosZ3		-			$F = 18.37, p = 1 \times 10^{-7}$			

For 2014, the F-crit value was 3.55, while it was 2.68 for 2015. Reported values correspond to significant results compared against a p-value of 0.05, while results failing to show significance are represented by a dash.

Table 3.3. ANOVA analysis of log scale results for yearly comparison amongst all sampling sites for statistically significant values reported only.

	Normalization to:	
	16S rDNA	**Soil dry weight**
cnorBB	-	$F = 5.69, p = 0.01$
narG	-	-
nirS	$F = 6.64, p = 0.01$	$F = 11.19, p = 0.001$
nirK	$F = 68.57, p = 1.7 \times 10^{-13}$	$F = 38.65, p = 7.1 \times 10^{-9}$
nosZ1	$F = 33.66, p = 5.1 \times 10^{-8}$	$F = 15.88, p = 0.0001$
nosZ3	-	$F = 7.20, p = 0.008$

Those represented by a dash indicate no statistically significant result. F-values were compared against the F-crit statistic with a value of 3.91. Significance was reported as a p-value lower than 0.05.

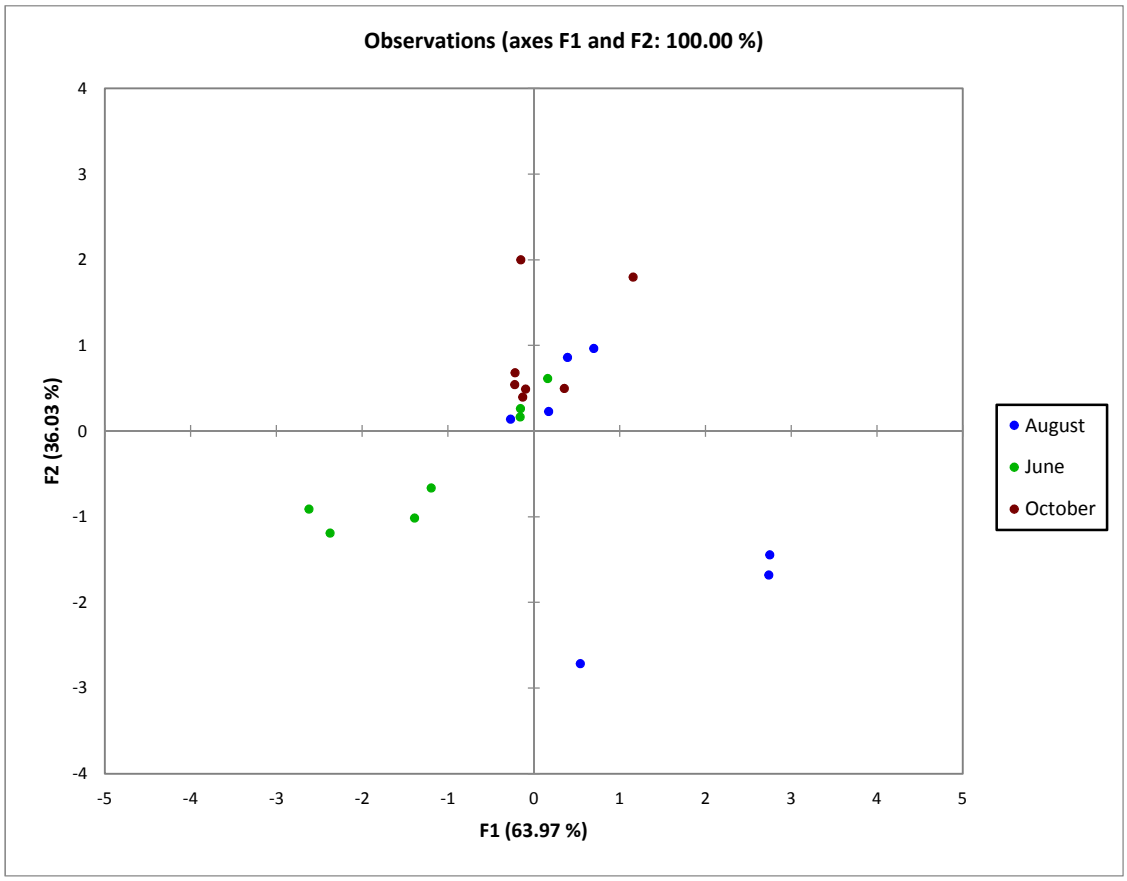


Figure 3.1. Discriminant analysis of the relative abundances of genes normalized to 16S rDNA copy number with respect to months sampled in 2014.

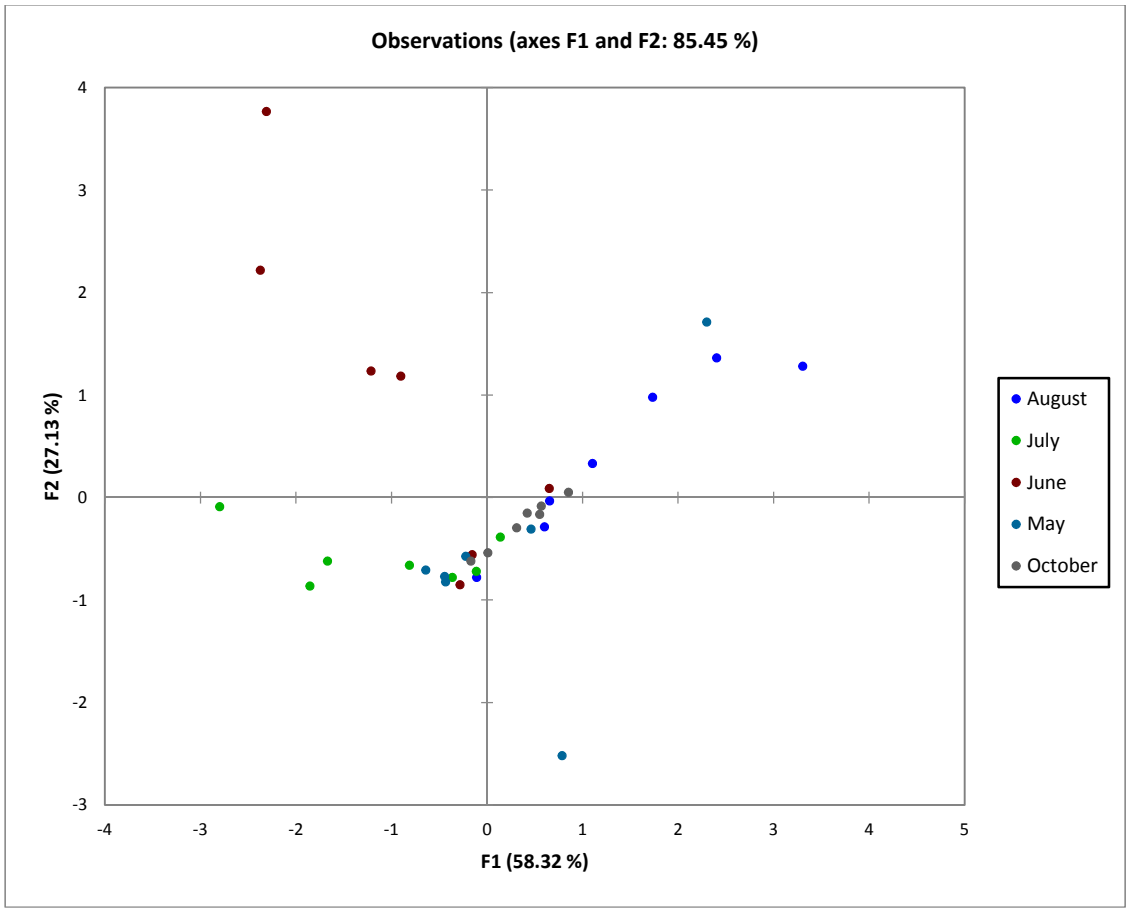


Figure 3.2. Discriminant analysis of the relative abundances of genes normalized to 16S rDNA copy number with respect to months sampled in 2015.

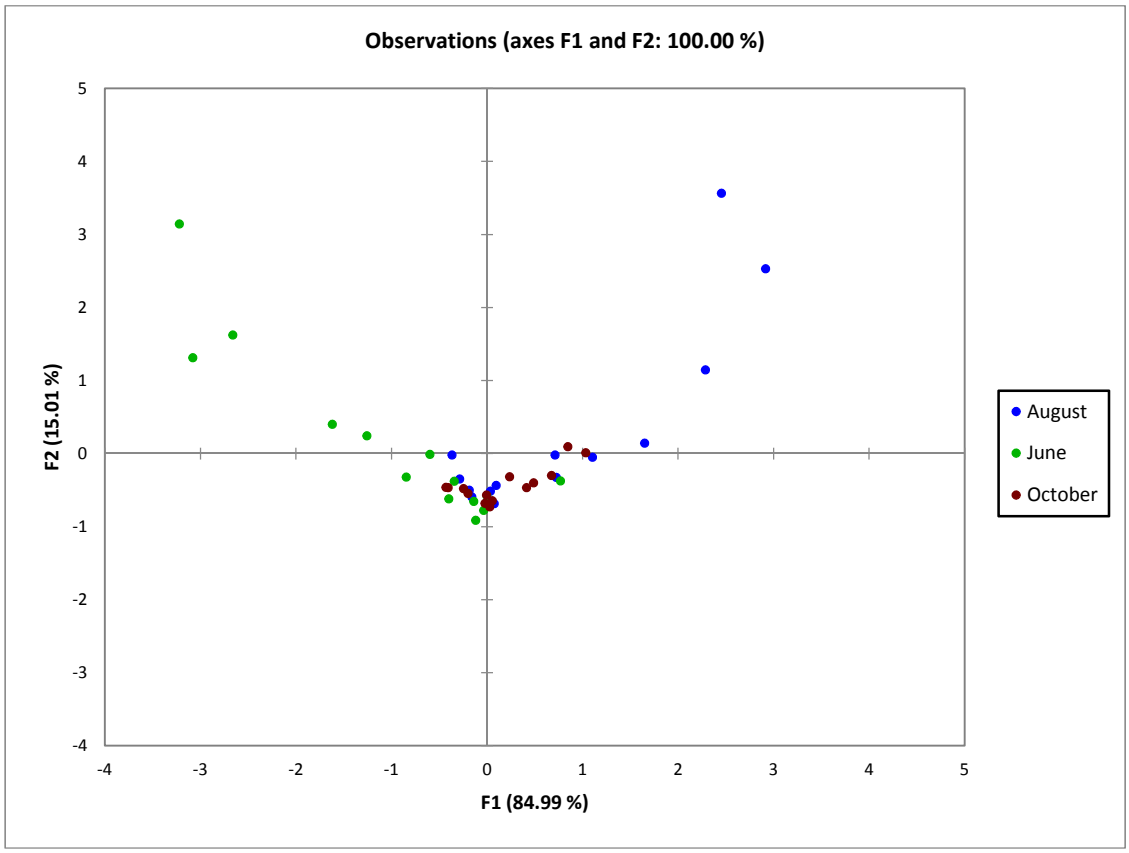


Figure 3.3. Discriminant analysis of the relative abundances of genes normalized to 16S rDNA copy number with respect months sampled over both years. The months of May and July were excluded due to no direct comparison in 2014.

2.3.2 Soil Moisture

Soil moisture levels, as measured by gravimetric water content, were not significantly different ($p=0.88$ for 2014 and $p=0.85$ for 2015) between months, staying within the same range of values. Similarly, when the values were considered over a timescale of years, there was no significant difference ($p=0.18$) between soil moisture content values (Table 3.4). No clear trends or discernable relationships between soil moisture and gene copy number were found, either with respect to 16S rDNA copy number as well as gram dry weight sediment. Data in Figures 3.4 and 3.9 show that in 2014 there was more consistency with soil moisture values vs. genetic metrics, compared to the 2015 data (Figures 3.5 and 3.10). Absolute copy numbers considered across years (Figure 3.6), showed that June had less variation per gene copy number values than did August (Figure 3.7), though the shifts in August did not correspond to soil moisture shifts. October results (Figure 3.8) were more similar to those of the month of June, with absolute gene copy numbers remaining less variable.

Although there appeared to be a slight upward trend with the various functional genes per gram dry weight sediment and soil moisture (Figures 3.4 through 3.8), the R^2 values (Table 3.5) are fairly low for all temporal scales, so that no claim can be made regarding soil moisture values correlating to functional gene copy numbers. The month of October is a notable exception, however, with R^2 values for each gene almost consistently in the 0.4 range.

Similarly, and with respect to 16S rDNA copy number, no trends were observed between soil moisture over the year scale (Figures 3.9 and 3.10). This is reflected in the R^2 values (Table 3.6). Thus, measured normalized copy numbers remained unchanging across all moisture levels, save for the occasional outlier. The same trend was independently found on monthly scales over each year (Figures 3.11, 3.12, and 3.13), and there were no differences in normalized functional gene copy numbers over the variety of measured soil moisture levels.

Table 3.4. Averaged soil moisture values as measured by gravimetric water content.

	Soil Moisture (Average)							
	June 2014	**Aug. 2014**	**Oct. 2014**	**May 2015**	**June 2015**	**July 2015**	**Aug. 2015**	**Oct. 2015**
SM1-1	0.29	0.26	0.31	0.39	0.40	0.39	0.27	0.25
SM1-2	0.65	0.61	0.30	0.39	0.62	0.39	0.42	0.43
SM1-3	0.49	0.31	0.35	0.49	0.45	0.58	0.51	0.46
SM2-1	0.50	0.49	0.61	0.77	0.56	1.64	0.47	0.54
SM2-2	0.49	0.93	0.70	1.04	1.09	1.45	1.64	1.06
SM2-3	0.46	0.25	0.36	0.48	0.54	0.50	0.55	0.68
Park	0.27	0.30	0.21	0.26	0.17	0.12	0.30	0.14

ANOVA analysis revealed no significant differences between months of each year as well as no significant differences between years (excludes the months of May and July 2015).

Table 3.5. R^2 values for soil moisture and copy number of functional genes per gram dry weight sediment over temporal scales.

	R^2 Values				
	2014	**2015**	**June**	**August**	**October**
cnorBB	0.14	0.30	0.18	0.36	0.46
narG	0.06	0.16	0.17	0.25	0.42
nirS	0.14	0.06	0.10	0.002	0.43
nirK	0.22	0.18	0.56	0.36	0.25
nosZ1	0.13	0.33	0.09	0.39	0.44
nosZ3	0.13	0.36	0.23	0.08	0.49

Table 3.6 R^2 values for soil moisture and copy number of functional genes per copy number 16S rDNA over temporal scales.

	R^2 Values				
	2014	**2015**	**June**	**August**	**October**
cnorBB	$8x10^{-5}$	0.003	0.003	0.37	0.40
narG	0.012	0.26	0.36	0.007	0.004
nirS	0.004	0.05	0.02	0.0003	0.001
nirK	0.001	0.03	0.008	0.38	0.02
nosZ1	0.09	0.16	$4x10^{-5}$	0.32	0.08
nosZ3	0.02	0.009	0.001	0.001	0.40

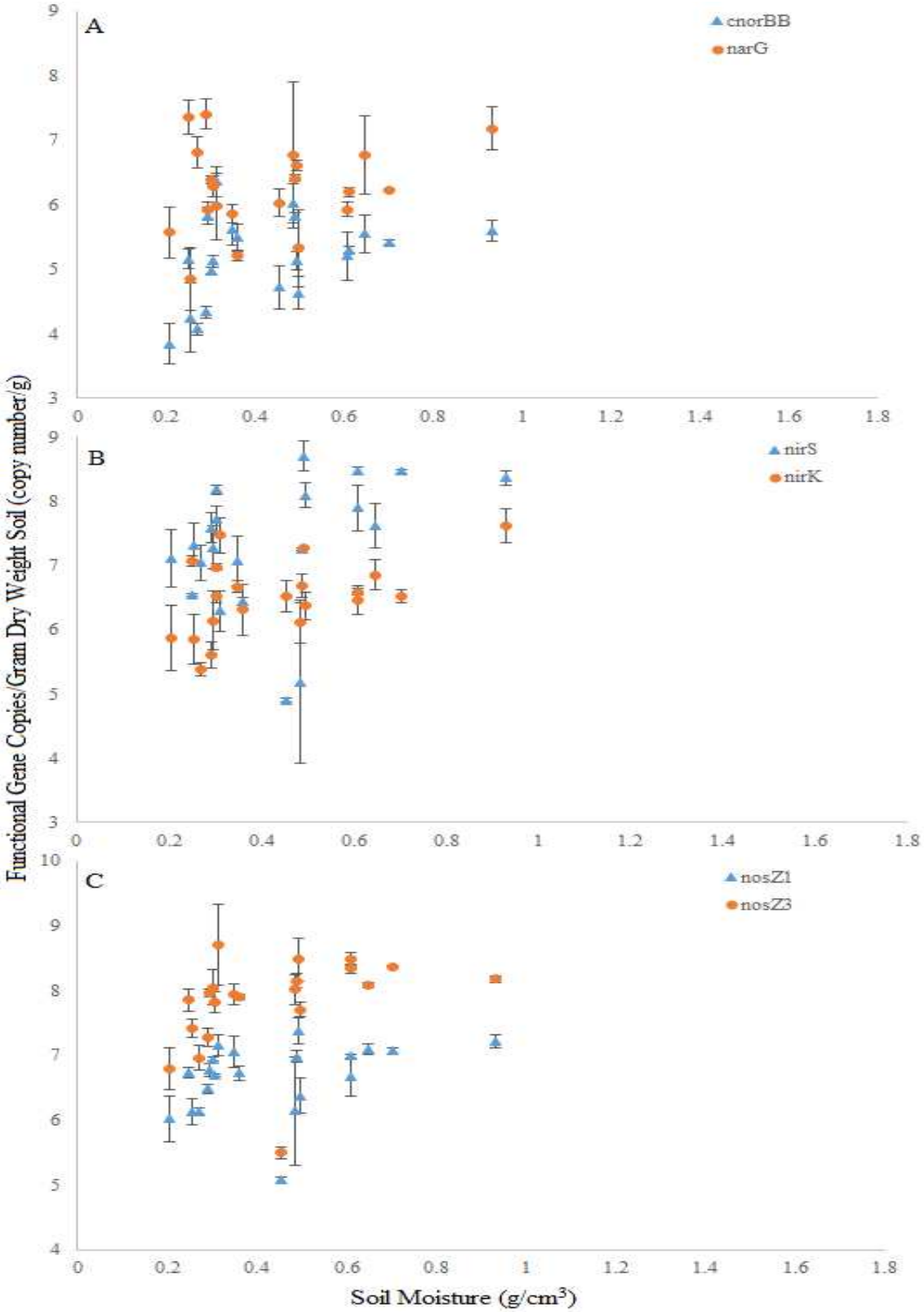


Figure 3.4. Soil moisture vs. functional gene copy numbers per gram dry weight soil from all months sampled in 2014. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

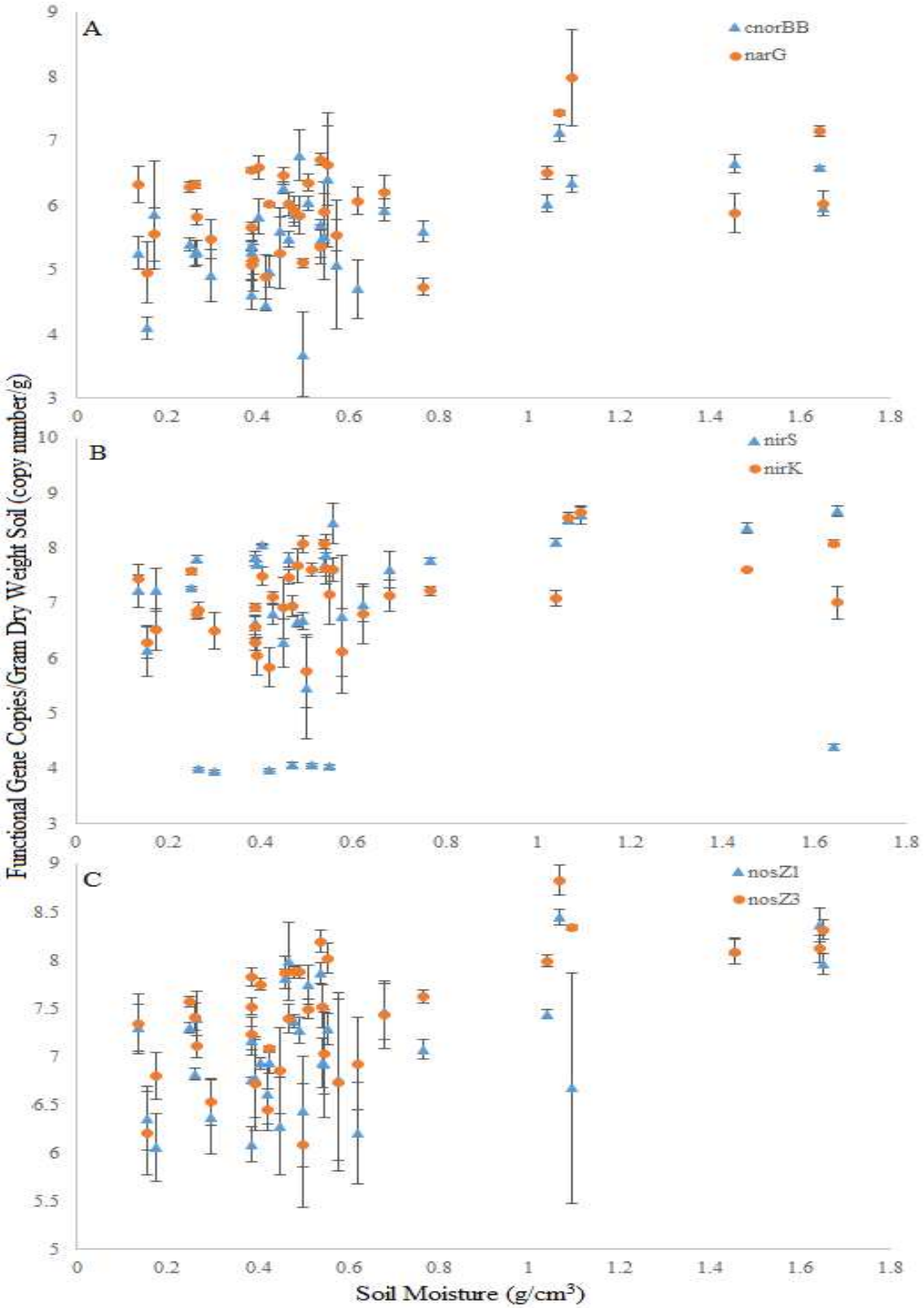


Figure 3.5. Soil moisture vs. functional gene copy numbers per gram dry weight soil from all months sampled in 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

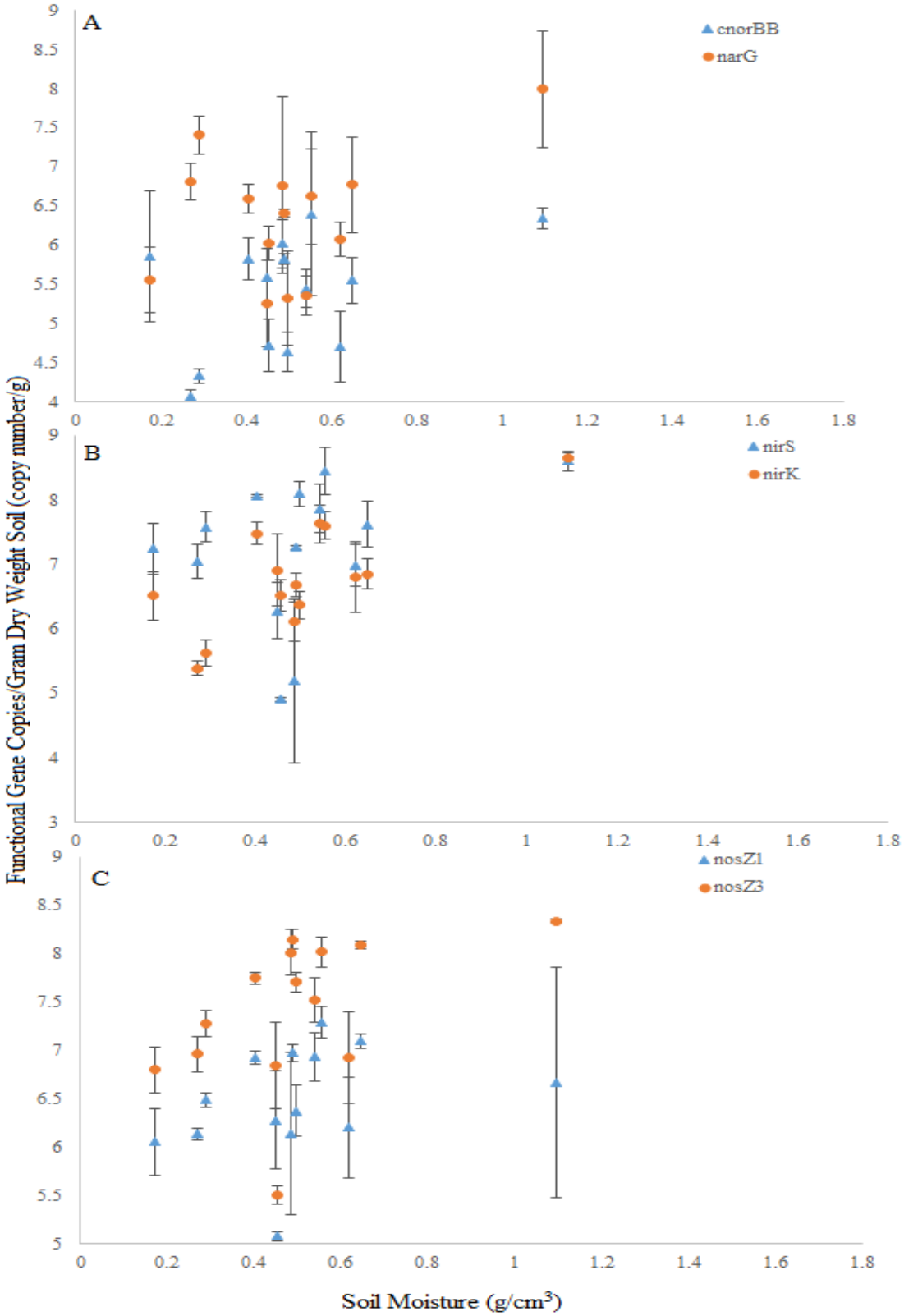


Figure 3.6. Soil moisture vs. functional gene copy numbers per gram dry weight soil in June of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

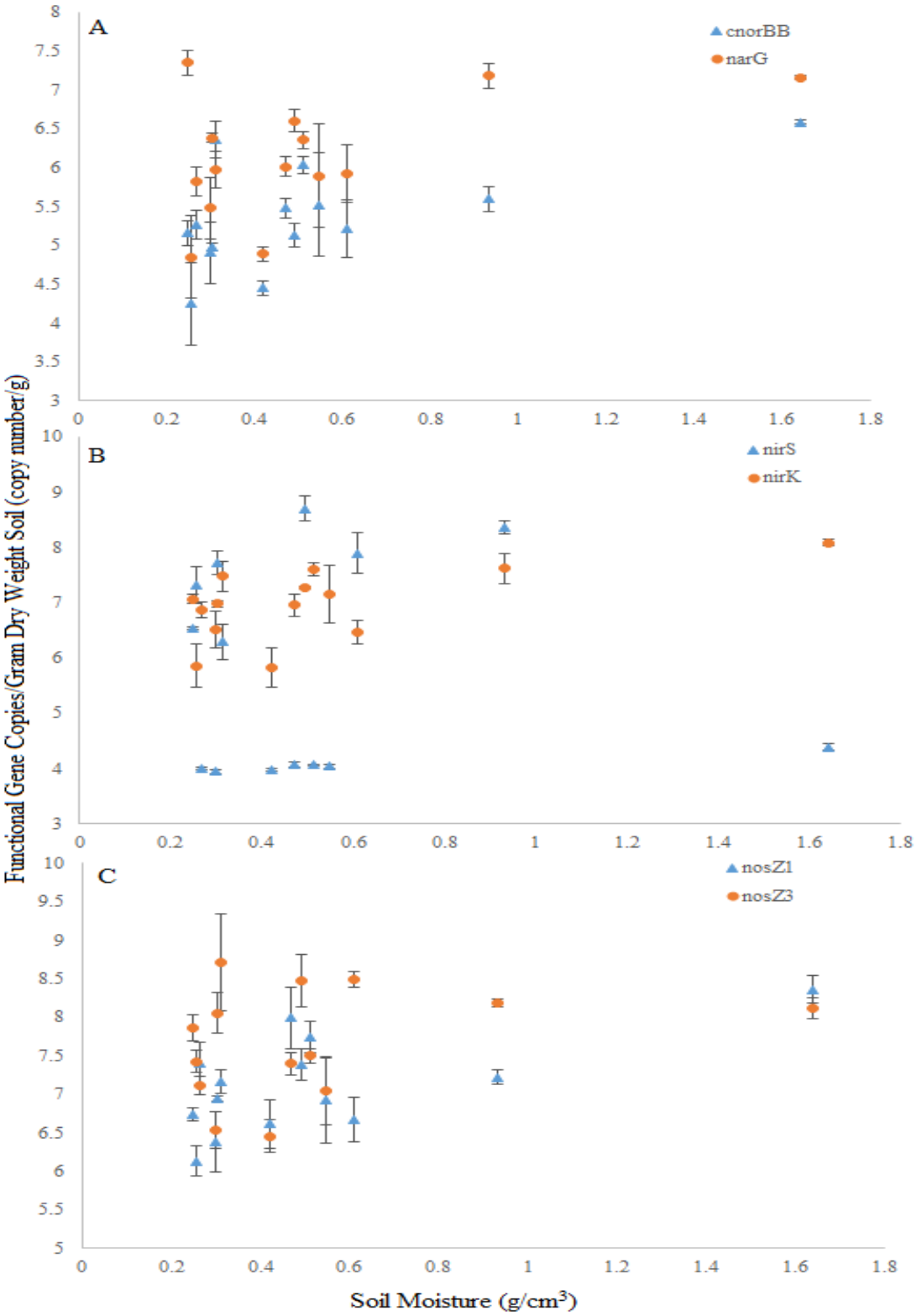


Figure 3.7. Soil moisture vs. functional gene copy numbers per gram dry weight soil in August of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

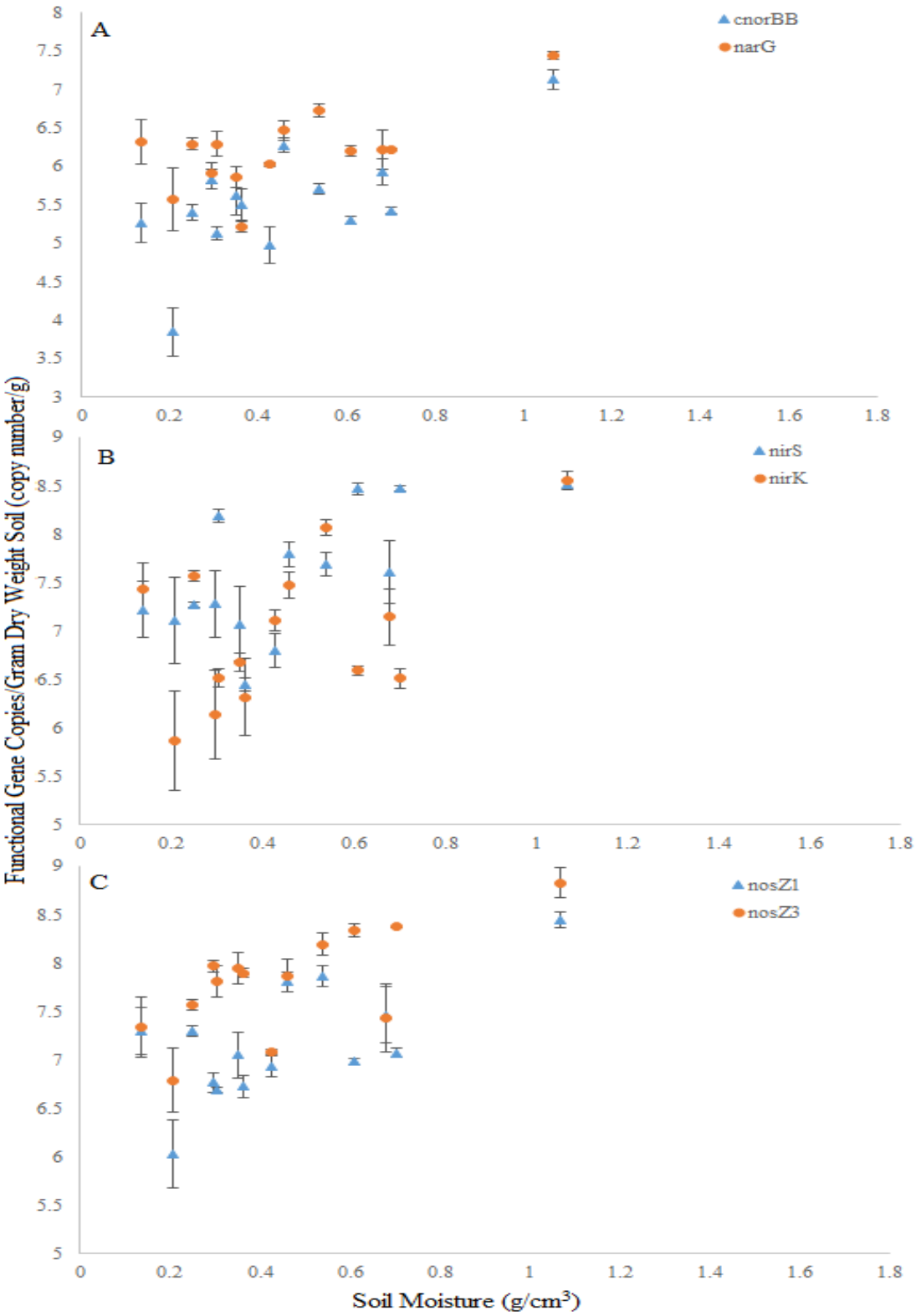


Figure 3.8. Soil moisture vs. functional gene copy numbers per gram dry weight soil in October of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

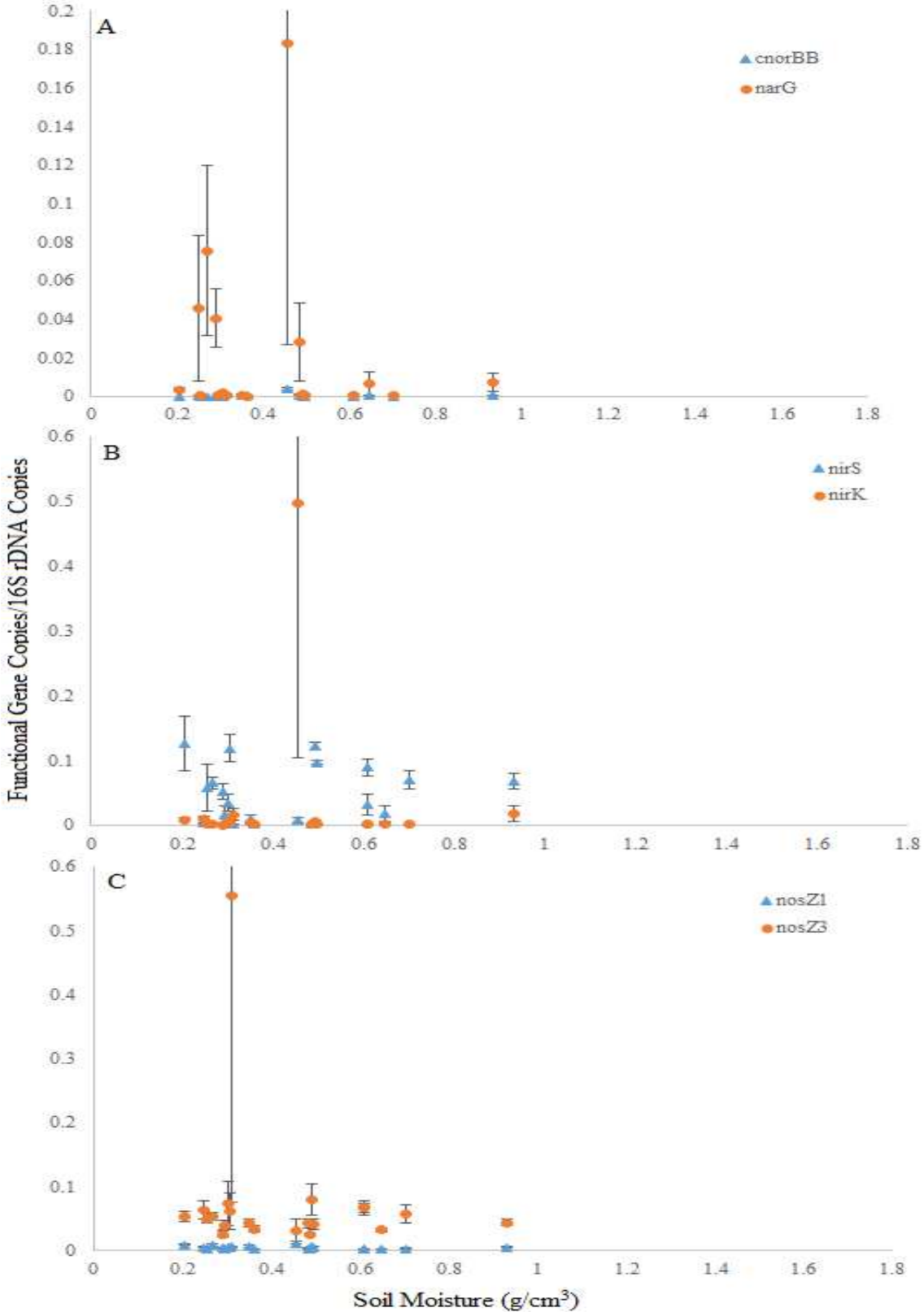


Figure 3.9. Soil moisture vs. 16S rDNA normalized functional gene copy numbers from all months sampled in 2014. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

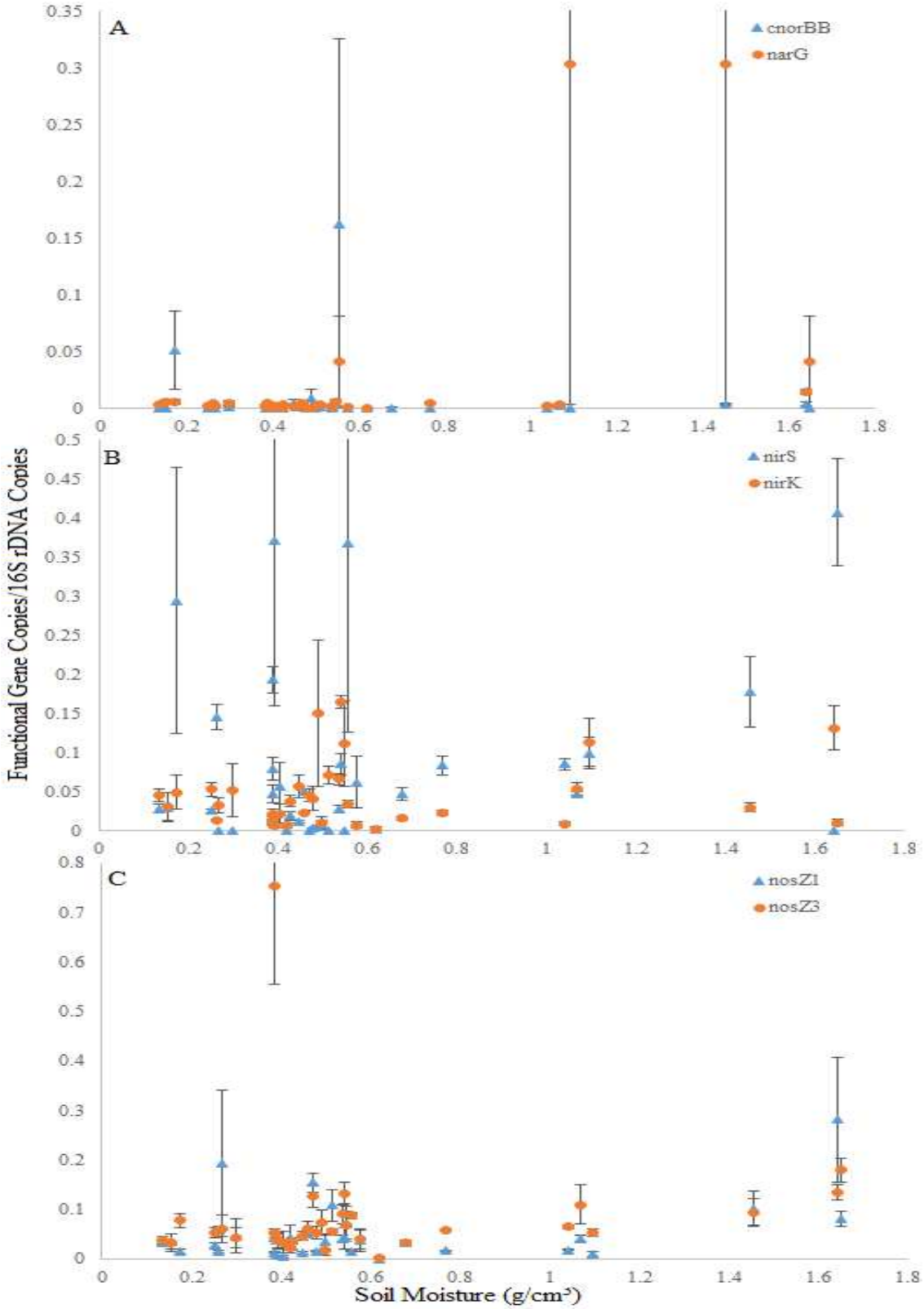


Figure 3.10. Soil moisture vs. 16S rDNA normalized functional gene copy numbers from all months sampled in 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

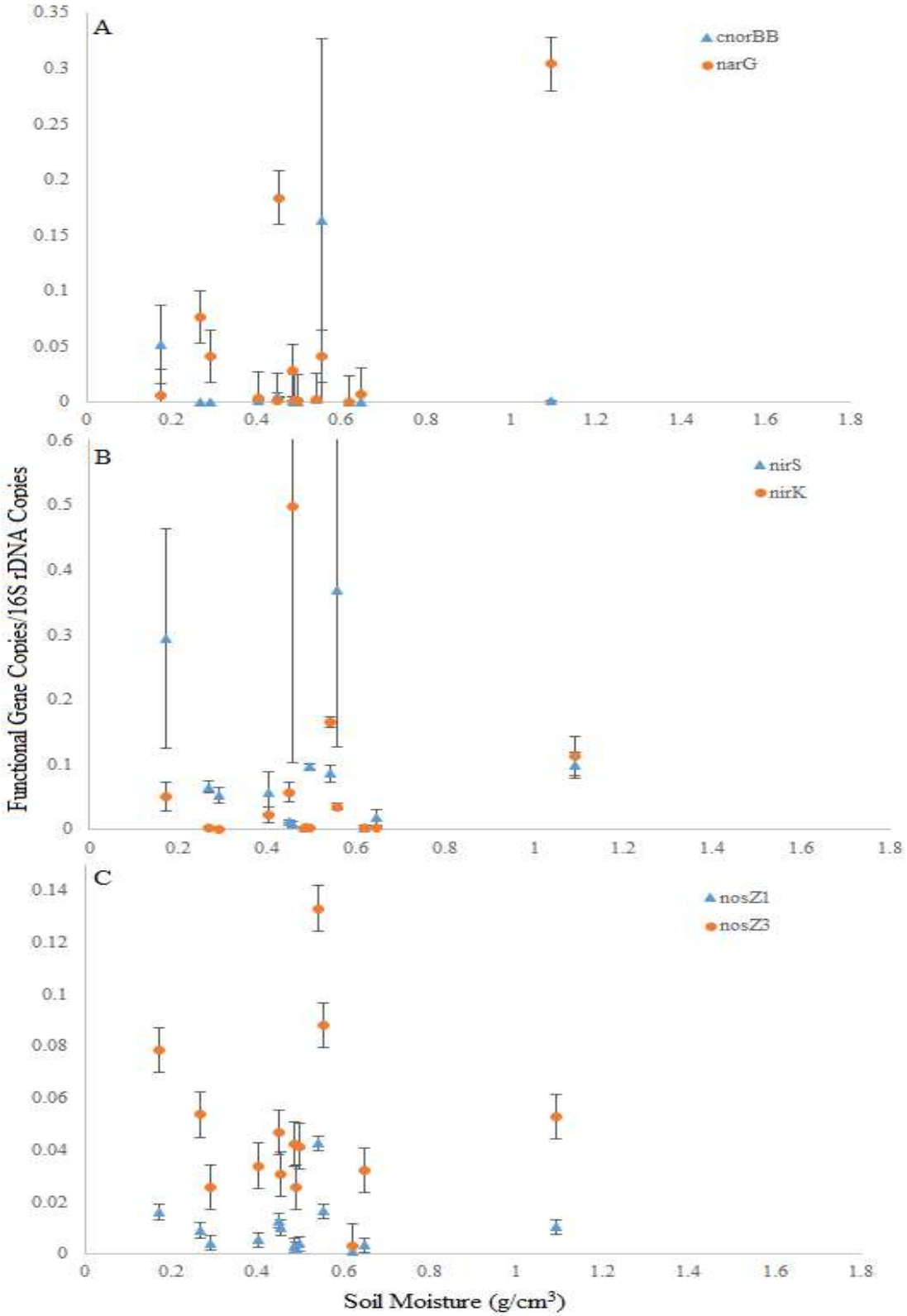


Figure 3.11. Soil moisture vs. 16S rDNA normalized functional gene copy numbers in June of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

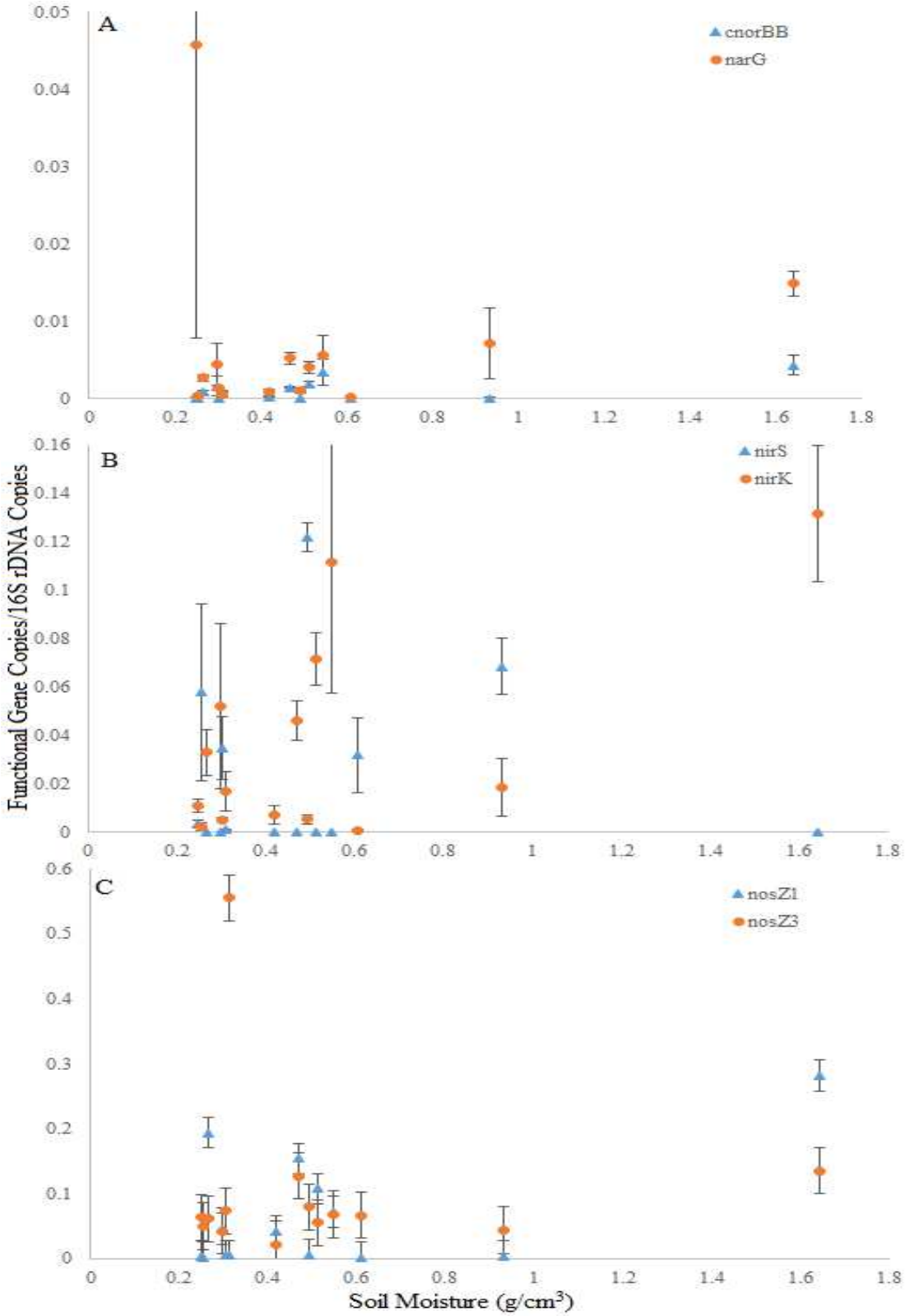


Figure 3.12. Soil moisture vs. 16S rDNA normalized functional gene copy numbers in August of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

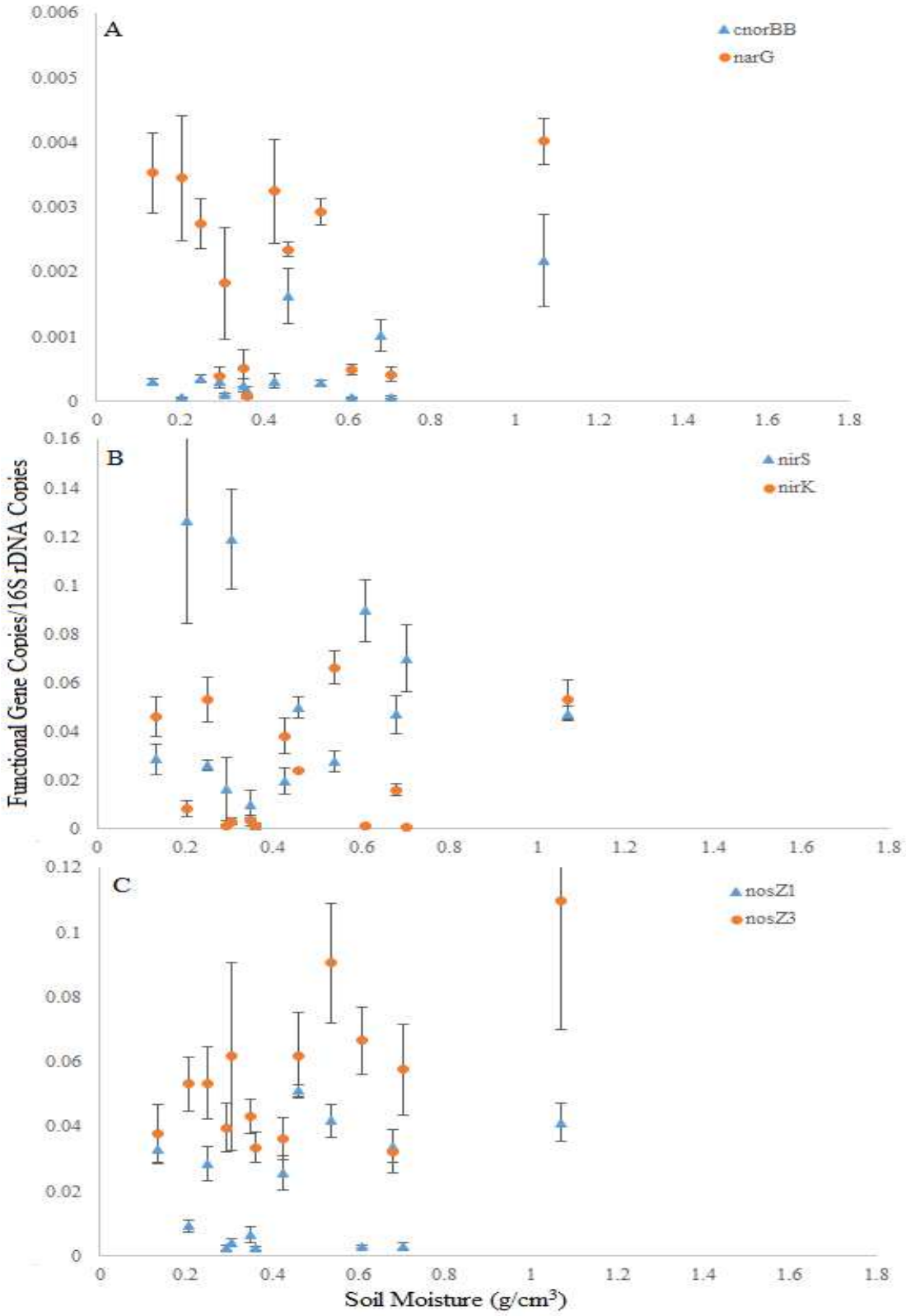


Figure 3.13. Soil moisture vs. 16S rDNA normalized functional gene copy numbers in October of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

2.3.3 Potential Denitrification

Values for potential denitrification varied over a large range, but revealed no significant differences on the monthly time scales for 2014 ($p=0.46$) or 2015 ($p=0.69$). Similarly, on the timescale of years, there was no significant difference ($p=0.67$) between the potential denitrification values from 2014 to 2015 (Table 3.7). Moreover, no significant relationships or correlations could be concluded between potential denitrification values and gene copy numbers, both with respect to dry weight soil (Table 3.8) and 16S rDNA copy numbers (Table 3.9) based upon R^2 values.

Though not a trend, it should be noted that in 2014 the data that compares gene copy number with potential denitrification values is more similar across functional genes (Figure 3.14) than that of the 2015 data (Figure 3.15), as was seen with soil moisture. Also of note, the month of June (2014 and 2015 combined data) had more tightly compacted, less variable data (Figure 3.16), while the month of August, for both years, had a larger spread of values (Figure 3.17), particularly the *nirS* gene.

In the year of 2015 only, there appeared to be a cycling behavior between potential denitrification values and *nirS* when normalized to soil dry weight only (Figure 3.15 B). The figure includes data from all site locations and is not chronological, but does show marked increases and decreases in *nirS* copy numbers per gram soil dry weight as potential denitrification values increase. The extreme fluctuation between high copy numbers and low resulted in a low R^2 value of 0.0294, that there is no correlation between the two.

Table 3.7. Average potential denitrification values (amended) as measured by the acetylene block method.

	Mean Potential Denitrification ($N\text{-}N_2O/M^2/day$)						
	June 2014	**Aug. 2014**	**Oct. 2014**	**May 2015**	**June 2015**	**July 2015**	**Aug. 2015**
SM1-1	575.0	148.23	373.9	441.45	1015.1	539.6	633.1
SM1-2	993.2	633.5	536.1	75.6	192.9	117.9	150.4
SM1-3	588.4	362.1	625.3	515.1	532.7	516.1	502.3
SM2-1	590.9	907.5	567.1	391.5	476.2	1813.7	668.4
SM2-2	1182.4	1011.6	352.1	647.7	428.4	1335.9	1555.8
SM2-3	440.8	306.7	393.7	400.9	568.9	282	448.5
Park	10.5	325.4	59.0	265.2	30.84	31.3	41.5

ANOVA analyses revealed no significant differences on either a monthly or yearly (June and August only) temporal scale.

Table 3.8. R^2 values for potential denitrification and copy number of functional genes per gram dry weight sediment over temporal scales.

	R^2 values			
	2014	**2015**	**June**	**August**
cnorBB	0.29	0.33	0.11	0.41
narG	0.10	0.15	0.013	0.43
nirS	0.07	0.03	0.023	0.015
nirK	0.26	0.22	0.051	0.52
nosZ1	0.24	0.63	0.29	0.62
nosZ3	0.17	0.39	0.26	0.25

Table 3.9. R^2 values for potential denitrification and copy number of functional genes per copy number 16S rDNA over temporal scales.

	R^2 values			
	2014	**2015**	**June**	**August**
cnorBB	0.044	0.0047	0.036	0.19
narG	0.0043	0.087	0.056	0.0052
nirS	0.026	0.042	0.13	0.056
nirK	0.0036	0.025	0.018	0.20
nosZ1	0.17	0.30	0.045	0.33
nosZ3	0.025	0.0001	0.047	0.0004

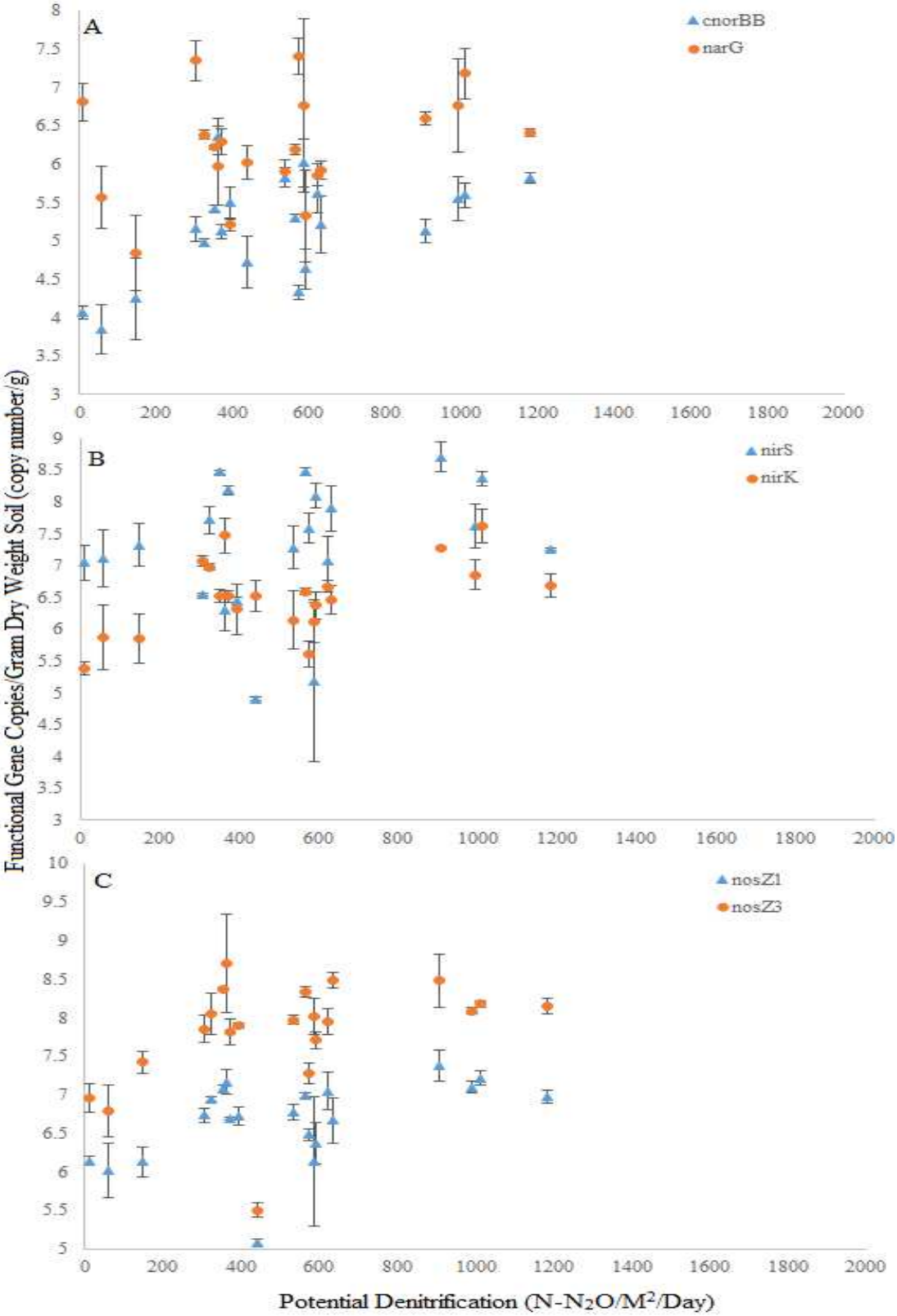


Figure 3.14. Potential denitrification vs. functional gene copy numbers per gram dry weight soil across all months sampled in 2014. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

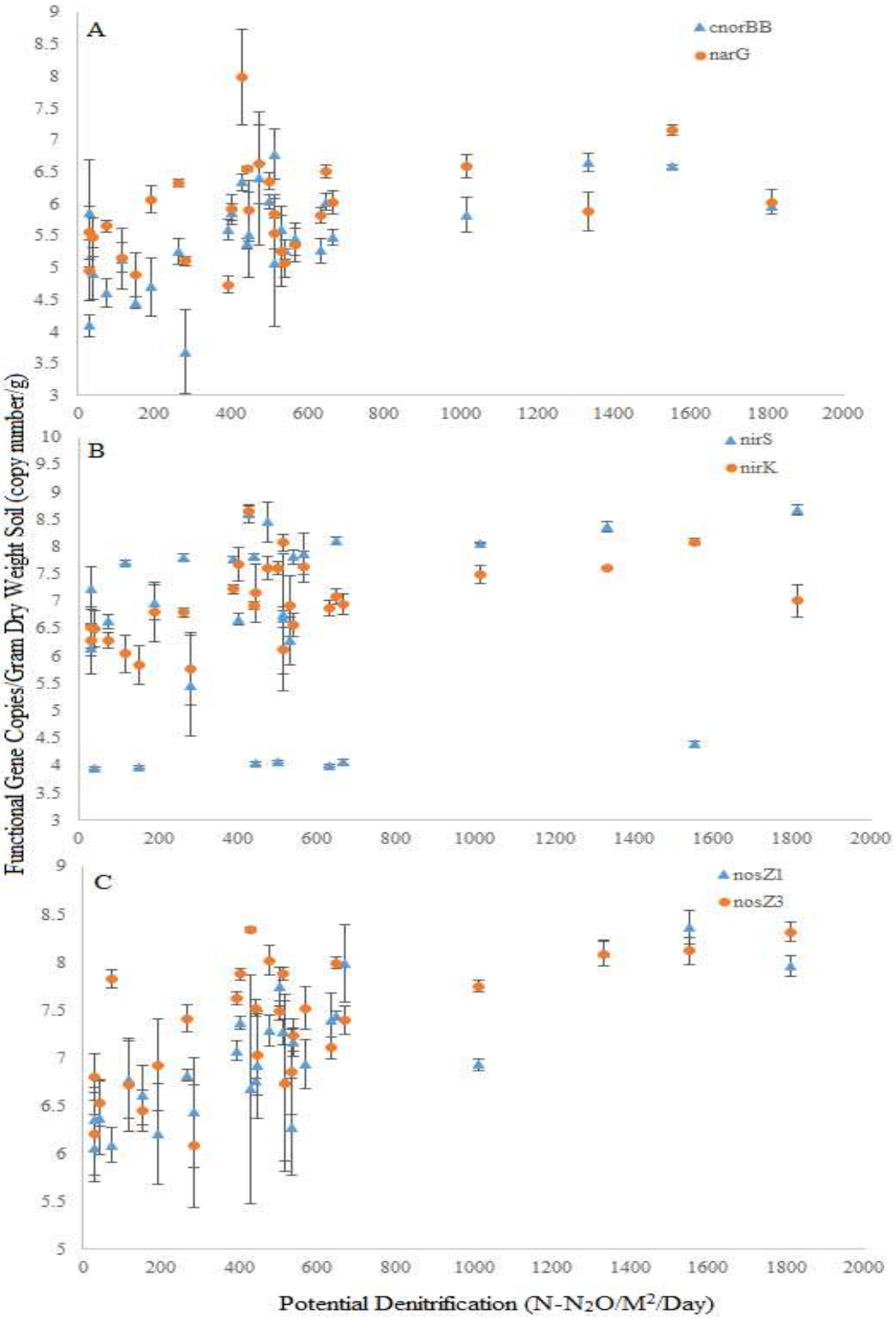


Figure 3.15. Potential denitrification vs. functional gene copy numbers per gram dry weight soil across all months sampled in 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

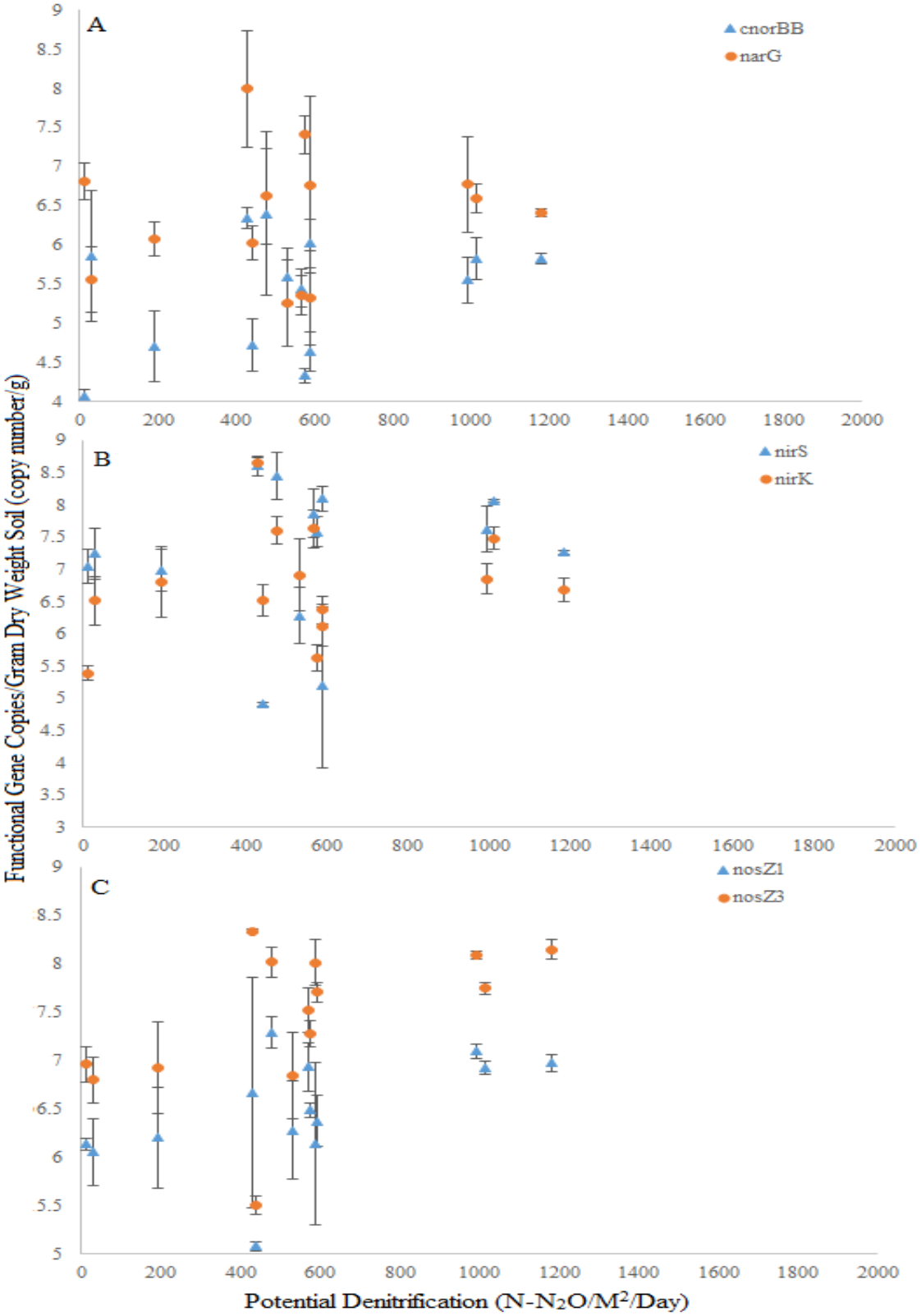


Figure 3.16. Potential denitrification vs. functional gene copy numbers per gram dry weight soil in June of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

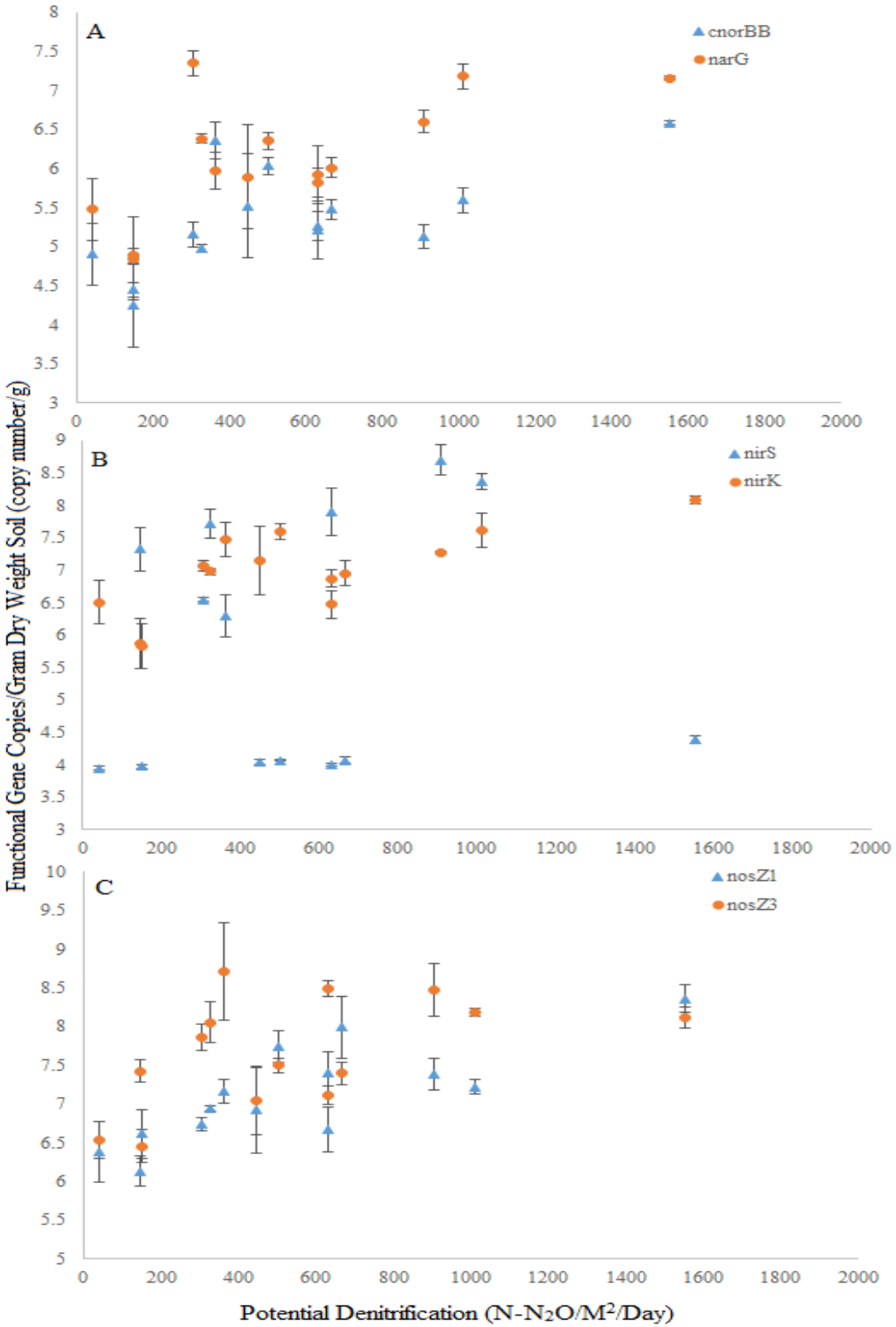


Figure 3.17. Potential denitrification vs. functional gene copy numbers per gram dry weight soil in August of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

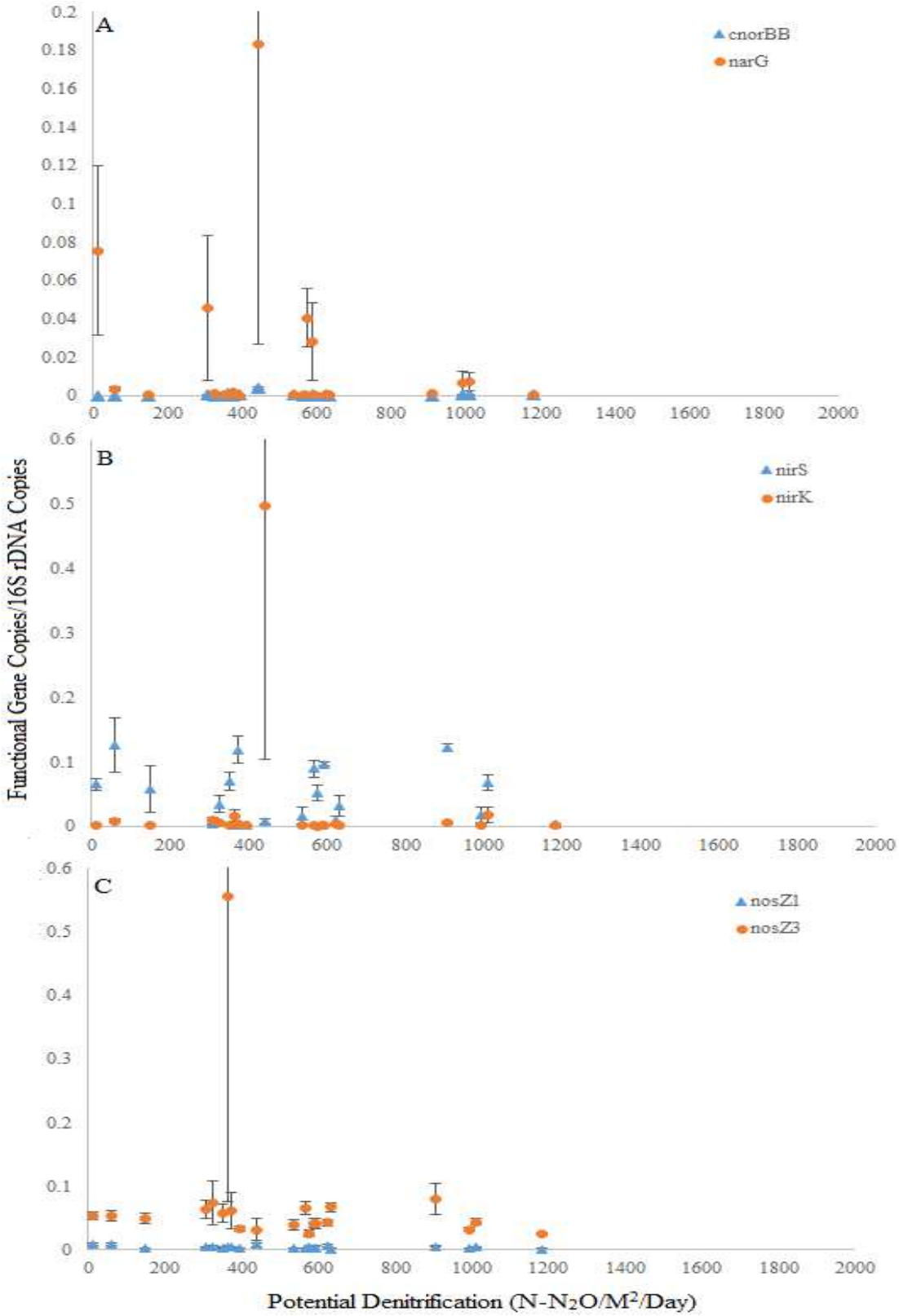


Figure 3.18. Potential denitrification vs. 16S rDNA normalized gene copy numbers across all months sampled in 2014. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

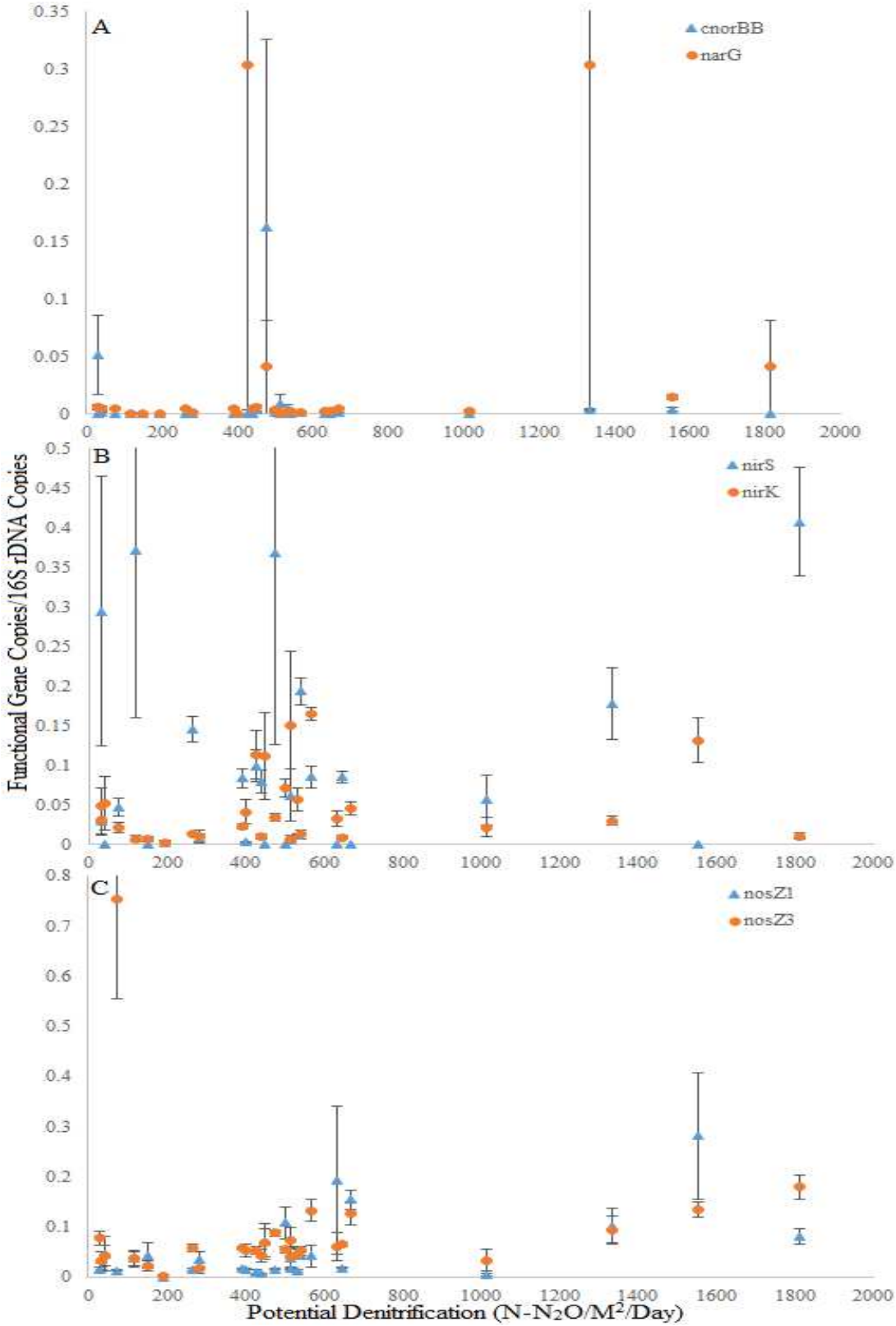


Figure 3.19. Potential denitrification vs. 16S rDNA normalized gene copy numbers across all months sampled in 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

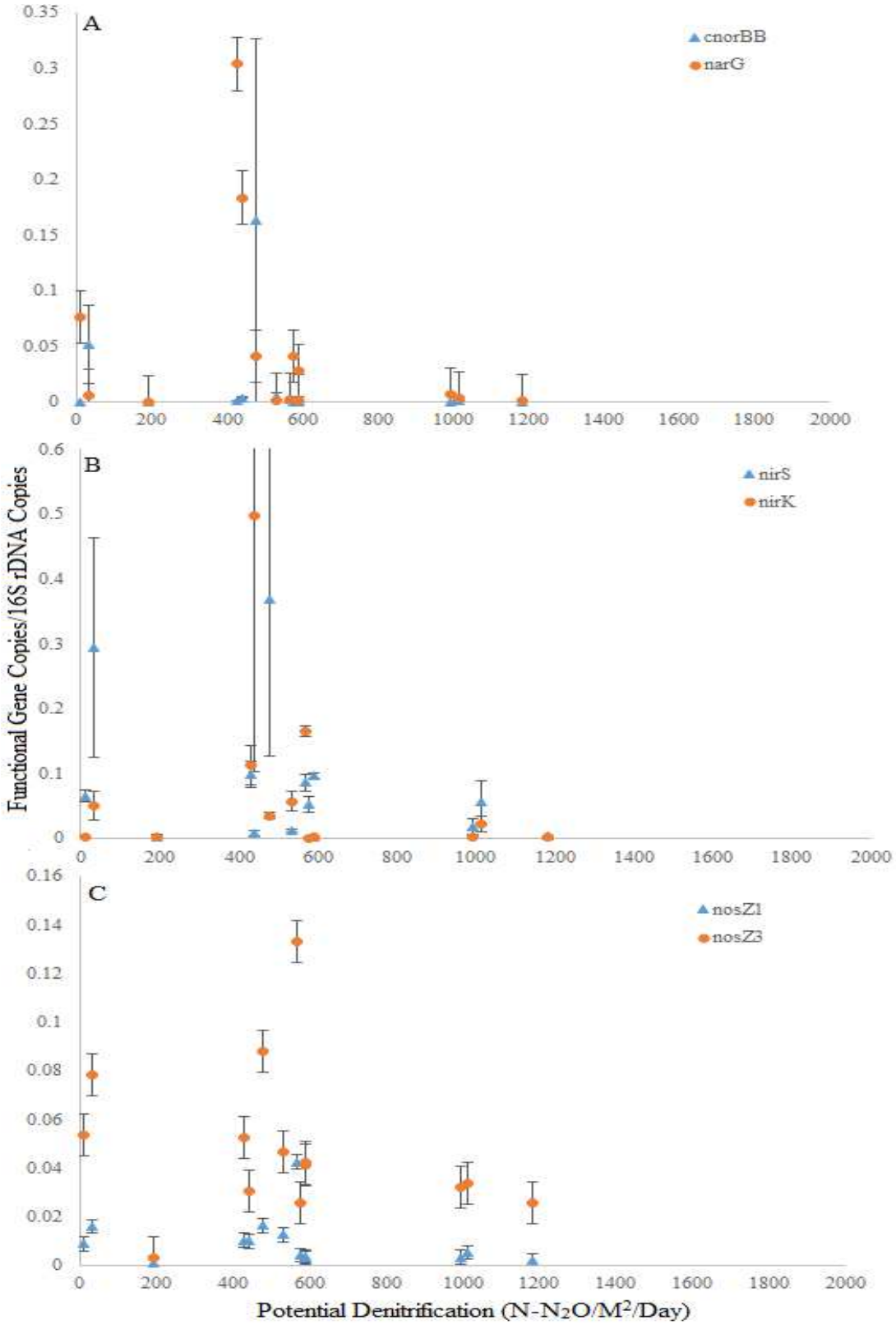


Figure 3.20. Potential denitrification vs. 16S rDNA normalized gene copy numbers in June of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

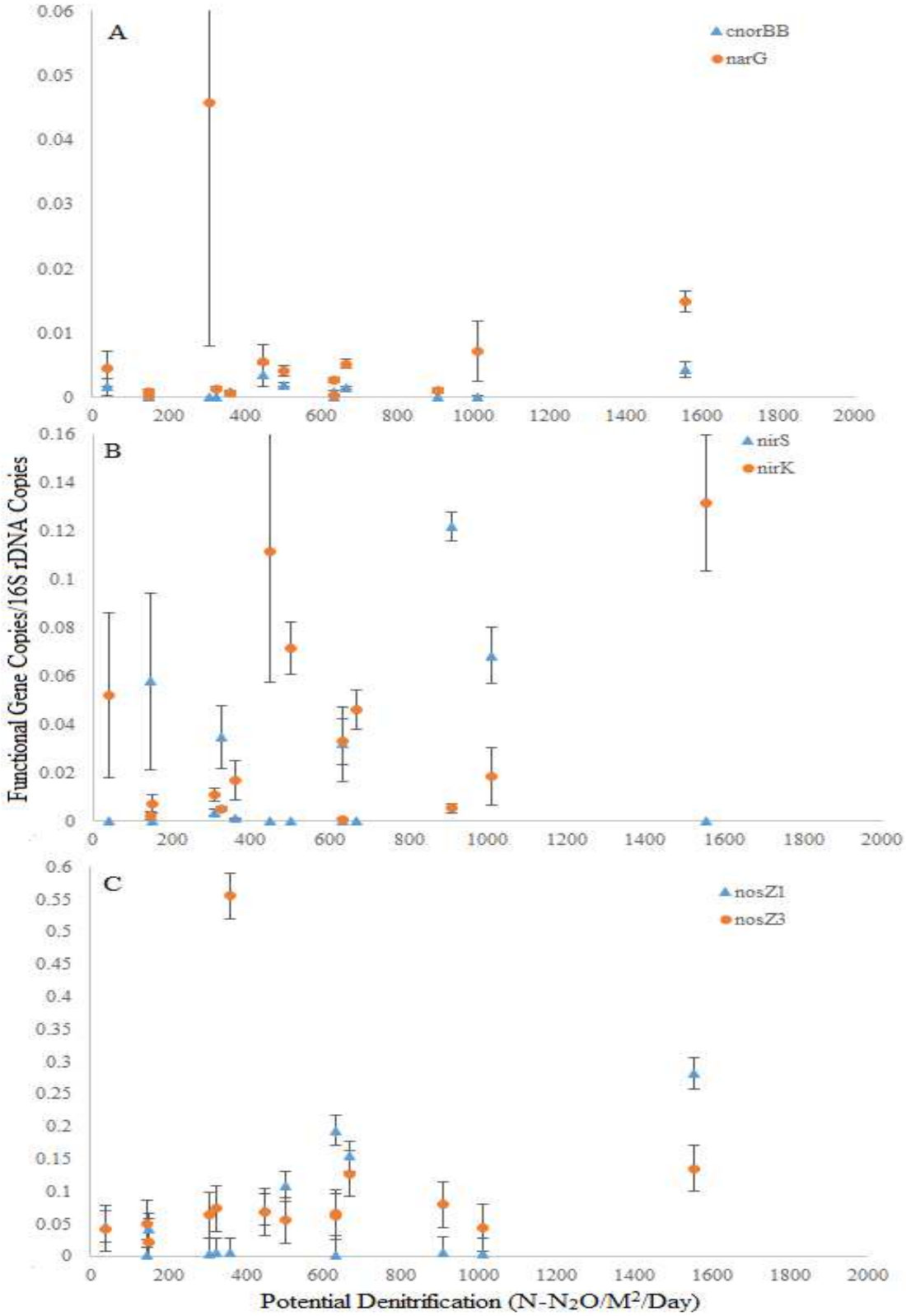


Figure 3.21. Potential denitrification vs. 16S rDNA normalized gene copy numbers in August of both 2014 and 2015. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

2.4 Results of Spatial Studies

2.4.1 Genetic Analyses

Spatial analyses were done with respect to intra and inter-site scales. Intra-site analyses involved examining the differences between channel locations (in channel, flooding zone, and non-flooding zone), while inter-site compared the three channel locations independent of one another, but between sites. Statistical analyses were done using ANOVA with a p-value < 0.05 needed to qualify as statistically significant. Analyses were done using the log scale of copy number of functional gene per gram dry weight sediment or soil, as well as to normalization to 16S rDNA copy number.

Results of channel position analyses were variable across the different genes (Table 4.1), with some showing no difference regardless of channel location, such as *narG* and *nosZ1*, for either metric of measurement. Differences in gene copy numbers normalized to either 16S rDNA or per gram dry weight sediment/soil, across different channel positions, were statistically significant (p=0.0002, $p=2.1x10^{-5}$, respectively) differences for *nirS*. However, *nirK*, *cnorBB*, and *nosZ3* were significantly different with respect to only one measurement, that of *nirK* and *cnorBB* being 16S rDNA normalization, and that of *nosZ* per gram dry weight soil/sediment.

Comparing individual genes at the same channel locations across sites differed results, depending upon which normalization method, either 16S rDNA or per gram dry weight sediment/soil, was considered in the analysis (Table 4.2). There were no statistically significant differences (p-values: *cnorBB*=0.31, *narG*=0.058, *nirS*=0.14, *nirK*=0.06, *nosZ1*=0.24, *nosZ3*=0.41) between sites by channel location with respect to 16S rDNA normalization for any of the functional genes analyzed. In contrast, copy numbers per gram dry weight sediment for all functional genes showed significant differences (p-values listed in Table 4.2), except *nirS* (p=0.08).

Discriminant analyses were done using averaged triplicate values for functional gene copy numbers that were normalized to 16S rDNA to give relative abundances by channel sampling location for sites SM1 (Figure 4.1), SM2 (Figure 4.2), as well as combined SM1, SM2, and Park site values (Figure 4.3). At SM1, the in-channel, flooding zone, and non-flooding zone profiles were distinctly different from one another, although the flooding zone and in-channel profiles were more similar to each other than either was

to the non-flooding zone (Figure 4.1). Site SM2 showed equally dissimilar profiles for each of the sampling locations, with each grouping approximately as distant from one another as the others (Figure 4.2). When considering all three sites together, the profiles of each sampling location were more similar to that of SM2, with each equally distant from one another, but with a dampened spread such that there is less difference between all three than that of SM2 (Figure 4.3).

Table 4.1. ANOVA analyses of gene copy number differences between bank location (in-channel, flooding zone, non-flooding zone) at the same date of sampling.

	Normalization Method	
	16S rDNA	**Per gram dry weight soil/sediment**
cnorBB	F = 6.20, p = 0.002	-
narG	-	-
nirS	F = 8.63, p = 0.0002	F = 11.62, p = 2.1×10^{-5}
nirK	F = 3.38, p = 0.03	-
nosZ1	-	-
nosZ3	-	F = 5.33, p = 0.01

F values were compared against an F-crit value of 3.06. Park data is excluded as no comparison is possible. Analyses were done with log scale data. Dashes represent a lack of statistically significant ($p<0.05$) results.

Table 4.2. ANOVA analyses of gene copy number differences compared by bank location for sites SM1 and SM2.

	Normalization Method	
	16S rDNA	**Per gram dry weight soil/sediment**
cnorBB	-	F = 5.28, p = 0.02
narG	-	F = 6.77, p = 0.01
nirS	-	-
nirK	-	F = 13.56, p = 0.0003
nosZ1	-	F = 7.87, p = 0.005
nosZ3	-	F = 5.77, p = 0.03

Park data is excluded. F values were compared against an F-crit of 3.90. Dashes represent a lack of statistically significant ($p<0.05$) results.

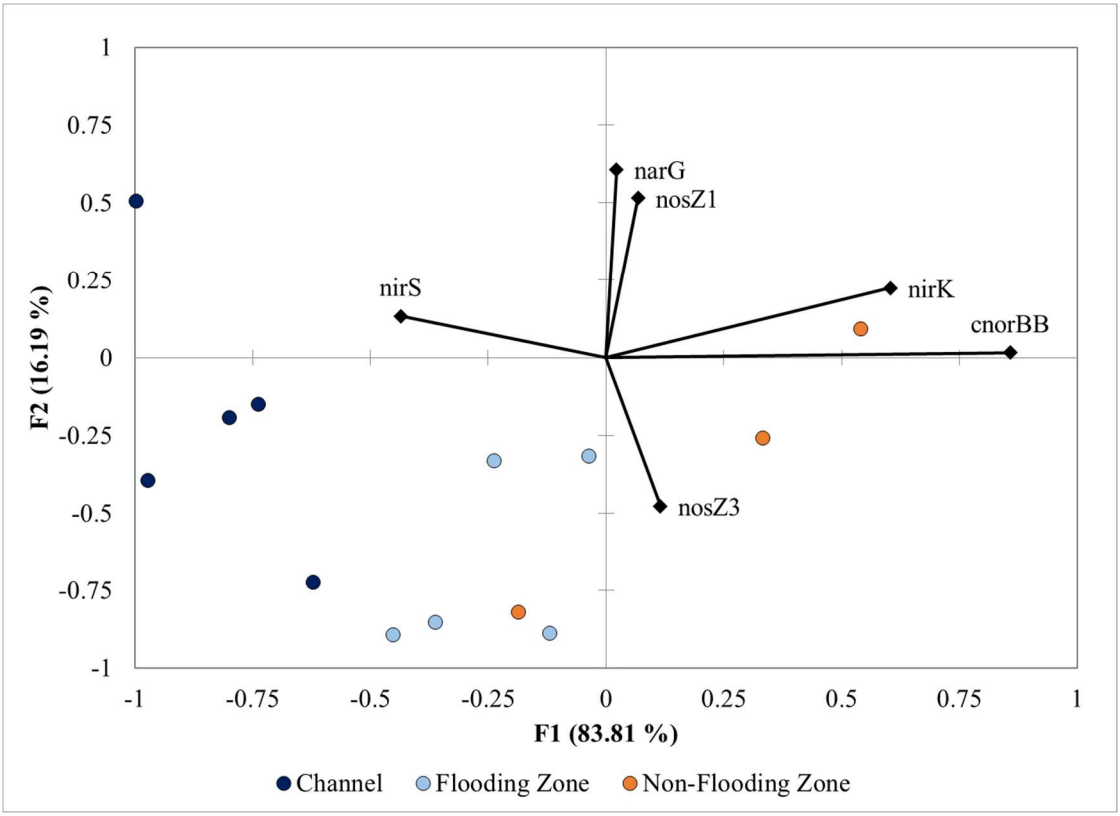


Figure 4.1. Discriminant analysis of the functional gene relative abundances normalized to 16S rDNA copy number by bank location at SM1.

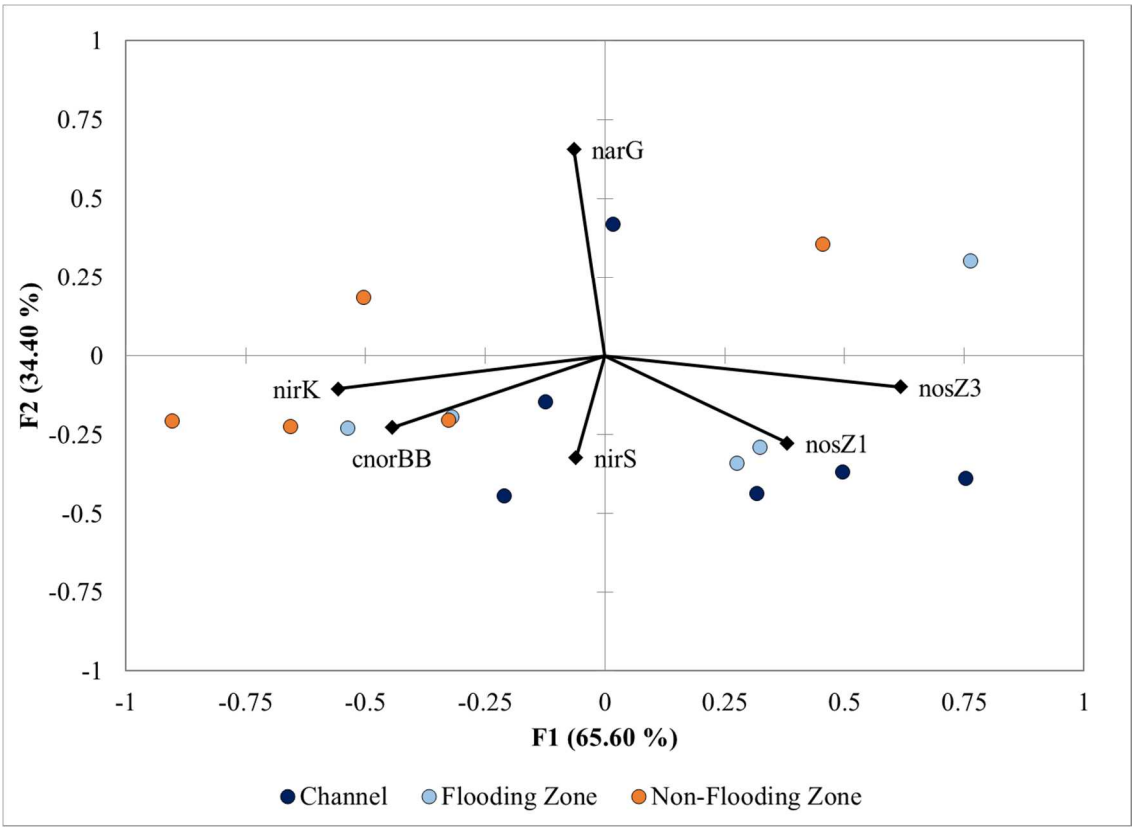


Figure 4.2. Discriminant analysis of the functional gene relative abundances normalized to 16S rDNA copy number by bank location at SM2.

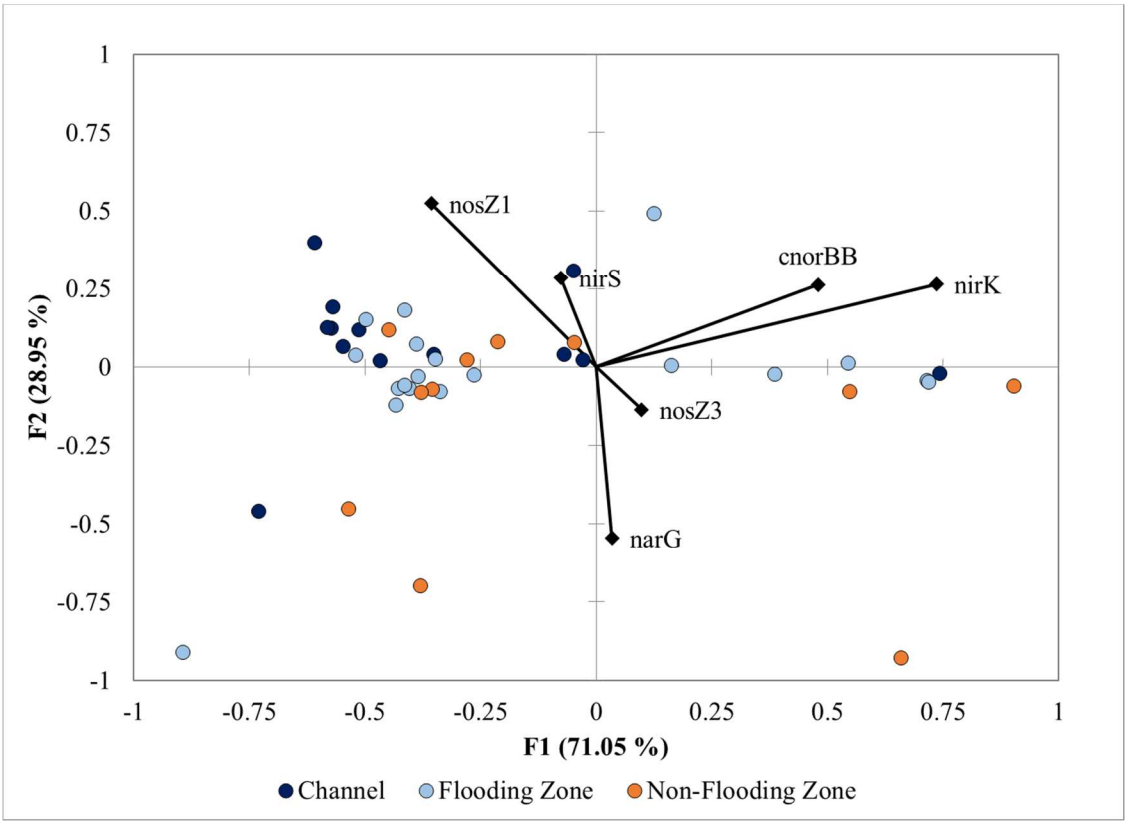


Figure 4.3. Discriminant analysis of the functional gene relative abundances normalized to 16S rDNA copy number by bank location at SM1, SM2, and the Park site. Park site values included with flooding zone values.

2.4.2 Soil Moisture

Gravimetric soil water content measurements done to determine soil moisture content revealed significant differences over both the inter-site ($p=0.0003$) and intra-site ($p=0.01$) scales. Significance for the inter-site, which is comparison between sites SM1 and SM2, is reported with an F statistic value of 15.281 against an F-crit of 5.15. Channel, flooding zone, and non-flooding zone, designated intra-site, is reported with an F statistic value of 5.36 against an F-crit value of 3.20.

Despite these spatial differences in moisture content across both scales, R^2 values (Tables 4.3 and 4.4) indicate that soil moisture does not significantly correlate with denitrification functional gene copy number, normalized to dry weight soil or 16S rDNA copy number. Exception to this conclusion can be seen in the flooding zone, however, where the R^2 values for both normalizations indicate that soil moisture values correlate with some significance to functional gene copy numbers, except for that of *nirS*. Generally, copy numbers over both metrics remained relatively stable over a range of soil moisture values, though there were increases in copy number with respect to 16S rDNA copy number (relative abundance) that did not appear to relate to changes in soil moisture (Figures 4.9 through 4.13). All observed functional genes showed what would appear to be random increases, as represented by outliers on the graphs, independent of soil moisture, and so no conclusion could be drawn between a specific gene and increases in abundance or relative abundance.

Soil moisture remained relatively constant at site SM1, while it was much more variable across SM2. The spike in moisture content occurred almost exclusively in samples taken from the flooding zone of SM2 (Figures 4.5, 4.10, 4.7, and 4.12), with one single outlying moisture reading occurring within the channel at SM2 (Figures 4.6 and 4.11). In contrast, the flooding zone at SM1 remained more similar to the in channel and non-flooding zone locations (Figures 4.4 and 4.9) such that there is little difference across the three locations.

Table 4.3. R^2 values for soil moisture and copy number of functional genes per gram dry weight sediment over spatial scales.

	R^2 values				
	SM1	**SM2**	**In Channel**	**Flooding Zone**	**Non-Flooding Zone**
cnorBB	0.024	0.37	0.22	0.59	0.0002
narG	0.012	0.084	0.0085	0.37	0.067
nirS	0.0002	0.047	0.10	0.0062	0.0002
nirK	0.018	0.20	0.042	0.63	0.0005
nosZ1	0.0005	0.33	0.24	0.62	0.021
nosZ3	$1x10^{-5}$	0.14	0.29	0.23	0.14

Table 4.4. R^2 values for soil moisture and copy number of functional genes per copy number 16S rDNA genes over spatial scales.

	R^2 values				
	SM1	**SM2**	**In Channel**	**Flooding Zone**	**Non-Flooding Zone**
cnorBB	0.072	0.13	0.0016	0.43	0.037
narG	0.012	0.007	0.24	0.59	0.011
nirS	0.010	0.056	0.48	0.057	0.24
nirK	0.038	0.0017	0.0057	0.48	0.013
nosZ1	0.011	0.32	0.011	0.48	0.26
nosZ3	$6x10^{-5}$	0.32	0.62	0.0087	0.14

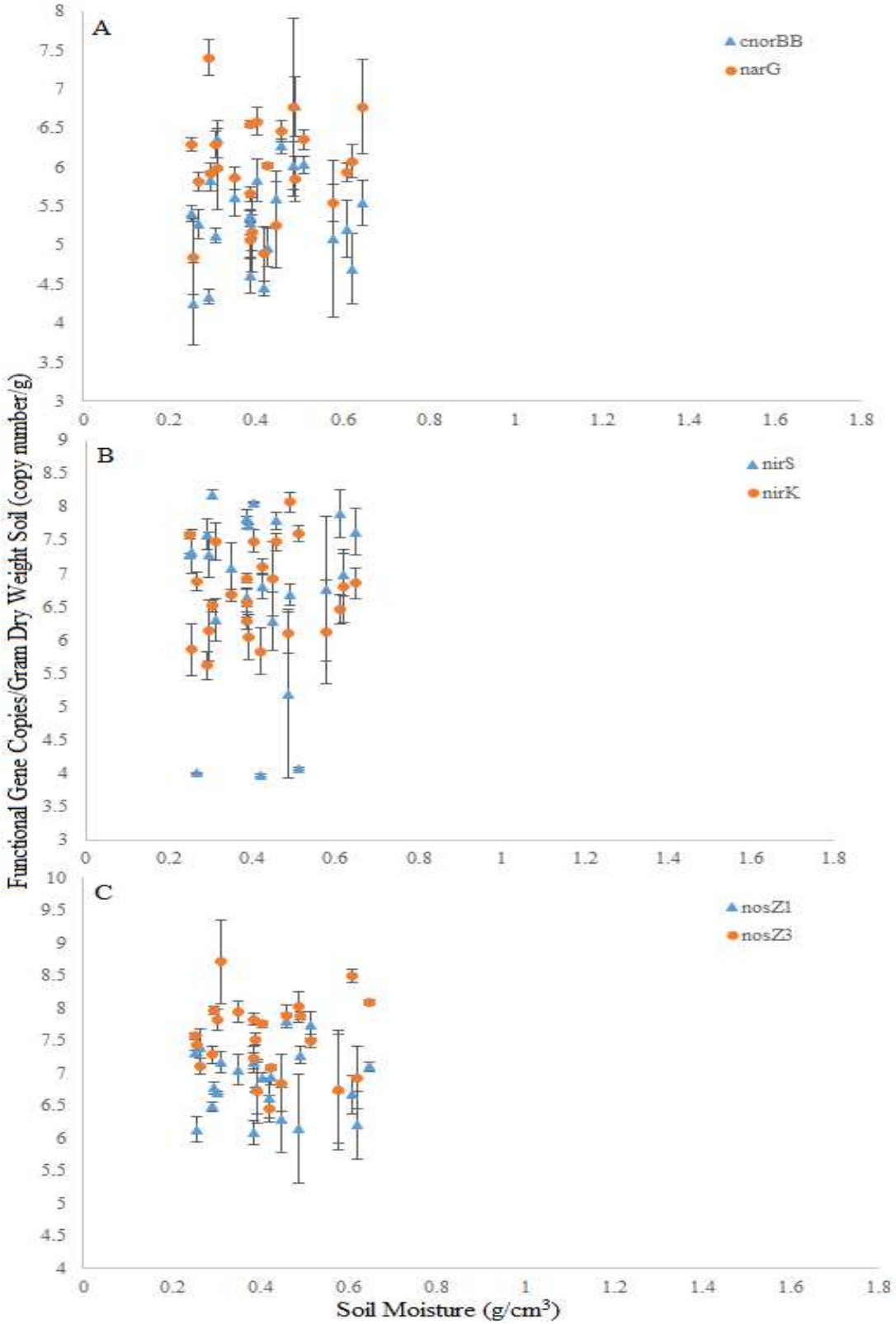


Figure 4.4. Copies of each functional gene per gram dry weight sediment/soil and soil moisture across all channel positions at Seven Mile Creek site 1 (SM1). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

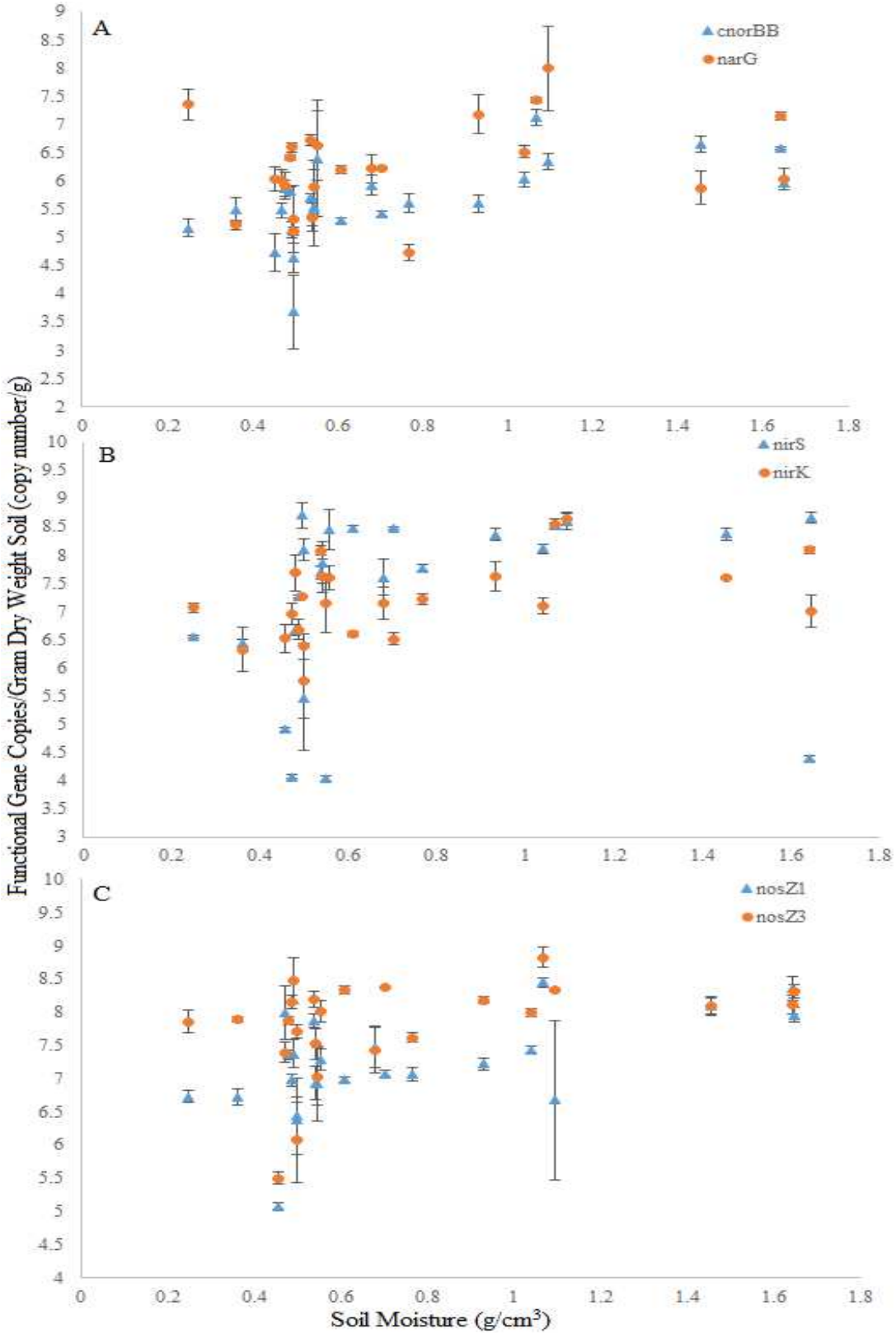


Figure 4.5. Copies of each functional gene per gram dry weight sediment/soil and soil moisture across all channel positions at Seven Mile Creek site 2 (SM2). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

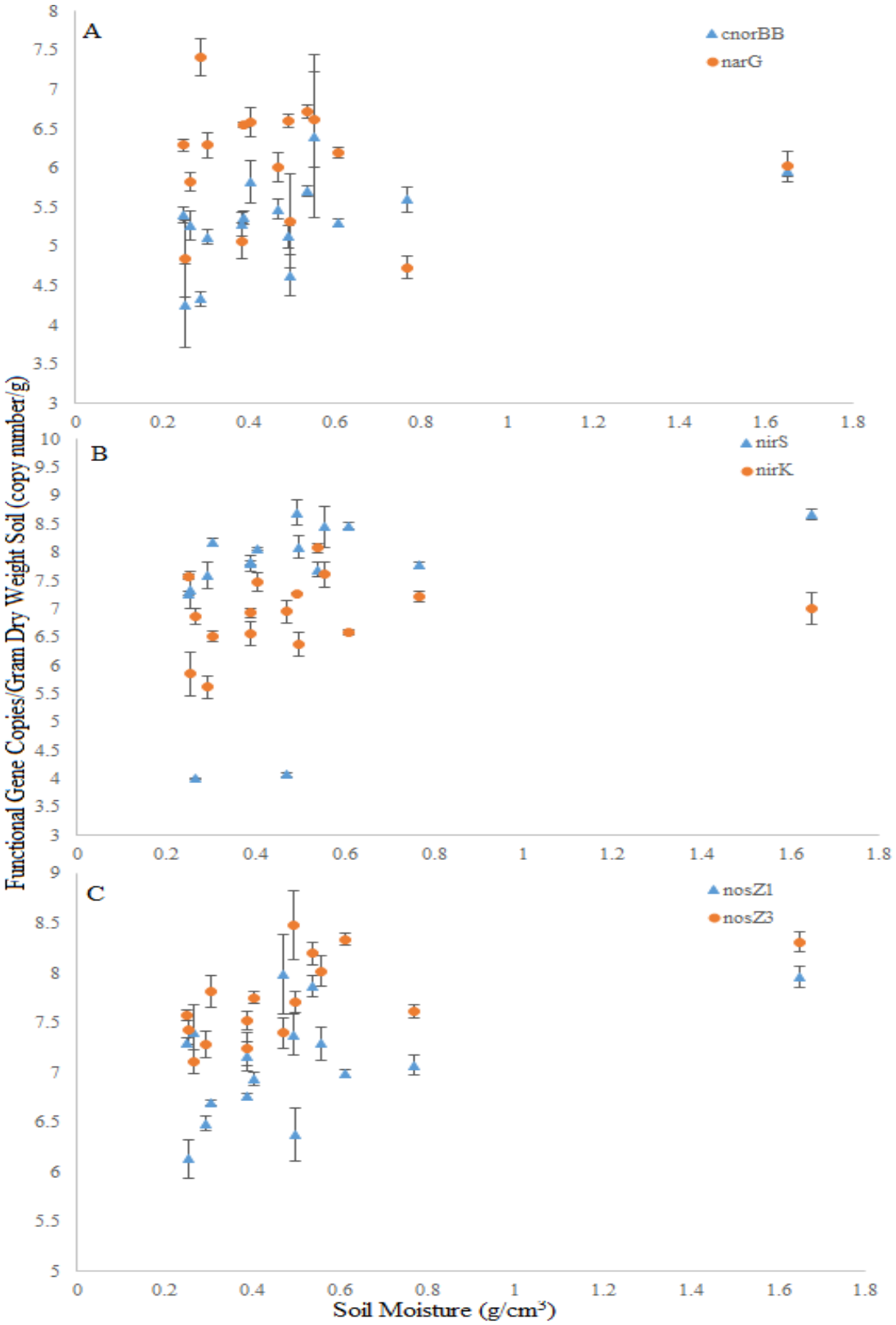


Figure 4.6. Copies of each functional gene per gram dry weight sediment and soil moisture for both SM1 and SM2 of samples taken in stream channel. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

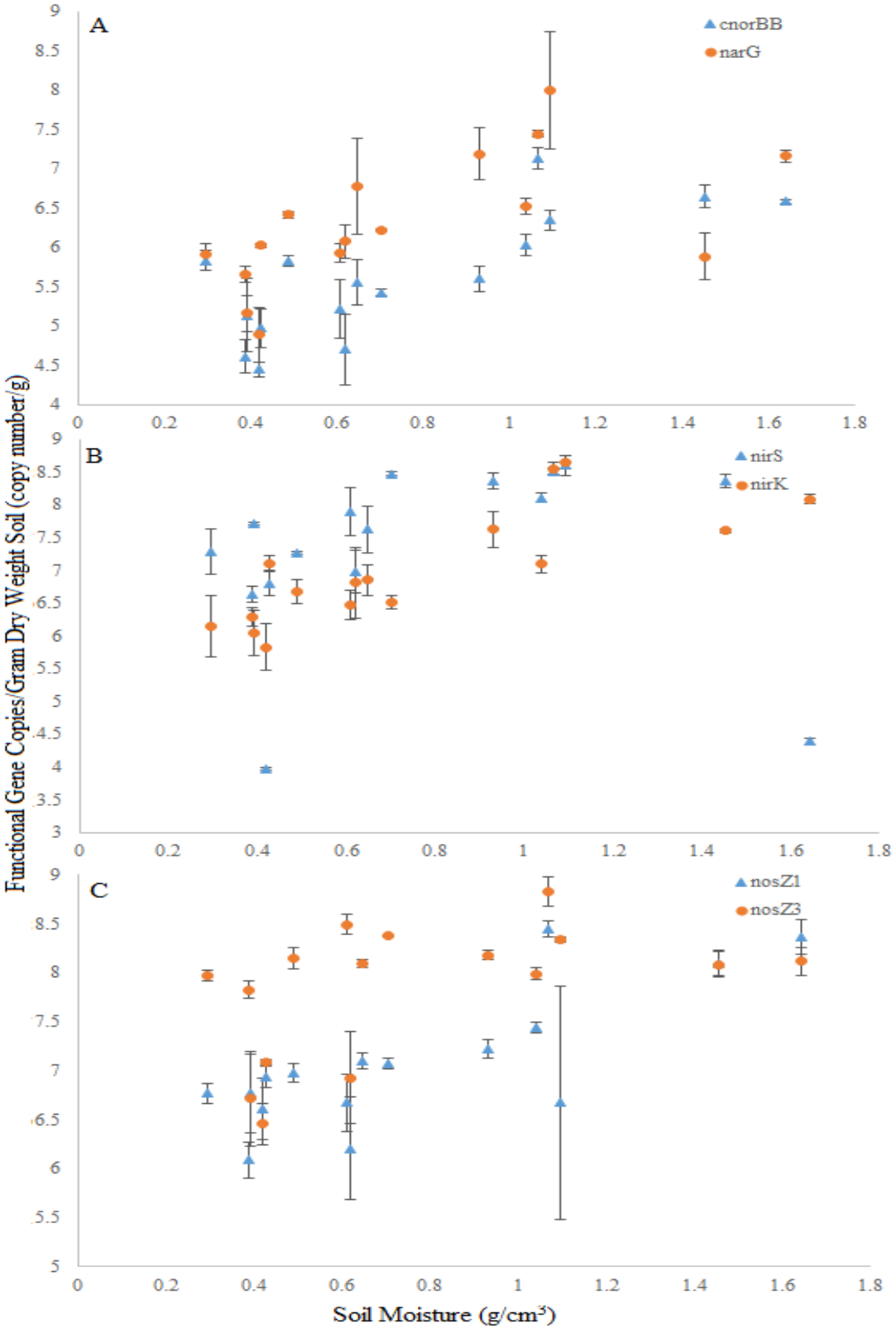


Figure 4.7. Copies of each functional gene per gram dry weight sediment and soil moisture for both SM1 and SM2 of samples taken from the flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

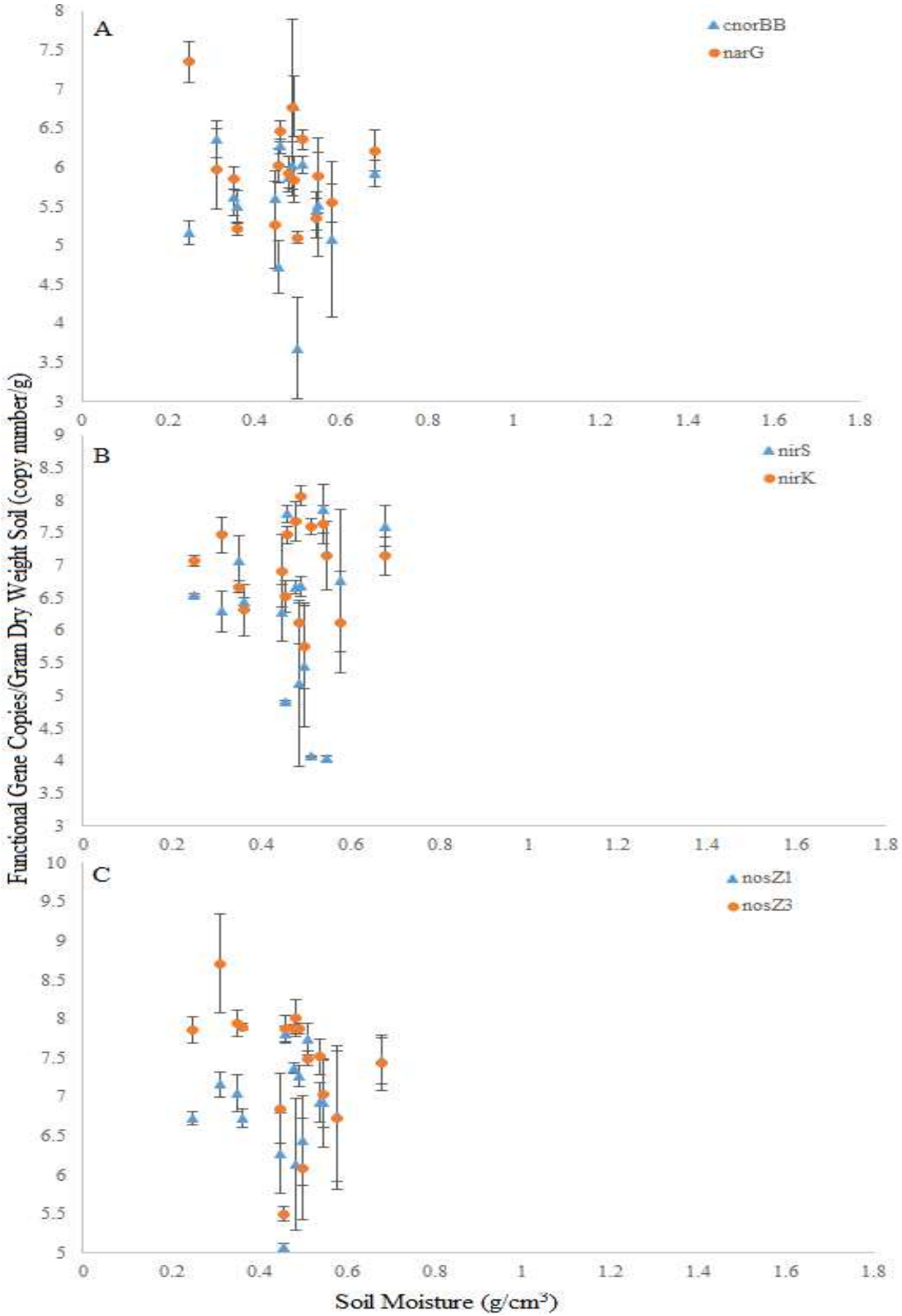


Figure 4.8. Copies of each functional gene per gram dry weight sediment and soil moisture for both SM1 and SM2 of samples taken from the non-flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

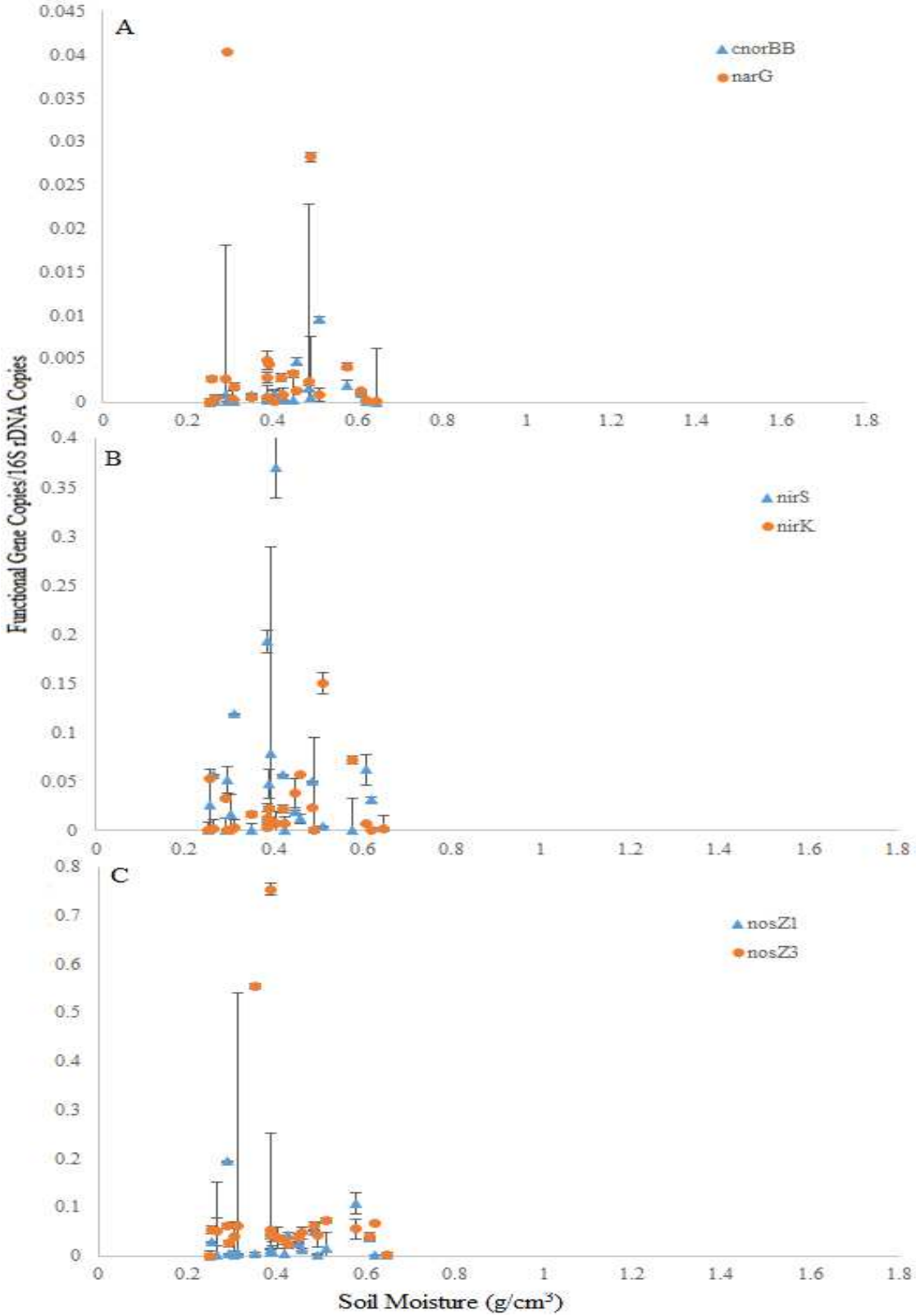


Figure 4.9. Copy number of each functional gene normalized to 16S rDNA copy number and soil moisture across all channel positions at Seven Mile Creek site 1 (SM1). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

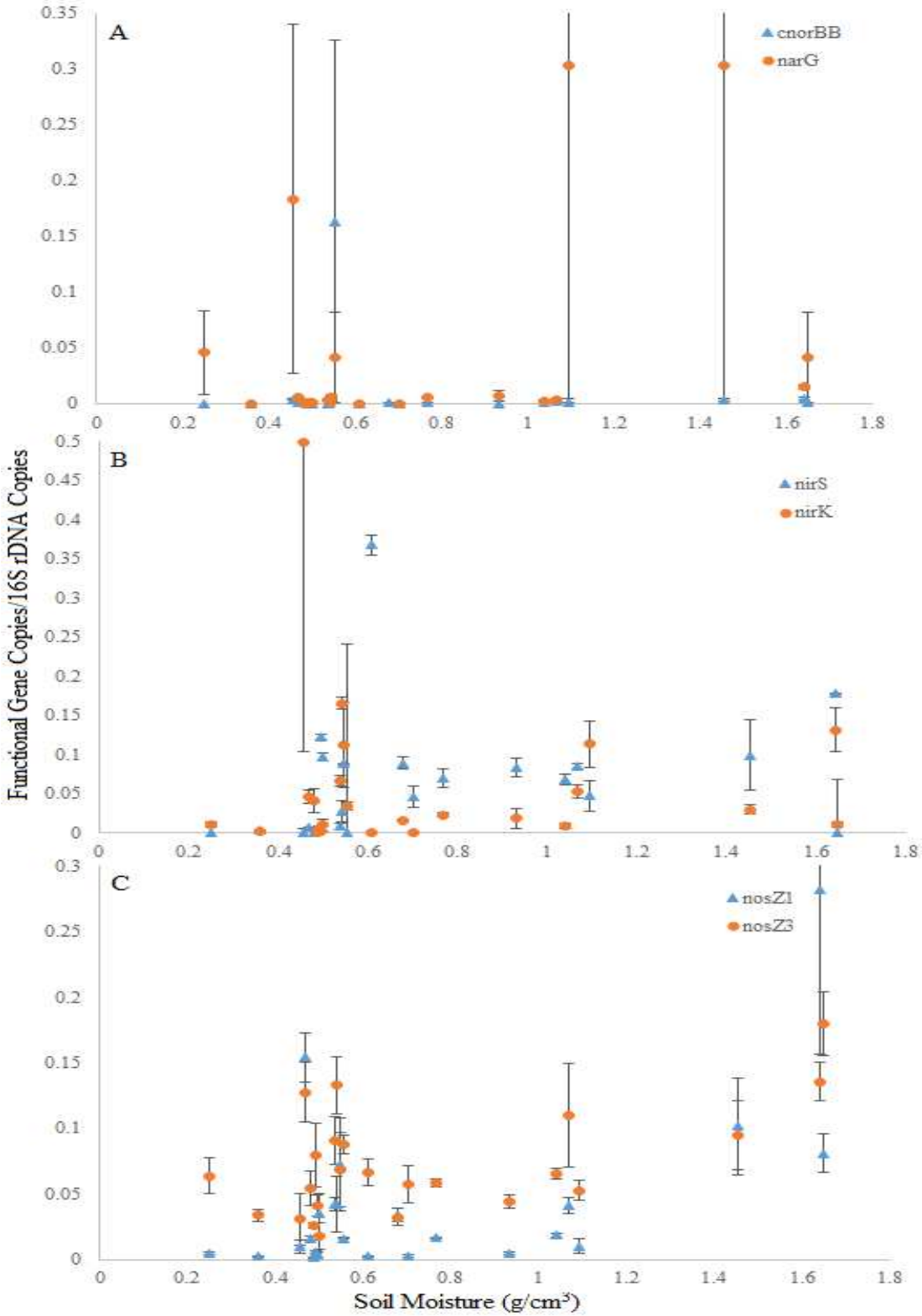


Figure 4.10. Copy number of each functional gene normalized to 16S rDNA copy number and soil moisture across all channel positions at Seven Mile Creek site 2 (SM2). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

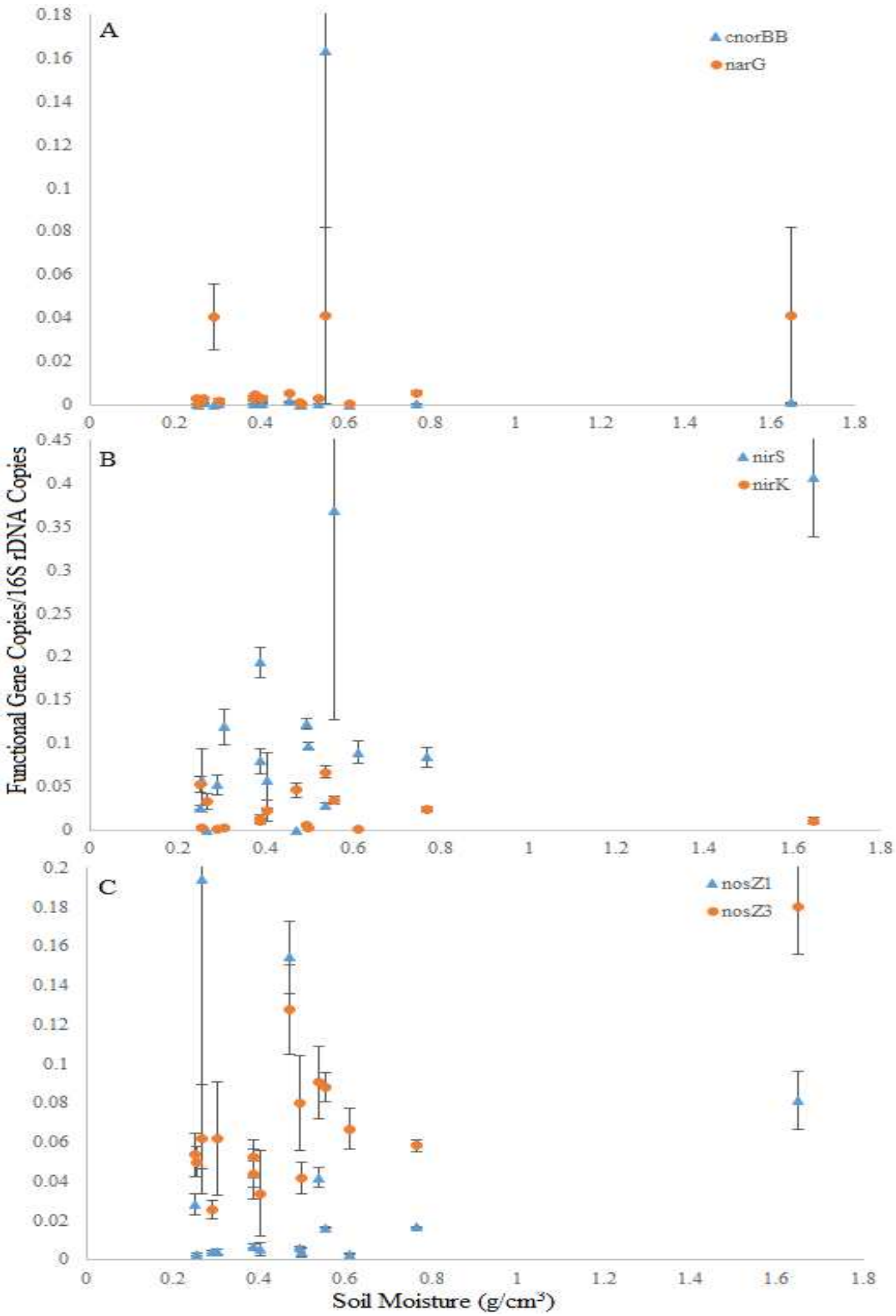


Figure 4.11. Copy number of each functional gene normalized to 16S rDNA copy number and soil moisture for both SM1 and SM2 of samples taken in stream channel. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

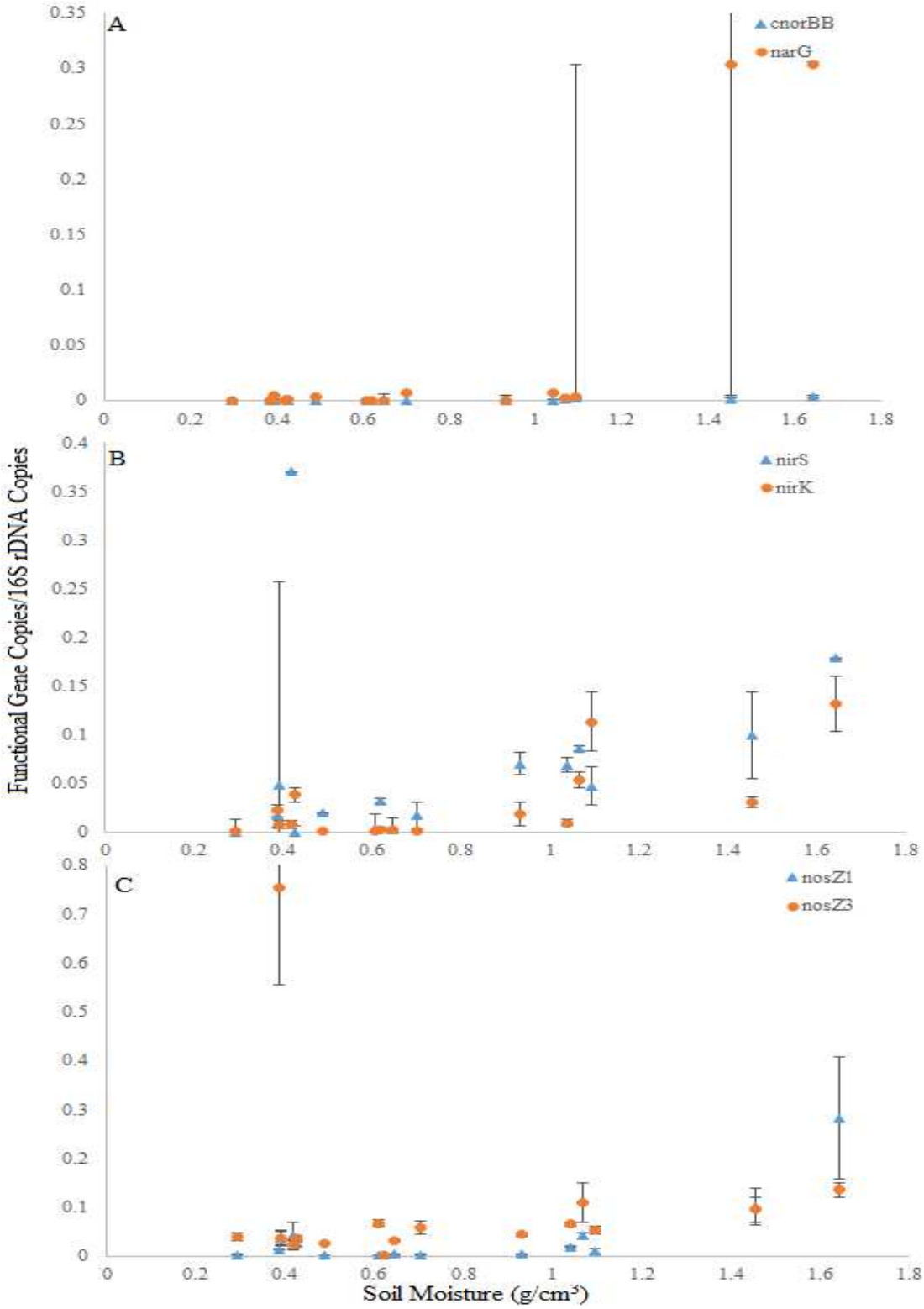


Figure 4.12. Copy number of each functional gene normalized to 16S rDNA copy number and soil moisture for both SM1 and SM2 of samples taken from the flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

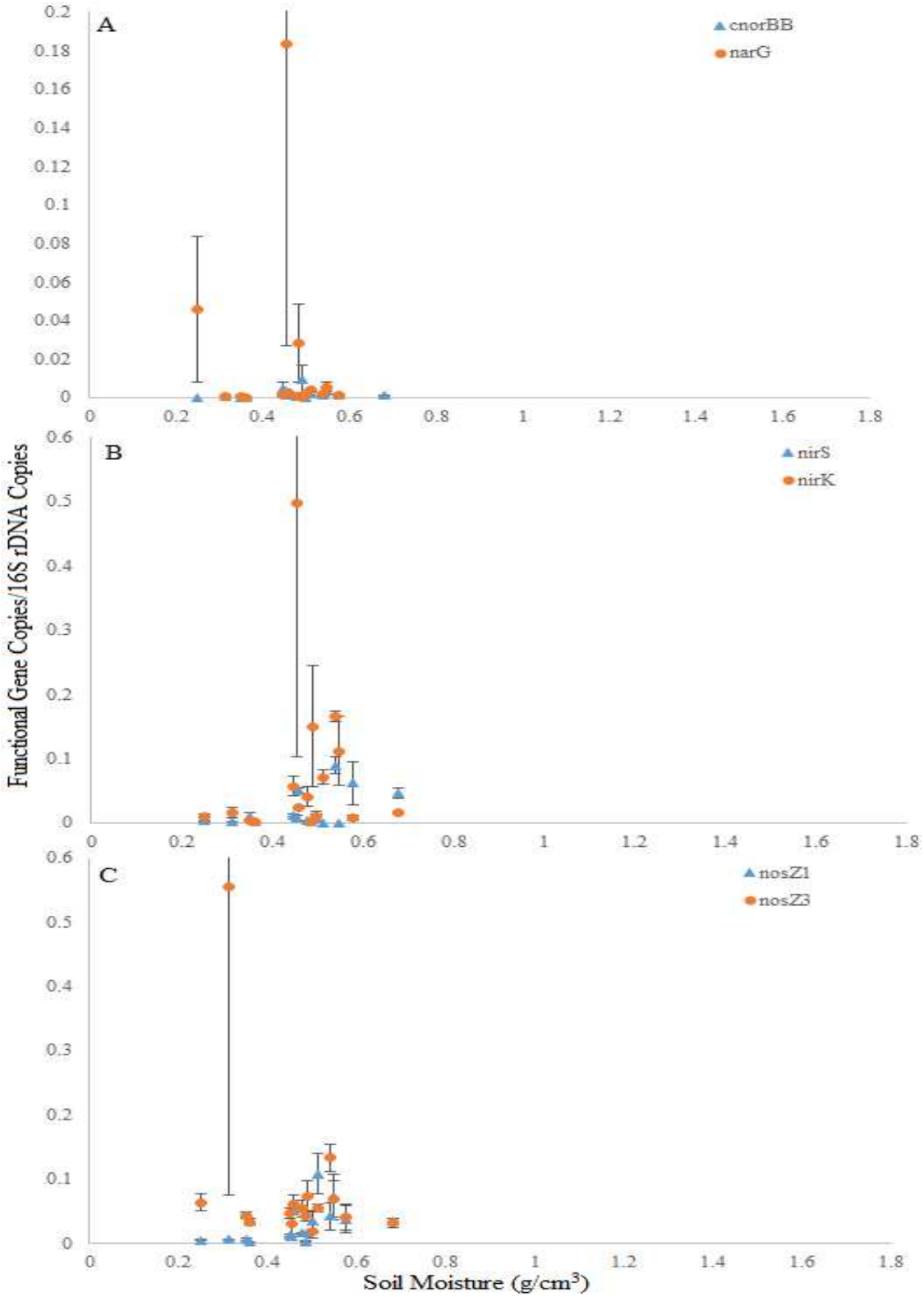


Figure 4.13. Copy number of each functional gene normalized to 16S rDNA copy number and soil moisture for both SM1 and SM2 of samples taken from the non-flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

2.4.3 Potential Denitrification

Measurements of potential denitrification showed that there were significant differences (p=0.04, F value=5.17, F-crit=4.08) between sites SM1 and SM2. In contrast, there were no significant differences in potential denitrification between channel, flooding zone, or non-flooding zone locations within each site respectively.

Functional gene copy number, normalized to 16S rDNA, as related to potential denitrification did not demonstrate any trends or potentially significant relationships over either spatial scale analyzed (Tables 4.5 and 4.6). The observed increases in copy number normalized to 16S rDNA failed to be explained by similar increases or decreases in potential denitrification, resulting in a low R^2 value. Instead a range of different potential denitrification values were found among all functional genes examined.

The same lack of significant relationship was observed between potential denitrification and copy number normalized to soil dry weight across both spatial scales, except for the flooding zone. The flooding zone R^2 values suggest that the observed potential denitrification values can be at least in part explained by copy number of functional genes, except for *nirS* (R^2=0.0001).

Both sites SM1 (Figures 4.14 and 4.19) and SM2 (Figures 4.15 and 4.20) displayed a wide range of potential denitrification values, with those at SM1 slightly lower than those of SM2 for all observed values. Flooding zone measurements of potential denitrification (Figures 4.17 and 4.22) were responsible for both extremes, the lowest observed values from SM1, and the highest observed values at SM2. While in-channel samples showed a reduced level of variation with respect to potential denitrification (Figures 4.16 and 4.21), there was a single outlier of increased potential denitrification value originating from site SM2 as was similarly reported with soil moisture content measurements. Non-flooding zone values were tightly clustered and displayed very little variation (Figures 4.18 and 4.23).

Table 4.5. R^2 values for potential denitrification and copy number of functional genes per gram dry weight sediment over spatial scales.

	R^2 values				
	SM1	**SM2**	**In Channel**	**Flooding Zone**	**Non-Flooding Zone**
cnorBB	0.22	0.23	0.19	0.59	0.19
narG	0.31	0.045	0.066	0.24	0.0037
nirS	0.026	0.017	0.027	0.0001	0.027
nirK	0.14	0.071	0.13	0.32	0.013
nosZ1	0.19	0.40	0.39	0.71	0.0022
nosZ3	0.13	0.15	0.23	0.30	0.017

Table 4.6. R^2 values for potential denitrification and copy number of functional genes per copy number 16S rDNA genes over spatial scales.

	R^2 values				
	SM1	**SM2**	**In Channel**	**Flooding Zone**	**Non-Flooding Zone**
cnorBB	0.002	0.011	0.016	0.22	0.069
narG	0.047	0.012	0.17	0.12	0.013
nirS	0.057	0.007	0.25	0.0003	0.13
nirK	0.0009	0.011	0.0023	0.14	0.0049
nosZ1	0.0042	0.31	0.061	0.33	0.0085
nosZ3	0.084	0.37	0.47	0.056	0.044

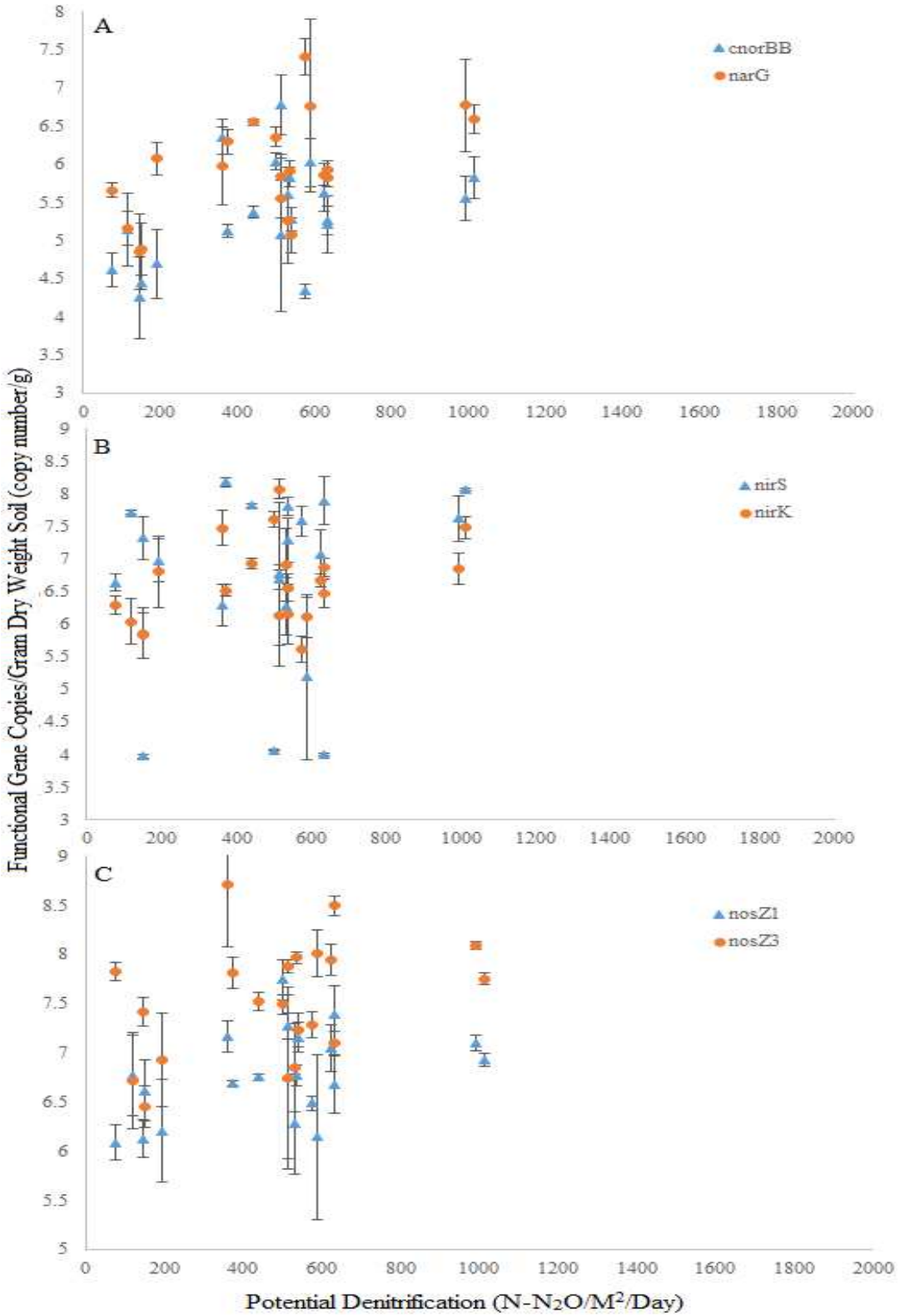


Figure 4.14. Copies of each functional gene per gram dry weight sediment/soil and potential denitrification across all channel positions at Seven Mile Creek site 1 (SM1). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

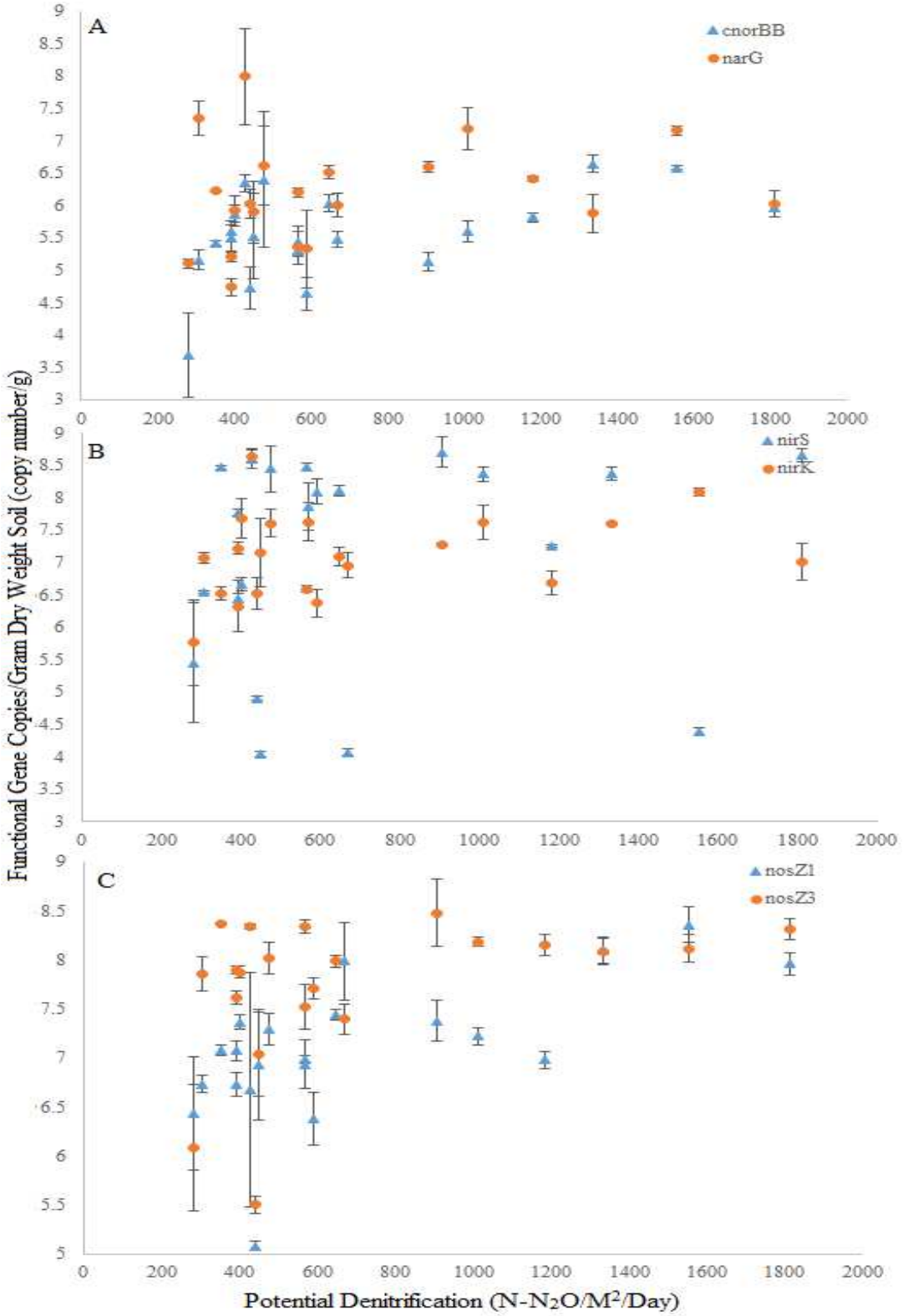


Figure 4.15. Copies of each functional gene per gram dry weight sediment/soil and potential denitrification across all channel positions at Seven Mile Creek site 2 (SM2). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

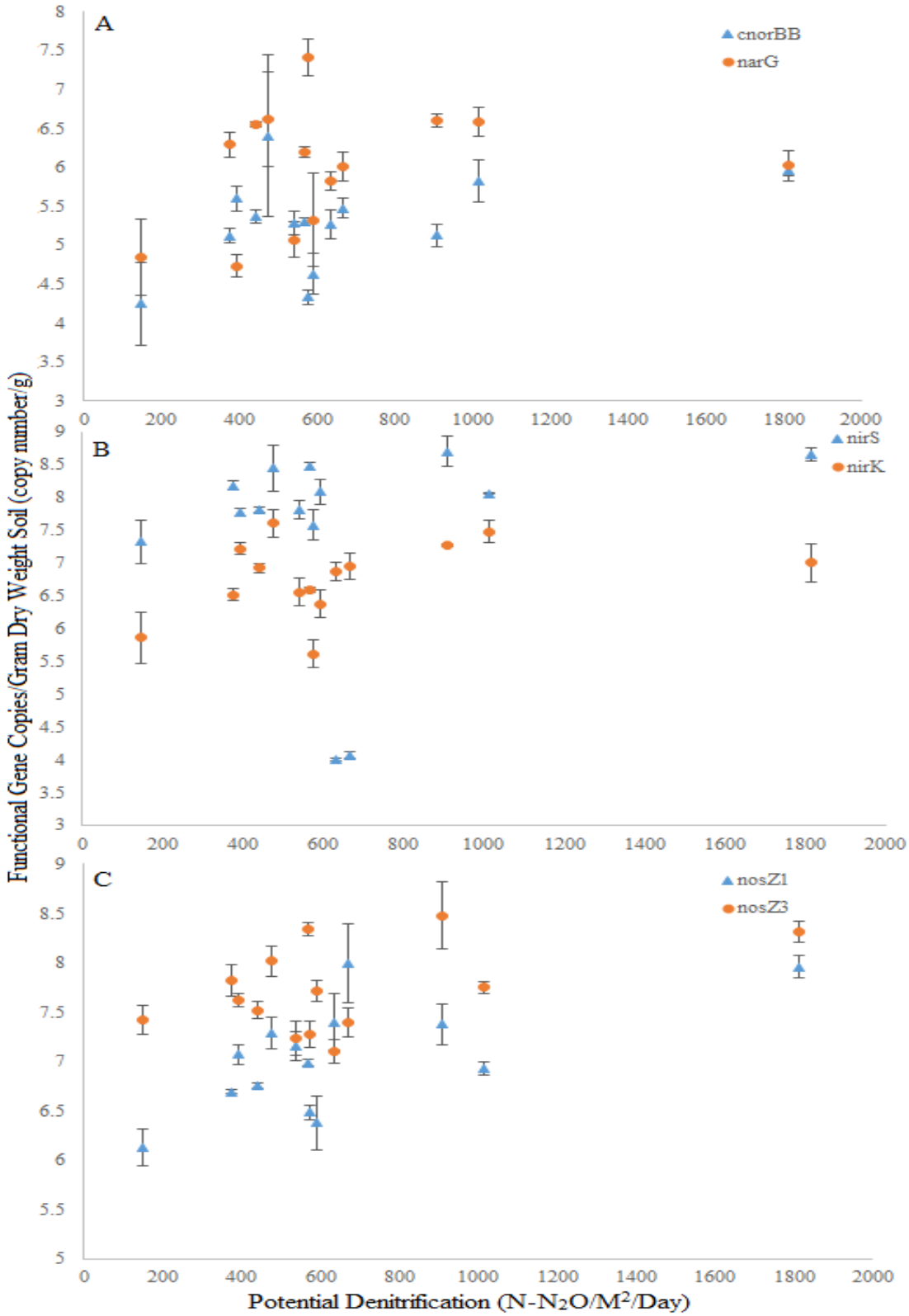


Figure 4.16. Copies of each functional gene per gram dry weight sediment and potential denitrification for both SM1 and SM2 of samples taken in stream channel. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

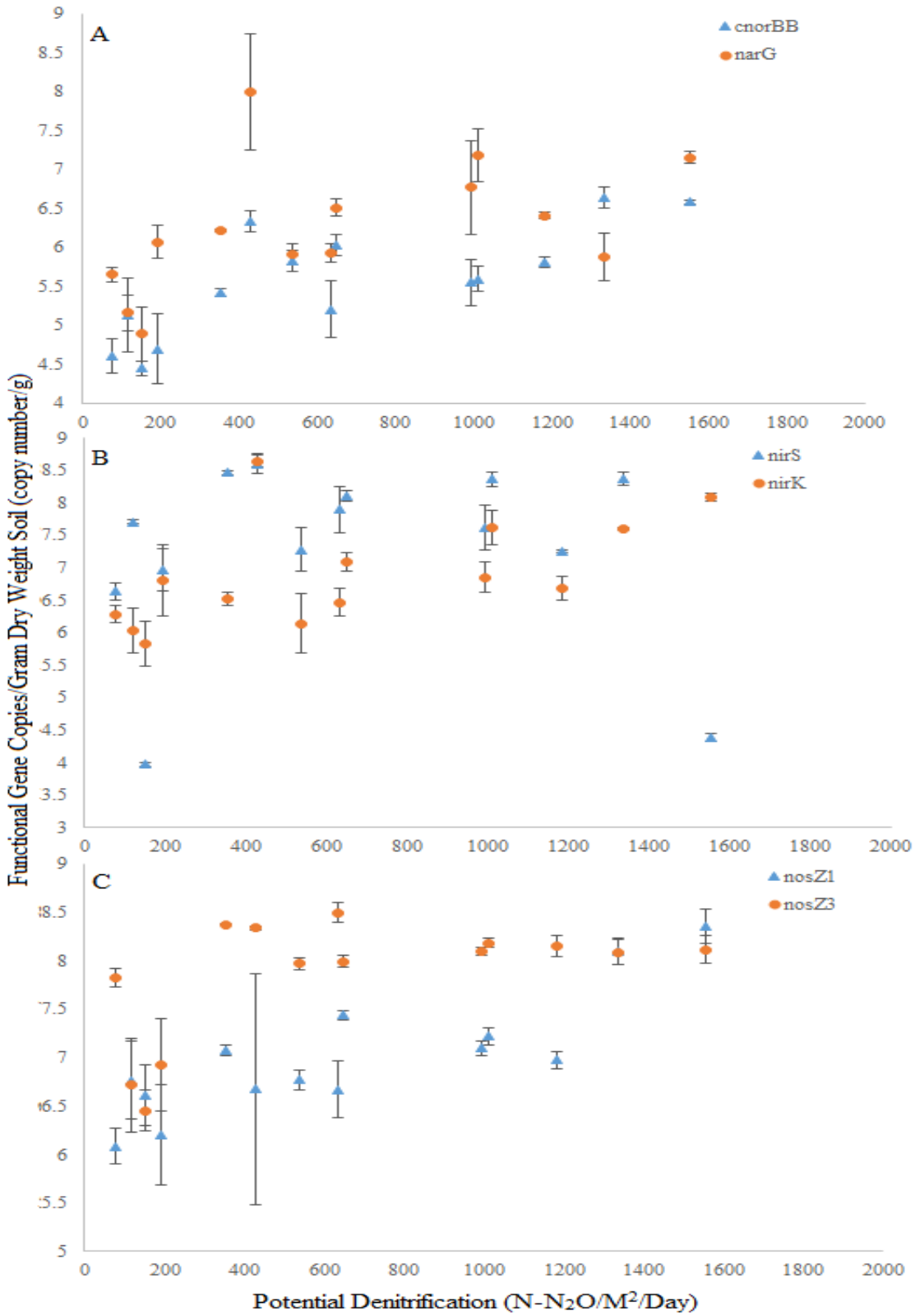


Figure 4.17. Copies of each functional gene per gram dry weight sediment and potential denitrification for both SM1 and SM2 of samples taken from the flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

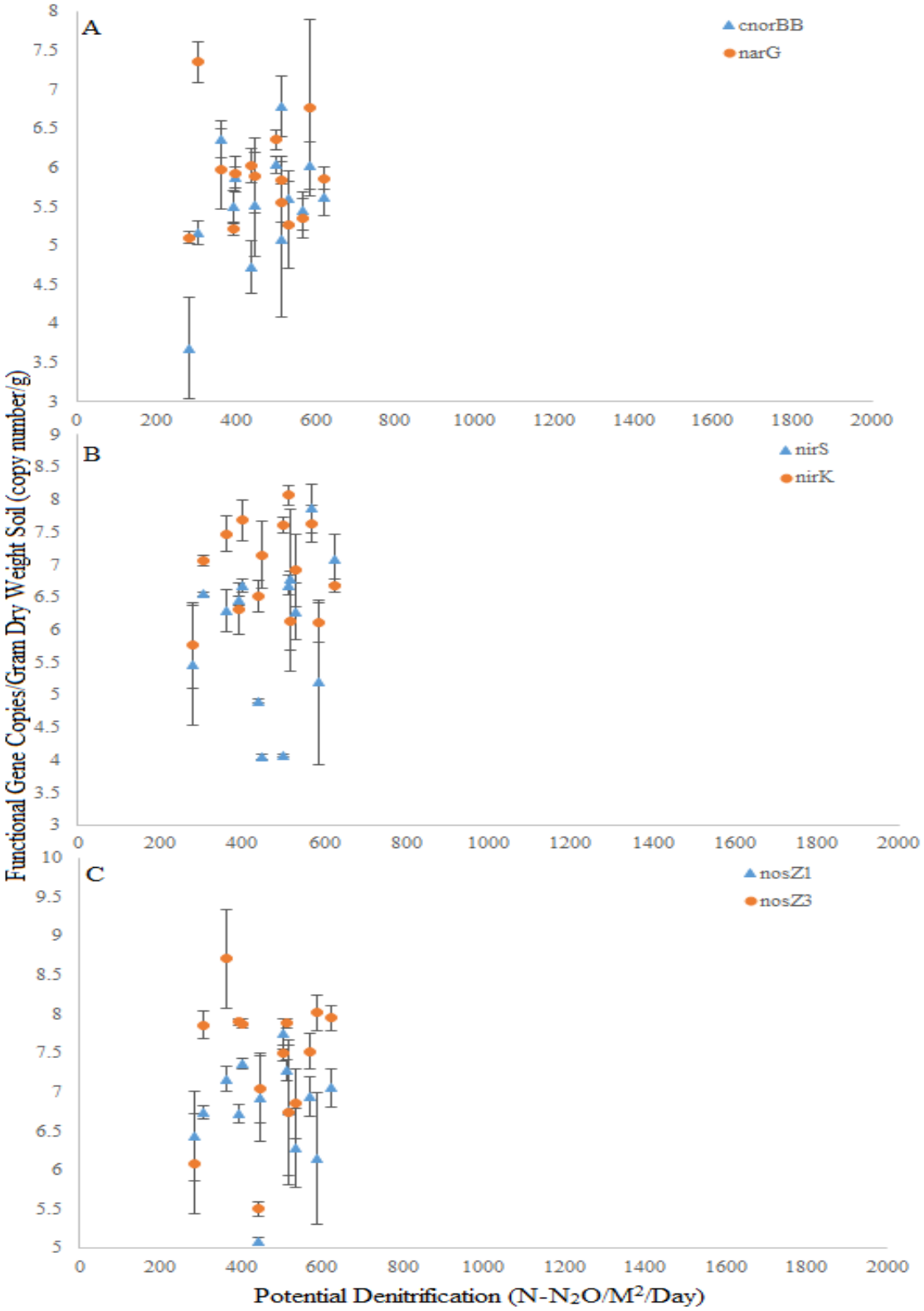


Figure 4.18. Copies of each functional gene per gram dry weight sediment and potential denitrification for both SM1 and SM2 of samples taken from the non-flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

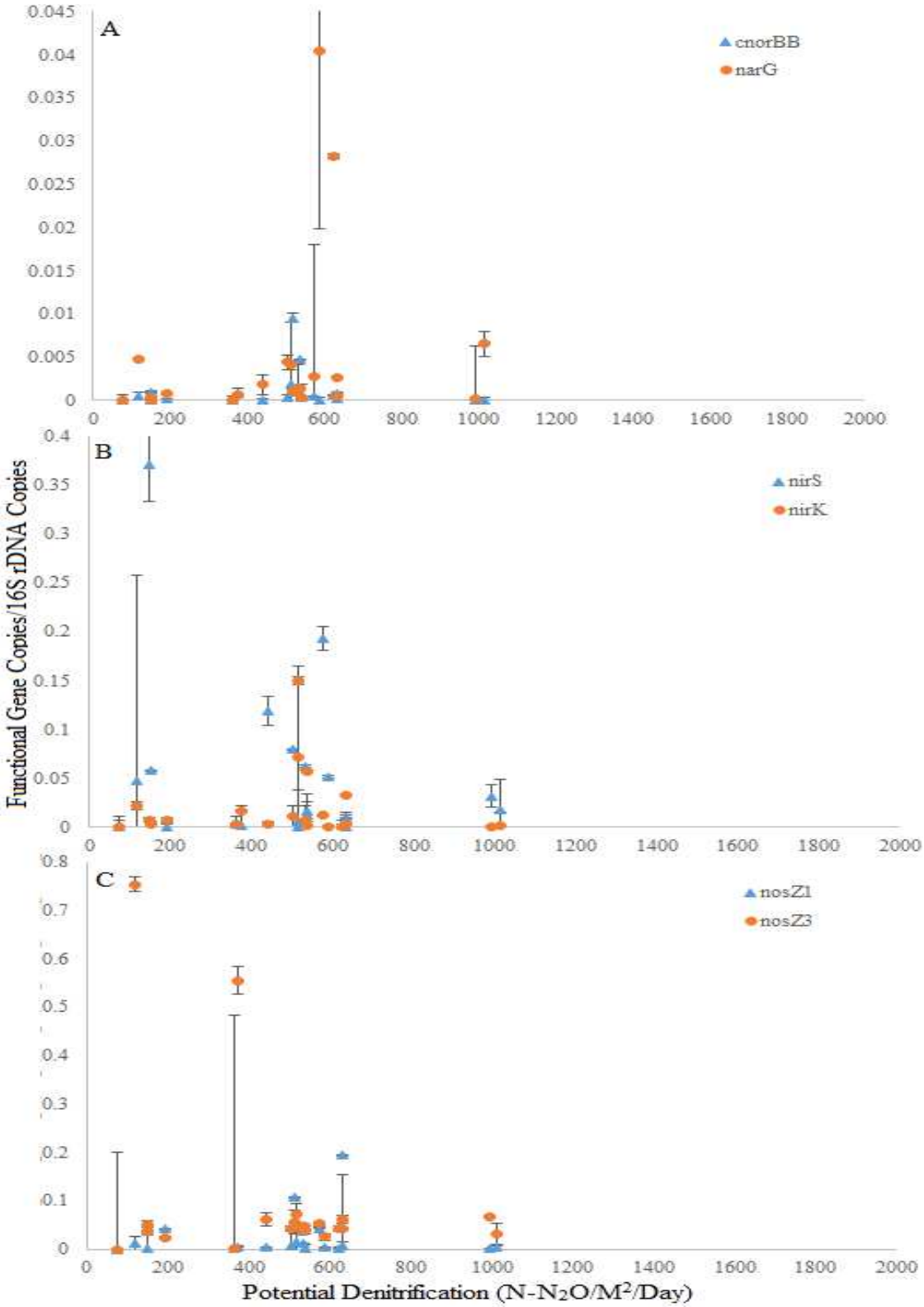


Figure 4.19. Copy number of each functional gene normalized to 16S rDNA copy number and potential denitrification across all channel positions at Seven Mile Creek site 1 (SM1). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

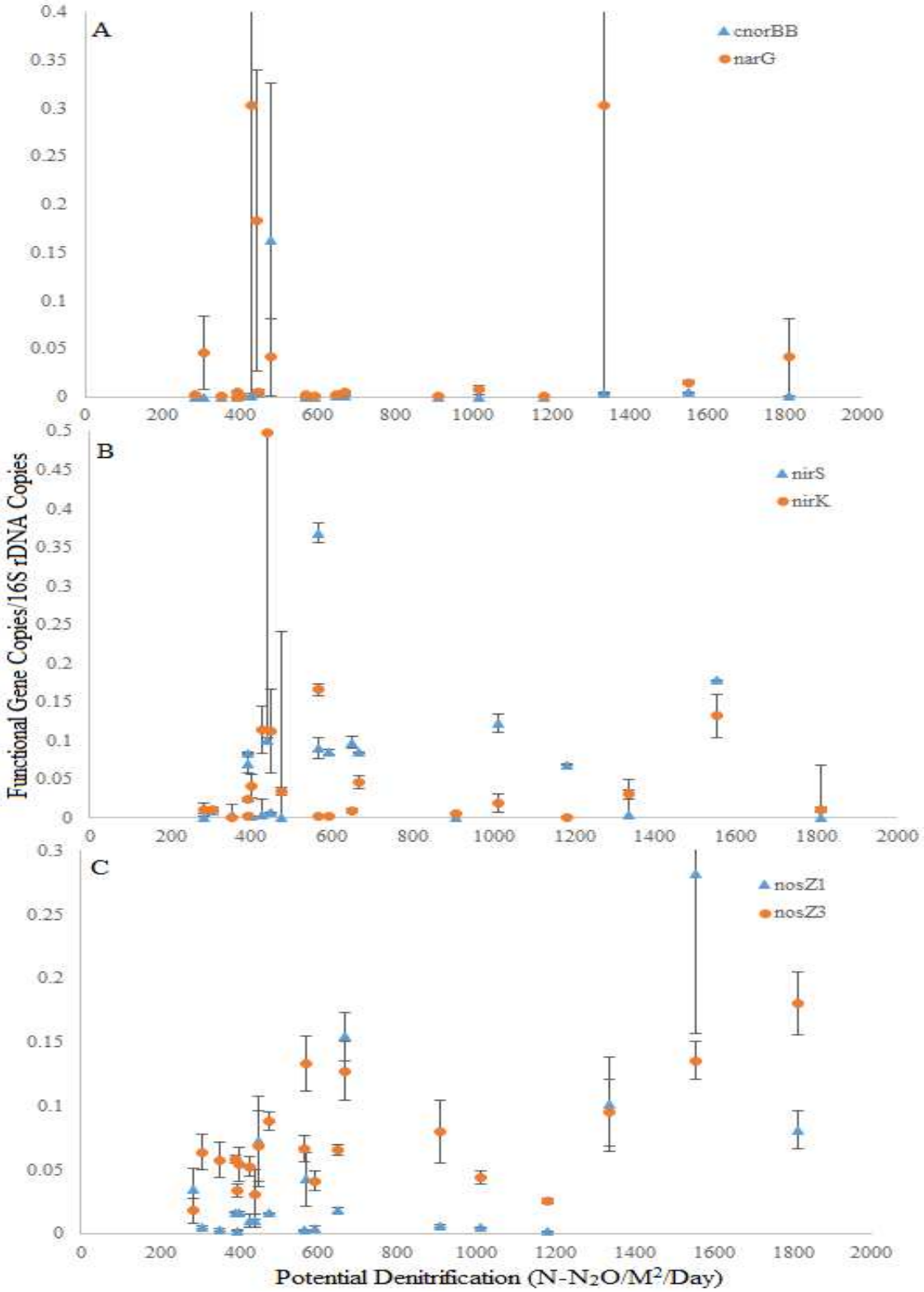


Figure 4.20. Copy number of each functional gene normalized to 16S rDNA copy number and potential denitrification across all channel positions at Seven Mile Creek site 2 (SM2). Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

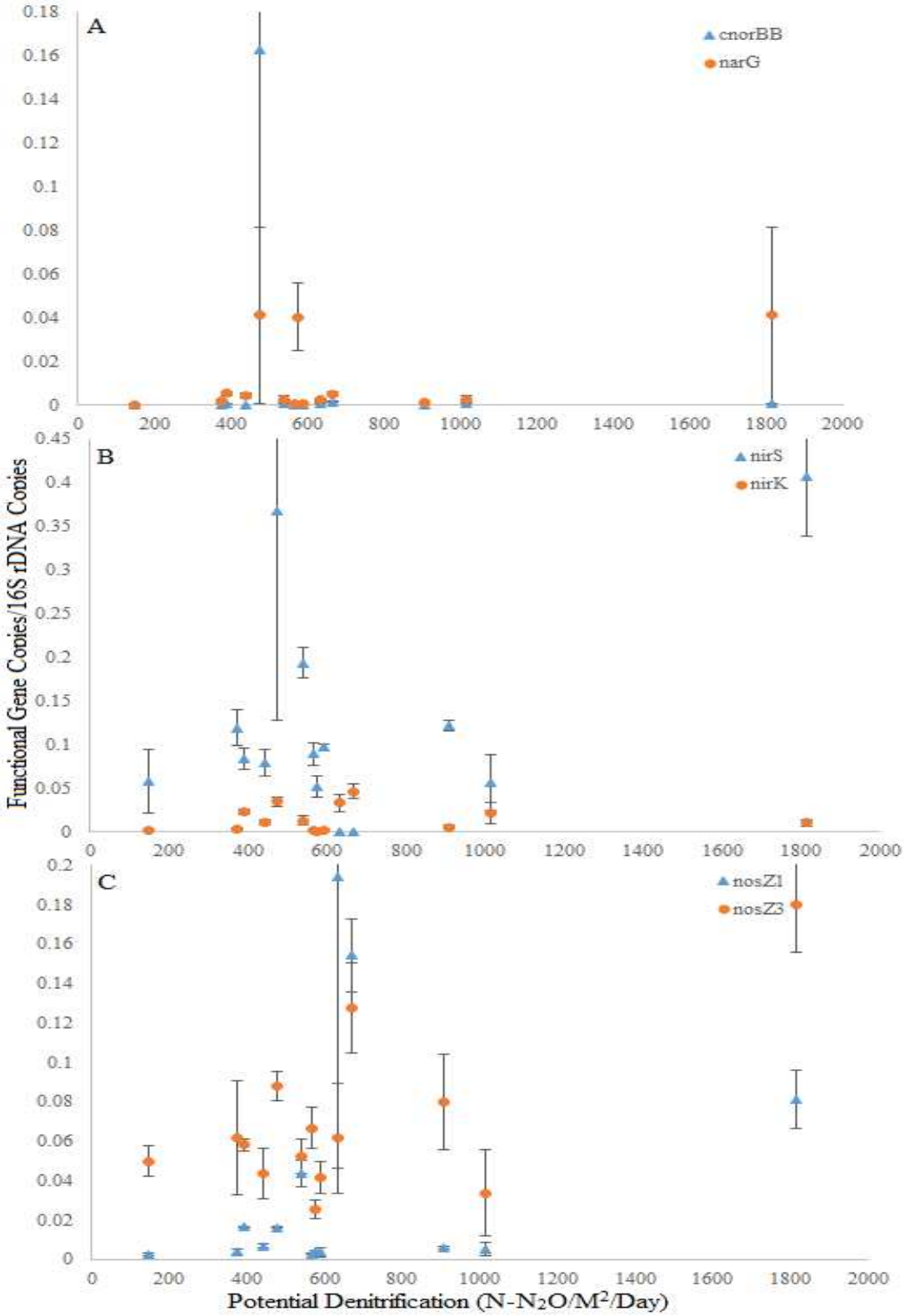


Figure 4.21. Copy number of each functional gene normalized to 16S rDNA copy number and potential denitrification for both SM1 and SM2 of samples taken in stream channel. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

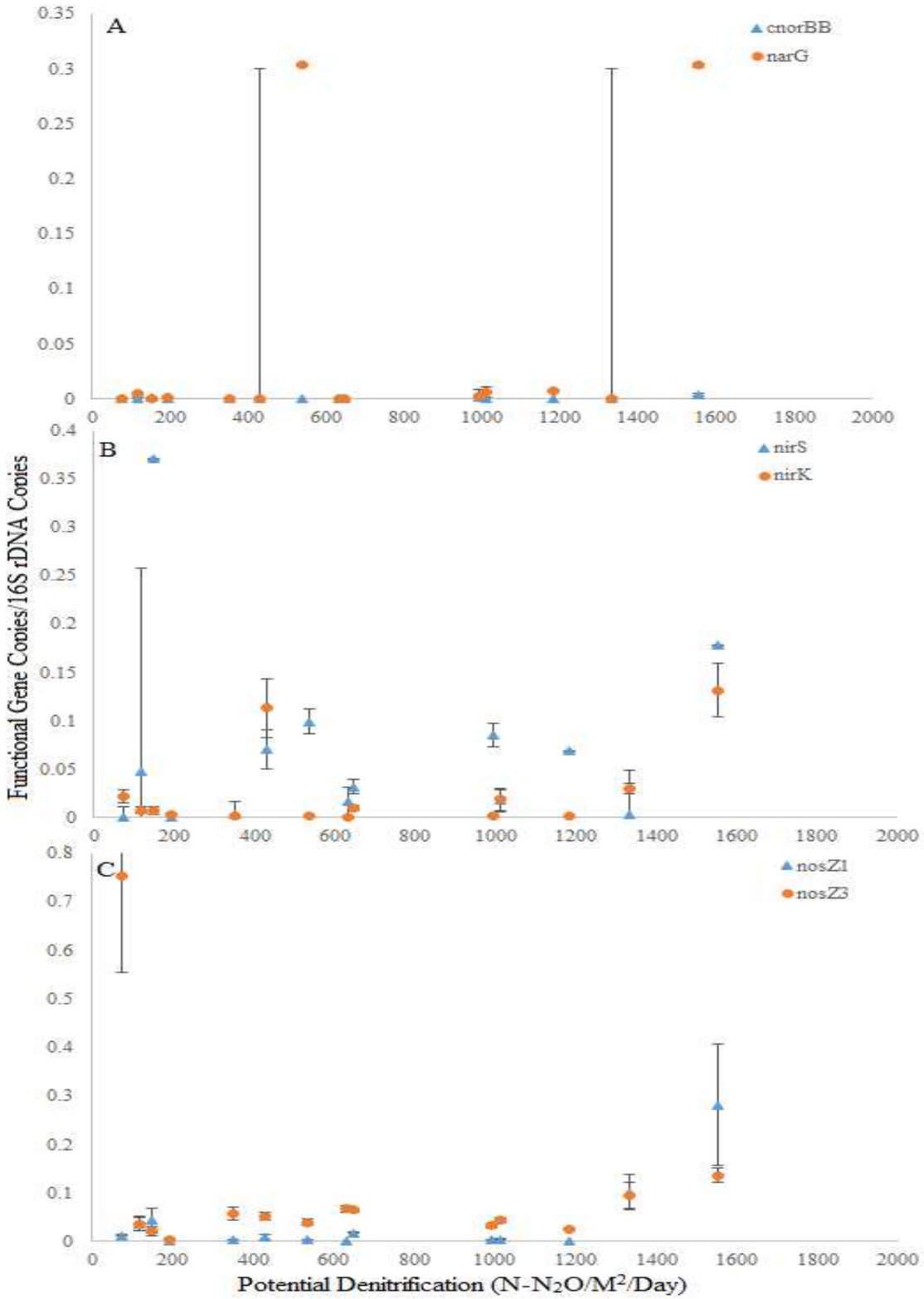


Figure 4.22. Copy number of each functional gene normalized to 16S rDNA copy number and potential denitrification for both SM1 and SM2 of samples taken from the flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

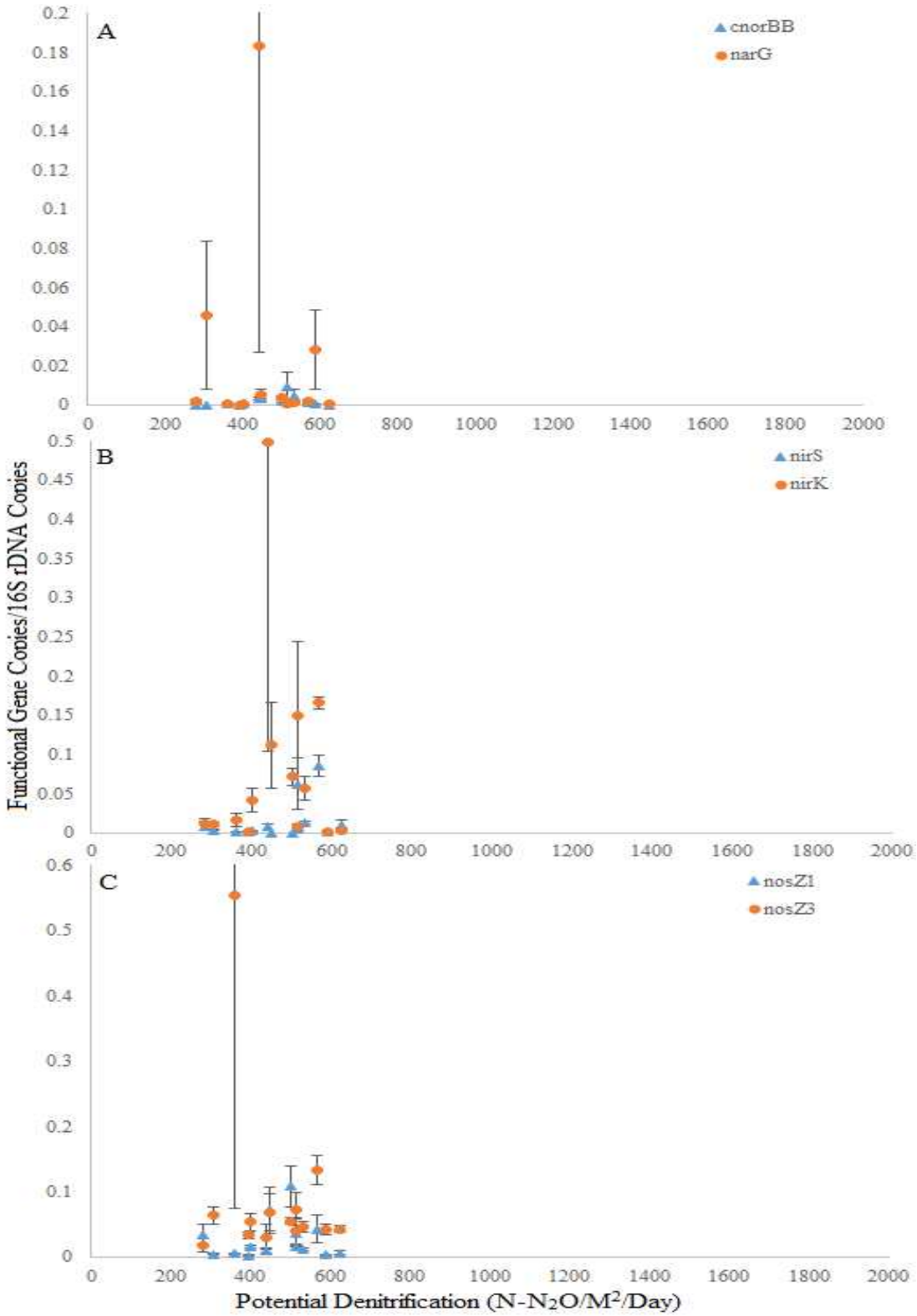


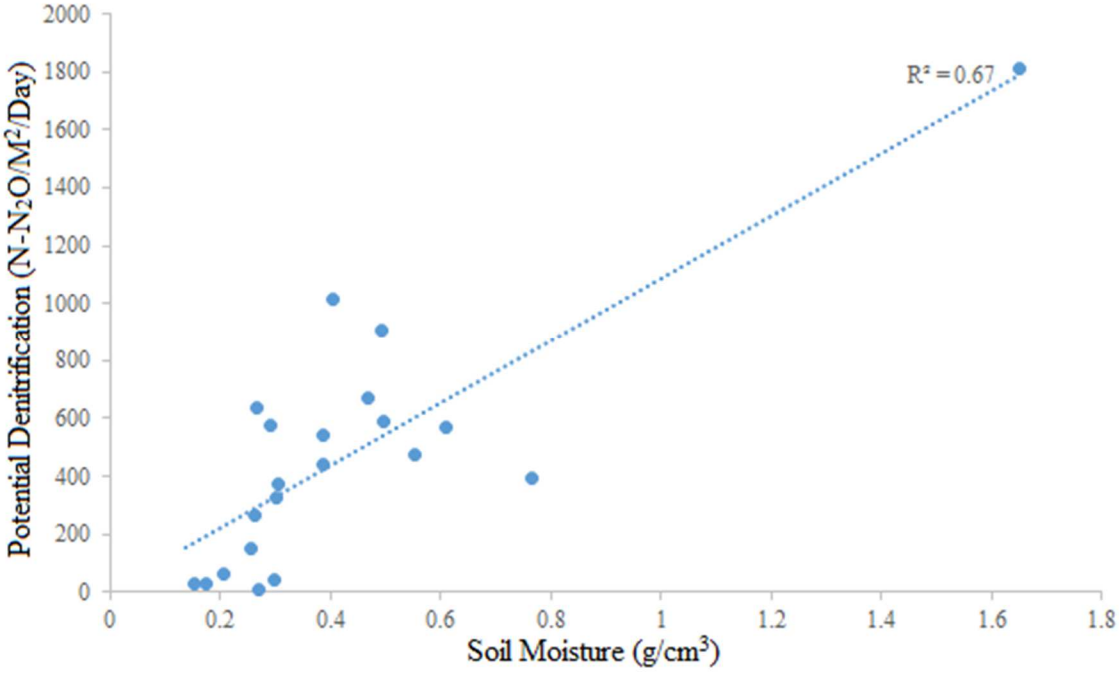
Figure 4.23. Copy number of each functional gene normalized to 16S rDNA copy number and potential denitrification for both SM1 and SM2 of samples taken from the non-flooding zone. Legend: A) genes *cnorBB* and *narG*, B) genes *nirS* and *nirK*, and C) genes *nosZ1* and *nosZ3*.

2.5 Abiotic Results

2.5.1 Potential Denitrification and Soil Moisture

The relationship between measured potential denitrification values and soil moisture content displays a strong, positive correlation (R^2=0.67) when the two variables are plotted against one another (Figure 5.1). This result reflects the genetic measures with respect to each variable, the graphical representations of which consistently mirrored each other.

Figure 5.1. Potential denitrification and soil moisture content variables plotted against one another.



Values are averaged from triplicate samples and include all three sites across all dates.

2.5.2 Temperature

Daily maximum air temperatures for each sampling date show no relationship with potential denitrification values for any sampling location (Figure 5.2). In channel, flooding zone, and non-flooding zone locations for both SM1 and SM2 were considered independently from one another in analyses and, while the non-flooding zone showed no relationship (R^2=0.0005), neither the in channel nor flooding zones yielded any

remarkable relationships (R^2=0.1166 and 0.0443, respectively). Water temperature measurements for in channel samples were not related with soil moisture (Figure 5.3, R^2=0.0002) or with potential denitrification measurements (Figure 5.4, R^2=0.0205).

Table 5.1. Maximum daily temperatures on the dates of sampling, excluding October 26 2015. All temperatures are reported in degrees Celsius.

	Temperature (Celsius)
June 12 2014	28.33
August 20 2014	27.77
October 20 2014	28.33
May 13 2015	14.44
June 15 2015	29.44
July 27 2015	30.55
August 17 2015	26.66

Figure 5.2. Air temperature maximums taken from the National Weather Service Reporting Station in St. Peter, MN and potential denitrification values for in channel, flooding zone, and non-flooding zone samples across all dates.

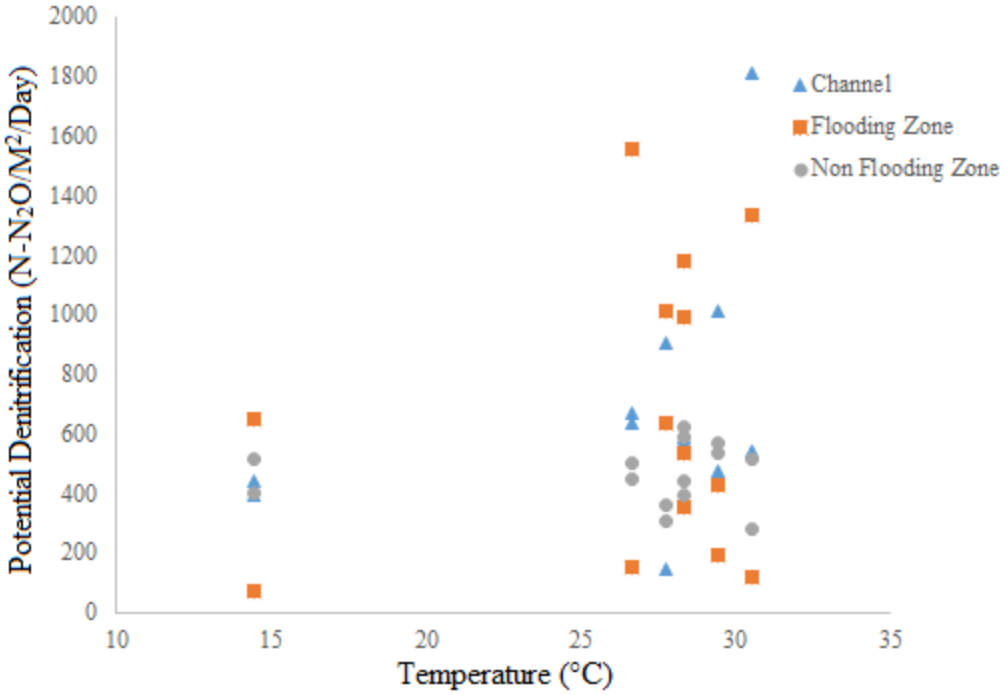


Figure 5.3. In channel water temperatures and averaged soil moisture values for all sampling dates.

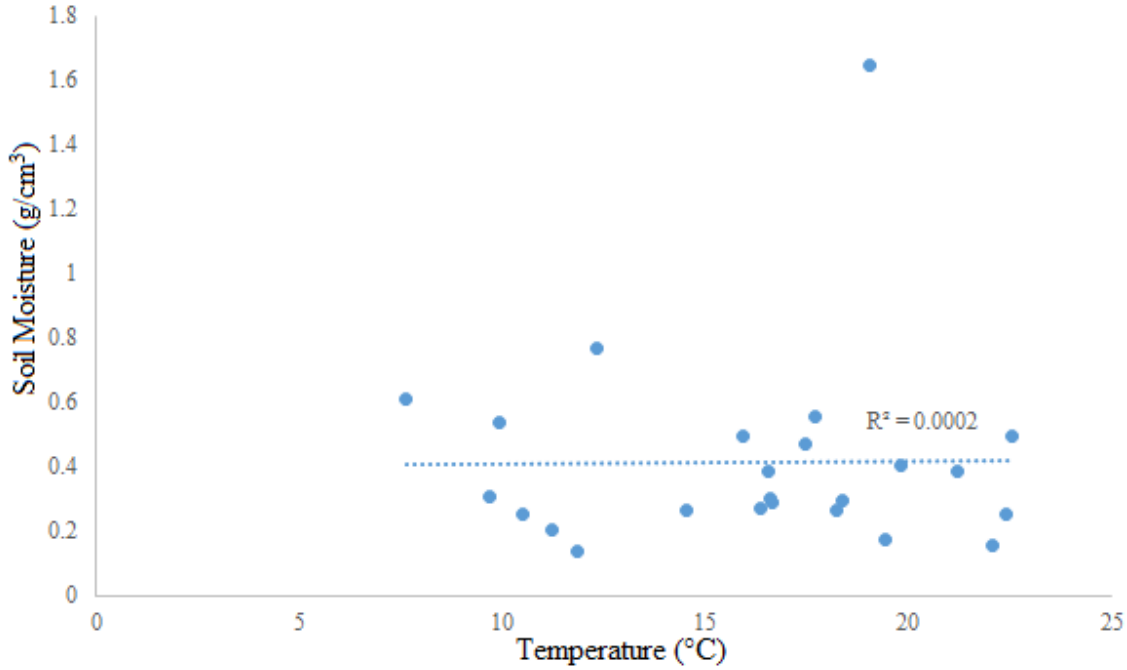
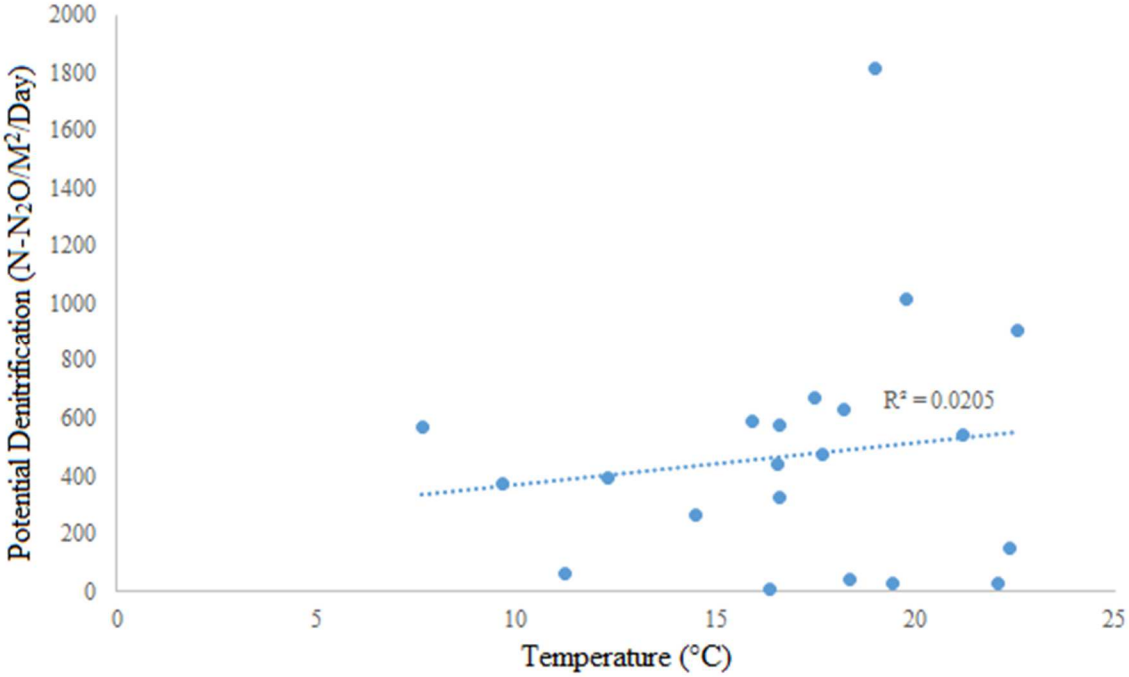


Figure 5.4. In channel water temperatures and averaged potential denitrification values for all sampling dates.



2.5.3 Dissolved Oxygen

Dissolved oxygen measurements taken from water in the channel showed negative relationships with both soil moisture (Figure 5.5) and potential denitrification (Figure 5.6), with the correlation stronger for that of soil moisture (R^2=0.3684) than potential denitrification (R^2=0.2902). The relationship between dissolved oxygen and soil moisture shows that as dissolved oxygen increases, soil moisture tends to decrease in the samples. Similarly, as dissolved oxygen increases in the water, potential denitrification tends to decrease. Correlational analyses include only data from sites SM1 and SM2 as the Park site was only sampled in the flooding zone, and therefore had no water data with which to obtain dissolved oxygen readings.

Figure 5.5. Dissolved oxygen of channel water and respective in channel soil moisture values for all sampling dates at SM1 and SM2.

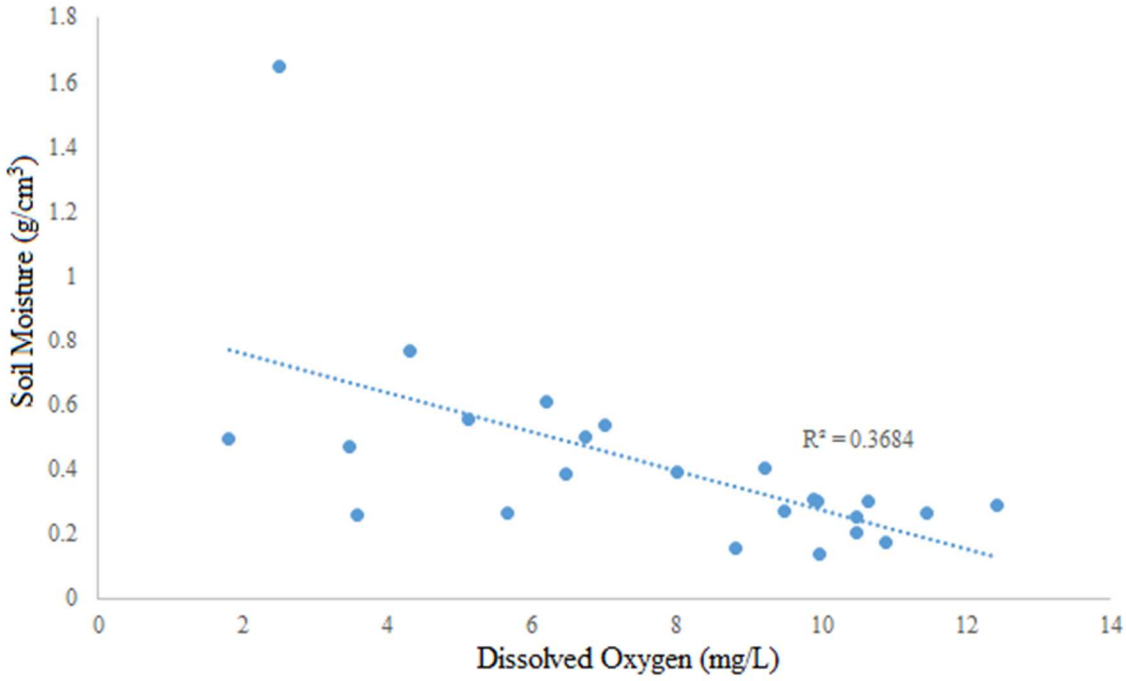
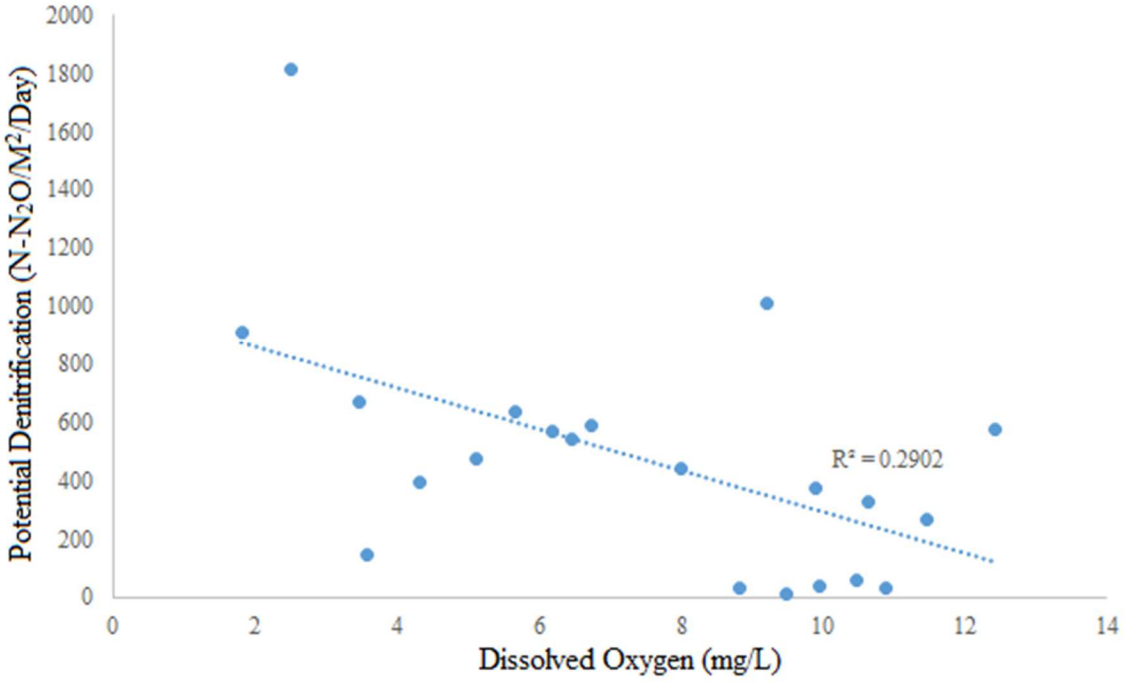


Figure 5.6. Dissolved oxygen of channel water and respective in channel sample potential denitrification values for all sampling dates at SM1 and SM2.



2.6 Redundancy and Correlation Analyses Results

2.6.1 Redundancy Analysis

The response variables potential denitrification and nitrate are notably removed from one another (Figure 6.1) despite the process of denitrification requiring nitrate, or subsequently reduced nitrogen species, to occur. They also demonstrate differential control over the independent and quantitative variables.

Potential denitrification is most strongly correlated with the functional gene abundances normalized to soil dry weight only, and with moderate strength. This is untrue for the 16S rDNA normalized functional gene abundances, which correlate more with nitrate, though this correlation is weak and lacks significance.

Additionally, potential denitrification correlates more strongly with site SM2 (Figure 6.1) and the values are also highest at SM2 (Table 6.1) and significantly different

from the Park site ($p = 0.008$). Nitrate correlates with the most strength with the month of June (Figure 6.1) and this month also demonstrates the highest observed nitrate values (Table 6.1) out of all months sampled. Independent of year or site, nitrate levels were significantly higher in June, and to a lesser degree July, than that of any other month ($p \leq 0.024$). Nitrate levels were the lowest in the month of May.

Soil bulk density was significantly different across all variables, but was primarily driven by site location ($p \leq 0.006$) with the highest values found at the Park site. Organic matter did not differ significantly across any spatial or temporal scale parameters. Soil moisture varied significantly between sites ($p \leq 0.004$), where SM2 demonstrated significantly higher values when compared to either the Park site or SM1.

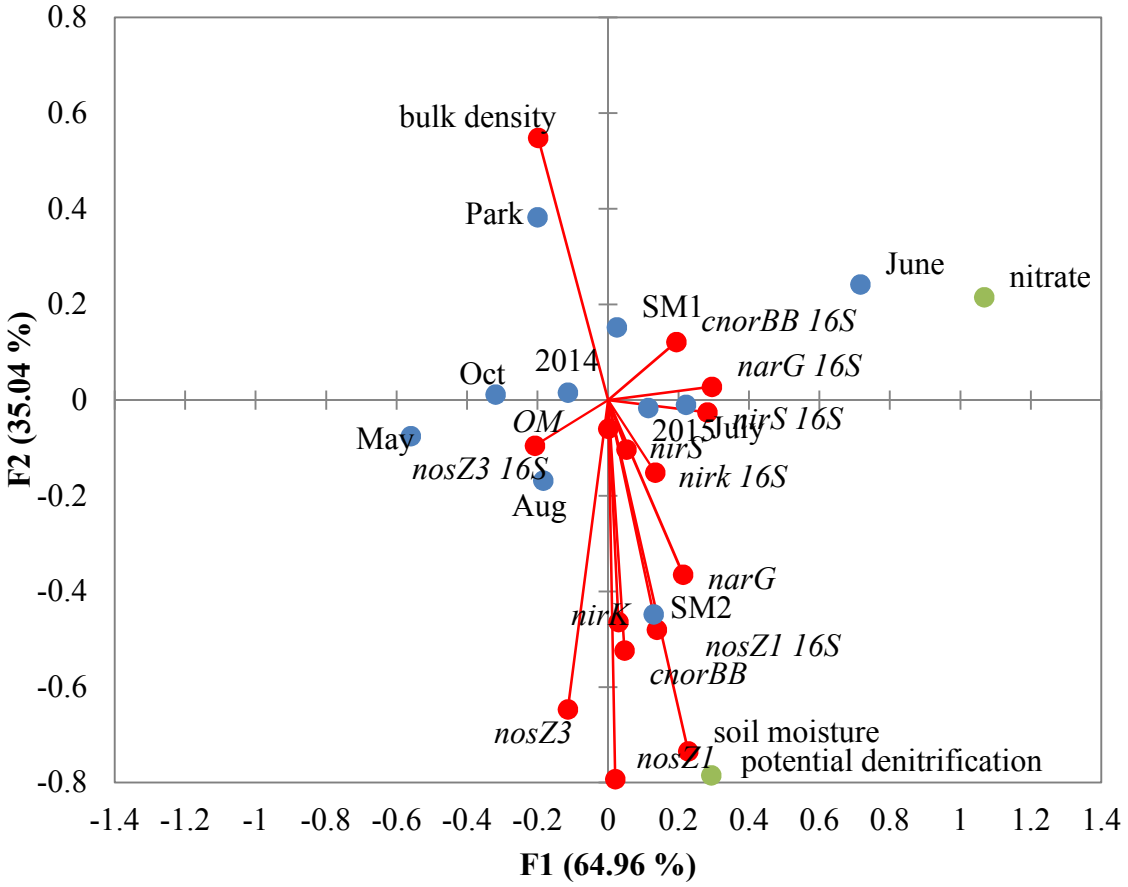
Table 6.1. Redundancy Analysis Table.

Variable	*P*-value	Month	Year	Site
Nitrate	< 0.0001	May[d] **June**[a] July[b] August[c] October[cd]	2014[a] **2015**[a]	SM2[a] SM1[a] Park[a]
Potential Denitrification	0.113	May[a] June[a] July[a] August[a] October[a]	2014[a] 2015[a]	**SM2**[a] SM1[a,b] Park[b]
Bulk Density	< 0.0001	May[a] **June**[a] July[a] August[a] October[a]	**2014**[a] 2015[a]	SM2[a] SM1[b] **Park**[c]
Organic Matter	0.115	May[a] June[a] July[a] August[a] October[a]	2014[a] 2015[a]	SM2[a] SM1[a] Park[a]
Soil Moisture	0.013	May[a] June[a] **July**[a] August[a] October[a]	2014[a] **2015**[a]	**SM2**[a] SM1[b] Park[b]
cnorBB	0.029	May[a] June[a]	2014[b] **2015**[a]	**SM2**[a] SM1[a]

		July[a] August[a] **October**[a]		Park[a]
narG	0.471	May[a] June[a] July[a] August[a] October[a]	2014[a] 2015[a]	SM2[a] SM1[a] Park[a]
nirS	< 0.0001	May[a] **June**[a] July[a] August[b] October[ab]	**2014**[a] 2015[b]	**SM2**[a] SM1[a] Park[a]
nirK	0.008	May[a] **June**[a] July[a] August[a] October[a]	2014[b] **2015**[a]	**SM2**[a] SM1[ab] Park[b]
nosZ1	0.004	May[ab] June[b] July[ab] **August**[a] October[ab]	2014[a] **2015**[a]	**SM2**[a] SM1[b] Park[b]
nosZ3	0.006	**May**[a] June[a] July[a] August[a] October[a]	**2014**[a] 2015[a]	**SM2**[a] SM1[b] Park[b]

Months, sites, or years in bold indicate when/where the indicated variable was highest, when significant differences were observed only. Genes reported are with respect to soil dry weight only.

Figure 6.1. Redundancy Analysis Chart.



All data normalized beforehand so that values fall between -1.4 and 1.4 as per the scale. Potential denitrification and nitrate as response variables (green dots), months, years, and sites as independent variables (blue dots), and all others quantitative (red dots with connecting lines). Genes denoted with a 16S are copy numbers normalized to 16S rDNA, while those without are soil dry weight normalized abundances.

2.6.2 Correlation Analyses

Functional gene copy numbers normalized to soil dry weights correlated significantly with other soil normalized denitrification functional gene abundances (R^2=0.35-0.86, $p \leq 0.017$). The exception to this observation is with the *nirS* gene, which correlated only with *narG* (R^2=0.383, p = 0.024) and *nosZ3* (R^2=0.639, p = 0.0001). The same correlational significance exists between 16S rDNA normalized denitrification

functional genes (R^2=0.343-0.811, $p \leq 0.044$) with few exceptions. The *nirS* gene, however, does not significantly correlate to any other gene when each are 16S rDNA normalized.

Soil moisture levels correlated significantly and with moderate strength for all genes except *narG* and *nirS*, normalized to soil dry weight only (R^2=0.469-0.55, $p \leq 0.005$). With respect to abiotic variables, soil moisture correlated significantly with potential denitrification (R^2=0.518, $p = 0.002$), soil bulk density (R^2=0.911, $p < 0.0001$), and organic matter (R^2=0.613, $p = 0.0001$) values.

Significant correlations of moderate strength were observed for potential denitrification and all genes except for *nirS* (R^2=0.44-0.71, $p \leq 0.009$), normalized to soil dry weight only. Additionally, a strong correlation was observed between potential denitrification and soil bulk density (R^2=0.506, $p = 0.002$).

Organic matter correlated significantly with the denitrification functional genes *cnorBB* (R^2=0.384, $p = 0.023$) and *nirK* (R^2=0.454, $p = 0.007$) when normalized to soil dry weight only, as well as to soil bulk density (R^2=0.727, $p < 0.0001$).

Soil bulk density correlated significantly and with moderate to high strength with each of the denitrification functional genes normalized to soil dry weight (R^2=0.362-0.710, $p \leq 0.033$), with the exceptions of genes *narG* and *nirS*.

There were no observed correlations of significance with nitrate levels.

2.7 Temporal Discussion

2.7.1 Genetic Analyses

When considering the results of the microbial genetic data, of equal importance to the time scale for analysis is the manner in which the genes were being analyzed – either with absolute numbers, as measured per gram dry weight soil, or relative, as normalized to 16S rDNA. Significant variations were observed between the two methods. Differences in reported significance were observed over both monthly and yearly scales between 16S rDNA normalization and with respect to soil dry weight, and the results

together are meaningful in that may help indicate which population is changing. For example, in the situation in which there is variation amongst data across sampling dates for the same functional gene normalized to 16S rDNA, it could be due to one of three possibilities: 1) a significant shift in general bacterial population where denitrifiers are not similarly affected, 2) denitrifier population shifts without accompanying bacterial population changes, or 3) that they both shift but in a dissimilar manner. To give the 16S rDNA normalized data meaning, the absolute copy numbers as measured per gram soil dry weight was considered. If a significant shift in denitrifier population is seen, it can be concluded that the shift in 16S rDNA normalized data can be, at least partially, explained by a shift in denitrifier population and not solely due to a general bacterial population increase.

Additionally, these results can be greatly impacted by amount of data collected. In this study, the 2014 season included three separate sampling events in the months of June, August, and October. In 2015, however, they included May, June, July, August, and October, and therefore provide a more reliable and likely accurate view of changes within the soil and sediment environments with respect to functional gene copy numbers.

The ANOVA analyses indicate that the functional genes studied varied differentially over temporal scales, with some changing significantly (for example, $p=0.005$ in 2014 and $p=0.05$ in 2015 for *narG* normalized to 16S rDNA) from month to month and others not (Table 3.1, 3.2). On the other hand, some only vary significantly over the span of years (*nirS* $p=0.01$ normalized to 16S rDNA, for example) while others do not (Table 3.3). The lack of consistency between finding significant difference among the variance of individual genes is both supported by the idea that different denitrifiers in soils/sediment do not respond in the same manner to environmental parameters (Ligi et al. 2013; Tatariw et al. 2013), as well as proves further evidence for the claim. It also makes sense intuitively, as it is known that different denitrifiers can come from vastly different clades and would thus have different nutritional and environmental requirements, likely thriving at different optimums as a result of these requirements.

Results showed that *narG* normalized to 16S rDNA changed significantly over monthly scales for both 2014 and 2015, as well as to normalized to soil dry weight in 2015 (Tables 3.1 and 3.2). In contrast, *narG* copy number did not significantly differ

when years were compared against one another (Table 3.3). This may be indicative of more seasonal parameters, given the similar variability between months in one year, but consistency between years.

To fully characterize the potential implications of the results, it would be necessary to have precise weather data including temperatures of the soil and water, as well as the occurrence of storm events and flooding or drought conditions. The tracking of storm events and other ephemeral fluctuations could provide insight when compared against these results by elaborating on the variables that were impacted, such as soil moisture and flooding in the event of storms, or nutrient loading as fertilizers wash off the fields and into the streams. Such information could provide insight into which parameters are more likely to have impacted the results at any given sampling date.

The differences between gene copy numbers calculated as copy number per gram dry weight soil/sediment varied from one another and did not mirror the results of normalization to 16S rDNA numbers. This suggests that denitrifier populations were impacted differently by environmental parameters from that of soil bacteria in general over the temporal scales examined. For example, while the *narG* copy number normalized to 16S rDNA was significantly variable over a span of months in both 2014 and 2015 (Table 3.1), *narG* copy number per gram dry weight soil/sediment was not significantly different over a monthly timescale in 2014 (Table 3.2). The two metrics consider the data from different perspectives as the former method considers relative abundance while the latter looks only at absolute abundance in the soil. These results suggest that there are fluctuations in the overall population of bacteria present thereby shifting the relative abundance of *narG*-type denitrifiers, while the absolute abundance of *narG*-type denitrifiers is more stable from month to month in 2014. This same lack of significant variation over both normalization methods held true for all functional genes considered in the study, though the exact temporal variations for each gene differ from that of *narG*-containing denitrifiers. These results provide support for the assertion that expression of genes among the denitrifier communities are influenced by environmental parameters differentially (Ligi et al. 2013).

Discriminant analyses highlighted how gene composition shifted over time as noted by the differences between 2014 (Figure 3.1) and 2015 (Figure 3.2). The analysis is

useful to consider due to the fact that denitrification is a community level process and temporal shifts in functional gene abundance relative to 16S rDNA likely impacts the overall denitrifying capability of an ecosystem. Given that each gene or set of redundant genes (i.e. *nirK* and *nirS*) is responsible for a different step in the denitrification process, fluctuations at any step, such as a decrease in only one gene that is not seen in the others, could have significant impacts for the overall process, and therefore result in differences in potential denitrification measurements such as those obtained from the acetylene block method.

Overall, no clear significant relationships or trends could be established by examining the gene copy numbers over both monthly and yearly temporal scales, and the ways in which the various functional genes observed respond over these scales differs from one gene to the next. These findings are in line with previous studies that attempted to characterize the genetic basis for denitrification, which also found that there are no seasonal trends that can be established with respect to denitrifiers (Chon et al. 2011). Additionally, the dissimilar changes between functional genes over different time scales has been observed by others (Levy-Booth, Prescott, and Grayston 2014; Ligi et al. 2013) and supports the findings of this study.

2.6.2 Soil Moisture

Across all data, there are no significant correlations between soil moisture and functional gene copy number across either normalization (Tables 3.5 and 3.6) method. The one exception is the month of October with respect to normalization to soil dry weight, where the R^2 values for the respective functional genes were as high as 0.49. While the soil moisture values for the month of October for either year (Table 3.4) did not differ significantly from that of the other months, it is possible that at this time soil moisture became a limiting variable where it was not previously, though it still was not the only factor to hold some control over the expression of the different functional genes. However, as this trend was observed only in the normalization to soil dry weight, and not in that of 16S rDNA normalization as well, it is likely that the total bacterial community was impacted similarly and the total proportion of denitrifiers did not change.

Despite a lack of significant variation in the soil moisture data alone, such as excessively dry or wet conditions, there is some similarity between the soil moisture and functional gene copy number (Figures 3.4 and 3.5) and potential denitrification and functional gene copy number (Figures 3.14 and 3.15) on a yearly scale. This similarity is evidenced by the spikes in both soil moisture and potential denitrification occurring at the same sampling date (Figures 3.5 and 3.15). The differences do not constitute formal trends but it does potentially provide evidence in support of the claim that soil moisture and potential denitrification rates are related.

2.7.3 Potential Denitrification

This study was unable to conclude that there was a relationship between genetic copy number of denitrifying functional genes and denitrification rates, which supports previous claims (Chon et al. 2011; Levy-Booth, Prescott, and Grayston 2014; Manis et al. 2014). There was no significant relationship between potential denitrification and genetic measures that could be established (functional gene copy number normalized to 16S rDNA copy number as well as per gram dry weight sediment) as per R^2 values (Tables 3.8 and 3.9). This lack of a correlation is demonstrated most clearly and consistently by the *nirS* functional gene, which revealed erratic shifts in low to high copy numbers normalized to soil dry weight across all potential denitrification values (Figure 3.15 B), resulting in a low R^2 value of 0.0294 (Table 3.8). Of all of the genes, *nirS* also resulted in the lowest R^2 values across all temporal measures, except where it was second lowest in the month of June across both years. This lack of significant correlation may suggest that the presence of this gene is less impactful with respect to denitrification rates.

It is possible, however, that the lack of findings is a result of the limitations of the process for determining potential denitrification itself rather than a lack of an existing significant relationship. Additionally, due to the extreme fluctuation in measured denitrification values between triplicate samples, values were averaged, possibly obscuring the patchy nature of these processes. Alternatively, it is possible that a temporal relationship exists, but is more complex than simply looking at the parameter of potential denitrification alone.

2.8 Spatial Discussion

2.8.1 Genetic Analyses

Results in Table 4.1 show that significant differences in copy numbers for the *cnorBB*, *nirK*, and *nosZ3* genes were dependent upon whether or not relative or absolute abundances were being considered. Significant differences for *cnorBB* and *nirK* were observed (p=0.002 and p=0.03 respectively) only when normalized to 16S rDNA, which suggests that there was a shift in total bacterial population but no corresponding shift in the denitrifier population. If the denitrifier population were the reason for the difference between normalized values, there would be a significant difference in absolute copy number (per gram dry weight soil/sediment) of the functional genes. This is an example of denitrifier population and other bacterial populations being disparately impacted by environmental variables (Peralta, Ludmer, and Kent 2013). Some variable or variables within the environment are leading to changes in overall bacterial population size while denitrifiers remained relatively unaffected. Conversely, *nosZ3* was observed to have significantly variable absolute copy number across channel locations, while there were no significant changes with respect to total bacterial population. This is explained by both general bacterial population and *nosZ3* denitrifier population being impacted similarly by the different environments.

In the case of *nirS*, which varied significantly (p=0.0002 for 16S rDNA and $p=2.1x10^{-5}$ for soil dry weight) over both measures of copy number (Table 4.1), it can be determined that the general bacterial and *nirS* denitrifier populations shifted differentially due to environmental parameters. This could potentially be explained, at least in part, by a shift in denitrifier population that is not mirrored in the total bacterial population, as discussed previously (Peralta, Ludmer, and Kent 2013). It is possible that total bacterial population varies significantly as well, but it would still be different than that of how the *nirS* denitrifier population shifts, otherwise one would expect normalization to show no significant difference.

Between sites, but with consistent channel locations, significant differences existed for all functional genes except for *nirS* only with respect to absolute copy number as indicated by copy number per gram dry weight soil/sediment (Table 4.2). No significant differences between sites were observed for any gene as normalized to 16S

rDNA copy number. Thus, while there were differences in *cnorBB*, *narG*, *nirK*, *nosZ1*, and *nosZ3* type denitrifier copy numbers, these differences may be explained by an overall difference in total bacterial population between sites, while proportion of denitrifiers remains constant. In order to demonstrate that the denitrifier populations were differentially impacted by environmental parameters from that of the total bacterial population, there would need to be significant differences between sites for reported 16S rDNA normalized data.

No significant differences in *cnorBB*, *narG*, *nirK*, *nosZ1*, and *nosZ3* normalized to 16S rDNA copy numbers between sites SM1 and SM2 were observed, meaning that while the two sites varied significantly in copy number of most functional denitrification genes, the abundance with respect to total bacterial population was the same. Consequently, it can be argued that the sites had differing levels of total bacteria, but the proportions of denitrifier groups to total bacteria remained the same.

Discriminant analyses (Figures 4.1, 4.2, and 4.3) suggested that functional genes profiles normalized to 16S rDNA differed over both inter-site and intra-site spatial scales. Denitrification is often a community process with each step controlled by different groups of microbes and, with fluctuations in any one of these steps, the overall process could be impacted such as in the case of a population decreasing enough to become limiting. These analyses demonstrate that different genetic profiles exist with respect to spatial sampling and also highlights the importance of considering all genes together rather than just one as it relates to denitrification, as changes in one gene's abundance that are not seen in the other genes may have significant impacts in overall denitrification and lead to confusing results. Nevertheless, results of this study support this idea in that there are distinct differences, highlighted by discriminant analyses, between the abundance of the different functional genes based upon location of sampling.

2.8.2 Soil Moisture

The lack of any significant correlation (Tables 4.3 and 4.4) between denitrifier gene copy numbers and soil moisture for both spatial scales, except for in the flooding zone, was not expected, but further demonstrates the complexity of the controls over the processes of denitrification. The graphical representations of both soil moisture and potential denitrification (Figures 4.6 and 4.16 or Figures 4.11 and 4.21, for example) show the same general trends of increasing at the same locations. This suggests some linkage between the two parameters. As potential denitrification is a microbially-driven measurement, it is likely that there is some relationship between soil denitrifier populations and soil moisture despite the exact nature of the relationship remaining unclear by way of direct comparison.

The statistically significant differences ($p=0.0003$ inter-site, $p=0.01$ intra-site) in values of moisture content between sites and between locations within each site can be explained by the disparity in range of values seen between the two sites and between channel positions, highlighted specifically by the flooding zone of SM2 in particular (Figures 4.5 and 4.7). The differences between flooding zone conditions at the time of sampling at both SM1 and SM2 may provide explanation for the wide range of values seen in SM2 but not SM1. SM1 was less likely to be temporarily saturated, never being submerged at the time of sampling. Conversely, the flooding zone location at SM2 was fully submerged at the time of at least one sampling date and likely at times throughout the year that were not sampled due to its much lower position on the bank.

The flooding zone is the only location to have resulted in relatively strong correlations (Tables 4.3 and 4.4) between soil moisture and functional gene copy numbers (except for *nirS*), which could be explained by these intermittent flooding conditions and shifts in soil moisture content. Previous research has demonstrated that irregular inundation of soils can partially explain higher denitrification rates (L. E. McPhillips et al. 2015), with denitrification being a process dependent upon microbes, which are measured using genetic methods (DNA, RNA). Given that soil moisture and potential denitrification were observed to be correlated ($R^2=0.67$, Figure 5.1), in the same way fluctuations from flooding events could explain the observed correlations between copy numbers of functional genes and soil moisture.

The disparity between flooding zone water content values can be further explained by a variation in soil and sediment texture, as properties such as pore space and surface area of the soil grains impact the ability to retain water (Castellini et al. 2015). While the channel at SM2 exhibited a coarse, sandy material overlaid by some silt, the flooding zone was of a dense and fine textured soil, leading to a differing ability to adsorb water. As the non-flooding zone exhibited the same soil properties as that of the flooding zone but dissimilar pattern of flooding in that it was never submerged, it must be concluded that the values can only be explained by both exposure to moisture and soil texture in conjunction.

2.8.3 Potential Denitrification

No distinct correlations or consistencies between potential denitrification and gene copy numbers, using either metric, were observed over either spatial scale, except for the flooding zone normalized to soil dry weight (Table 4.5), except for in the case of *nirS*. The flooding zone showing increased correlations between copy number per soil dry weight and potential denitrification may be explained by the assertion that intermittent flooding leads to increased denitrification (L. E. McPhillips et al. 2015) if copy number is, indeed, a significant control over denitrification rates. If this is true, however, the notable exception would be that of *nirS*, the copy number of which shows essentially no correlation with potential denitrification.

However, results of potential denitrification and functional gene copy number analyses closely mirror the trends observed in the analyses of soil moisture and functional gene copy numbers. For example, results in Figures 4.17 and 4.22 depict flooding zone potential denitrification and genetic measures reflect the soil moisture graphical counterparts demonstrated by Figures 4.7 and 4.12 with respect to range of and outliers in data. This is further reflected in the in-channel and non-flooding zone data, which both show more compact data ranges as with soil moisture data. Additionally, the large range of potential denitrification values observed in the flooding zone was observed only at site SM2 (Figures 4.15 and 4.20), which is mirrored by the soil moisture data.

While this comparison may prove useful in relating soil moisture and potential denitrification measurements, it should be regarded with caution as the acetylene block

method used for obtaining potential denitrification values comes with some associated error. However, despite this error, it would seem unlikely to see such well aligned results between the two parameters. In order to determine reliability, it would be prudent to attempt to repeat the measurements with other soil samples.

The ability to draw any conclusions with respect to relationship between genetic copy number and denitrification rate over spatial scales is confused by the conflicting results of studies on the topic, lending to the assertion that results of one location of study cannot easily be extrapolated over other locations (Giles et al. 2012). In this study there were three separate sites and, though they are all of riparian nature, it is known that riparian areas are highly heterogeneous (Burgin, Groffman, and Lewis 2010) and that this heterogeneity can lead to significantly different properties, such as soil texture, which relates directly to soil moisture, a property directly implicated in denitrification rates (Ducey et al. 2015). These facts explain why, even though the sites SM1 and SM2 were both the same type of location, there were notable differences between them in terms of potential denitrification rates which led to the inability to determine any spatial based relationships.

2.9 Abiotic Discussion

2.9.1 Potential Denitrification and Soil Moisture

Though the observed potential denitrification values cannot be fully explained by soil moisture values alone, the R^2 value of 0.67 suggests that the soil moisture content of soils and sediments is an important determinant in the ability of the system of denitrify.

Water holding capacity (WHC), or the ability of a soil to retain water, is one way to describe the soil moisture property of a soil and is impacted by an array of factors. Soil organic carbon (SOC) and total porosity (TP) are two such properties of a soil which impact the amount of water that is retained (Korkanç 2014), which also vary significantly by soil type. When soils become inundated, the ability of oxygen to penetrate past the uppermost layers decreases, which can lead to anoxic conditions, required for denitrification to occur. In this way, it makes logical sense that the moisture content of a soil or sediment is correlated with denitrification rates. The findings of this study are also

supported by previous work, which similarly observed positive relationships between soil moisture and denitrification rates measured by acetylene block methodology (Liu et al. 2016; Xiong et al. 2015)

2.9.2 Temperature

Temperature is an important variable to consider with respect to microbiologically mediated processes such as denitrification (Adouani, Limousy, and Sire 2015; Carrera, Vicent, and Lafuente 2003) due to the potential impacts on enzyme activities and nutrient availability. Each of the daily high temperatures were obtained and compared against averaged potential denitrification rate measurements for each sampling location per date, yielding no identifiable relationship. It should be noted, however, that there is little fluctuation in temperatures on the sampling dates (Table 5.1), save for a single drop on May 13 2015. It is possible that the particular temperature is high enough so as not to prove limiting to the process of denitrification. A more reliable conclusion could be made if sampling were done in winter months so as to obtain a range of widely varying temperatures.

To further elaborate on the impacts of temperature, the water temperature data was utilized to compare against only in channel samples with respect to both soil moisture and potential denitrification parameters. Water temperatures appear to have little influence over potential denitrification measurements for in channel samples, despite temperatures ranging from 7.6°C to 22.6°C. The lack of any correlation between water temperature and in channel potential denitrification could mean that, despite the importance of temperature on denitrification (Adouani, Limousy, and Sire 2015), it is not the limiting parameter in the system at the times of sampling.

2.9.3 Dissolved Oxygen

Given the importance of oxygen with respect to the process of denitrification, it is logical that oxygen availability has some impact on the observed potential denitrification results. The analyses, which compared dissolved oxygen with soil moisture values (Figure 5.5) and potential denitrification values (Figure 5.6), support the claim that oxygen availability can partially explain potential denitrification results.

The concentration of dissolved oxygen in a stream is impacted by numerous other factors, both biotic and abiotic. Biotic factors, such as photosynthesis and decomposition, are impacted by the plants, algae, and bacteria present that are able to produce oxygen, as well as microbes present which are responsible for the decomposition of organic material, thereby removing oxygen. Abiotic factors include but are not limited to turbulence, which is impacted by the streambed properties, wind, and velocity of the moving water, and temperature. The ability of gasses, such as O_2 to dissolve in water, is dependent in part upon temperature, with colder temperatures able to dissolve greater concentrations of oxygen.

The observed negative relationship between dissolved oxygen and soil moisture seems reasonable in that as soil or sediment becomes saturated the ability of oxygen to penetrate through the soil is markedly diminished (Cook, Knight, and Kelliher 2013; Uteau et al. 2015). The ability for oxygen to penetrate is not the sole determinant of oxygen availability within streambed sediment, however, as oxygen may be evolved through microbial processes, or through sheering of the sediment layers depending upon the velocity of the water current.

It is known that denitrification is greatly diminished by, or does not occur, in the presence of oxygen, though many denitrifiers are able to carry out denitrification as well as aerobic respiration, so the presence of denitrifying genes is likely to be ubiquitous in both oxic and anoxic environments. Denitrification potential determines the capacity for denitrification to occur only, and does not give the true amount of denitrification that is occurring at the time of sampling. Additionally, denitrification can still occur in soil/sediment environments even in the presence of oxygen in small aggregates of soil, which create anoxic conditions despite oxic bulk soil conditions. Factors such as these may provide insight into why the observed relationship between oxygen and potential denitrification rates is not a more strongly negative one, as would be expected.

2.10 Redundancy and Correlation Analyses Discussion

2.10.1 Redundancy Analysis

While nitrate or other reduced nitrogen species are required for and produced by the process of denitrification, nitrate was not observed to correlate with any significance with measured potential denitrification rates. Most likely, this is because nitrate is not the limiting variable over denitrification rates in the system. Given that the watershed is an agricultural one, nitrate levels may remain high enough in the soil from agricultural runoff that it is never the most limiting reactant.

Potential denitrification rates correlate with moderate strength with the denitrification functional gene copy numbers as they are normalized to soil dry weight only. This same correlation is absent with respect 16S rDNA normalized functional gene copy numbers. This is not surprising given that the two methods of normalization report the gene copy numbers as either absolute abundance, in the case of soil dry weight, or as relative as with 16S rDNA normalization. Reporting the proportion of the bacterial community that are denitrifiers is not necessarily meaningful with respect to denitrification rates because the proportion can change, while the absolute abundance of denitrifiers remains the same. In the case that the non-denitrifying bacterial community increases or decreases where the denitrifying community does not, the proportion will reportedly change, but the denitrification rate should not be impacted.

The site locations showed differential strength in terms of correlation to potential denitrification rates, SM2 being the strongest and the Park site with no correlation and this can be explained by the vastly different conditions at each site, though which conditions have the most control are not yet understood. SM2 and the Park sites differ from one another in terms of soil types present, with the Park having had a sand and gravel sampling location, and SM2 with a more finely textured silt.

The month of June having the highest correlation with measured nitrate levels can easily be explained by the timing of application of fertilizer on the surrounding agricultural fields, which is done in the month of May. This is further supported with the measured nitrate concentrations being the lowest in the month of May, with sampling

times likely occurring before fertilizer application, or at least before any significant amount could leach from the fields.

Differences in soil bulk density over both temporal and spatial scales can be explained by the sites being drastically different and high heterogeneity within some of the sites. That is, while the Park site tended toward sandier stream bottoms, some samples were more filled with gravel. As well, at site SM1 the bottom of the channel was both filled with gravel and silt, and so different sampling dates likely had slightly different bulk densities. Site location being the most impactful factor in determining bulk density of a sample makes sense, however, given the vast differences between locations with respect to soil or sediment.

Soil moisture levels varying between sites mimics the potential denitrification observations, which makes sense given the correlation between the two that has already been discussed. SM2 having the highest moisture content values could be due in part to both the flooding zone being intermittently submerged, which did not occur with the flooding zone at SM1 at the time of sampling, and also in part due to soil differences. While SM1 and the Park site had some gravel or sand, SM2 was dominated by very fine grain sediment and soils. These soil type differences can and likely did lead to differences in water holding capacity, and subsequently water content.

Surprisingly, there was no significant difference ($p = 0.115$) between the sites with respect to organic carbon measurements. This was unexpected because the Park site did not appear to have much detritus or biological matter in the samples, while each of the other sites did. In fact, the measured organic matter fluctuated greatly in the Park samples, with them being markedly lower than the other locations with few exceptions.

2.10.2 Correlation Analysis

The varying strength in correlations between the different functional genes with respect to other functional genes over both normalizations can potentially be explained in two ways, depending upon the two genes being compared. The first explanation involves two genes that are functionally redundant, in this case *nosZ1* and *nosZ3*. The correlation between the two when normalized to soil dry weight ($R^2=0.542$, $p = 0.001$) suggests a

relatively strong relationship and this may be because the two are mutually exclusive within microbes. That is, if *nosZ1* is possessed, then *nosZ3* is not. In such a case, the two will relate to one another based upon which populations exist in a sample assuming that the populations are differentially successful under different conditions.

The other explanation is that perhaps some genes occur together with different frequently than others, leading to stronger correlations. For example, *nirK* and *cnorBB* had a notably strong correlation (R^2=0.86, $p < 0.0001$) when normalized to soil dry weight, suggesting that the two tend to fluctuate in abundance in the same manner.

Over either normalization, *nirS* was the exception to the correlations observed between most other genes. For example, it might have been expected that *nirS* have some correlation of significance with *nirK* given that they are functionally redundant and possessed by discrete populations, such that when one is abundant the other is not. There was no significant or strong correlation between these two genes, however. This is potentially due to problems in the protocol for *nirS*, leading to potentially erroneous measurements.

The correlations between soil moisture and functional gene copy numbers normalized to 16S rDNA being weak and lacking significance makes sense because it seems unlikely that soil moisture would have direct control over the proportion of the bacterial population that are denitrifiers. There could be some indirect control by means of oxygen availability, however many denitrifiers are facultative anaerobes and do not require the absence of oxygen to grow at all. This means that denitrifying microbes will still possess denitrification functional genes, even if they are not being translated and transcribed. The correlation that exists between soil moisture and several of the functional genes could be due to those particular gene bearing populations being more sensitive to water availability.

As discussed, soil moisture and potential denitrification correlated significantly and this makes sense given that soil moisture has the potential to impact oxygen availability and denitrification does not occur to any sizable degree in the presence of oxygen. Soil moisture and bulk density are strongly correlated as bulk density is

influenced by the type of soil or sediment and its properties, such as pore size, which control the ability of the soil to hold water.

Potential denitrification correlating significantly with the functional denitrification genes is intuitive simply because the genes being present are required for denitrification to occur, and the capacity of a sample to denitrify was expected to be impacted by the gene copy numbers. The same was not true with respect to denitrification gene abundances normalized to 16S rDNA, likely because the absolute copy number is more impactful than the proportion of the population bearing the genes.

The strength in correlation between potential denitrification and bulk density of the soil/sediment makes sense in that bulk density is influenced by the same properties of the soil which likely impact the amount of oxygen available. More densely packed soil, for example, should have less oxygen penetration and this would mean that denitrification is more likely to occur given all other conditions for the pathway to proceed are present.

Organic matter content of the soil/sediment samples correlated with significance to only *cnorBB* and *nirK* when normalized to soil dry weight, and the reason for this is not entirely clear. It is possible that the *cnorBB* and *nirK* bearing species have different demands for carbon than that of other functional gene bearing denitrifiers. The strength of the correlations should be noted as being only moderate, however.

The moderate to strong correlations between bulk density of the soil and the soil dry weight normalized functional genes can be explained by bulk density having a hand in controlling the amount of available water, as well as organic matter.

The lack of correlation between any of the functional gene copy numbers over either normalization and nitrate levels, as well as any of the other abiotic factors and nitrate levels, comes as a surprise. These agricultural systems are flooded with nitrate at various times of the year, and the Park site is historically high in nitrate, so it is possible that it is simply because nitrate is not a limiting variable. The lack in correlation between potential denitrification and nitrate specifically can potentially be explained by the potential denitrification measurements done with samples amended with nitrate,

phosphate, and carbon sources so as to get the maximum ability of the samples to denitrify under the condition that no nutrient is limiting. In such a case, the ambient levels of nitrate are less meaningful when comparing to the potential denitrification measurements.

2.11 Possible Hotspots

The flooding zone of site SM2 was the source of the consistently highest potential denitrification readings, flagging it as a potential hotspot for denitrification. Overall, site SM2 (Figure 4.15) had notably higher potential denitrification rates (Figure 6.1) than that of SM1 (Figure 4.14), and the flooding zone (Figure 4.17) was the source of almost all observed increases, except for one increase that occurred in the channel location (Figure 4.16).

While the single in channel increase was the highest potential denitrification value observed, it sits as an outlier against all observed in channel values and cannot be considered a hotspot. It would be more correct to consider the location a hot moment, or moment in which the conditions are such that there is a marked increase in denitrification. The exact conditions that lead to this increase are not known, though soil moisture values reflected the same increase in value. Given the relatively strong relationship between the two as reflected by the R^2 value of 0.67 as well as previous work reporting similar findings (Liu et al. 2016; Xiong et al. 2015), soil moisture can likely explain a portion of the increased value. It would be useful to examine the sediment properties for any temporal changes that would lead to a shift in the soil moisture, such as increase in organic matter, and explain the increase in potential denitrification that occurs at only one date for this site.

The flooding zone sampling location of site SM2 was the most variable of all sites and locations due to erratic flooding patterns in this drainage ditch. Several times during sampling it was observed that the sampling area was entirely submerged due to a rising stream level. This rise and fall of the water that lead to complete saturation could at least partially explain the consistently higher values of potential denitrification, which has been suggested by previous work (L. McPhillips and Walter 2015). When the location becomes flooded, it is possible that new nutrients are introduced that allow the microbes

there to thrive. If this influx of nutrients is met with a similar decrease in oxygen, the conditions would be right to potentially see an increase in denitrification.

It should be noted that, while the Park sampling site in the channel experienced similar intermittent flooding events, so that the location became fully submerged, there was no corresponding increase in potential denitrification values. In general, the Park results showed low potential denitrification and soil moisture values, as well as less denitrifier gene presence, potentially explained by the larger particle size of the sediment material (Jia et al. 2016). The sediment was primarily composed of small rocks and gravel as well as sand, the properties of which are not conducive to higher water holding capacity and therefore soil moisture levels.

2.12 Conclusions

In summary, the results of the study presented were unable to pinpoint a universally applicable relationship regarding denitrification functional gene copy number normalized either to soil dry weight or 16S rDNA copy number and measured potential denitrification or soil moisture values. However, there are both temporal and spatial scales that resulted in significant correlations, depending upon the normalization method used.

In the month of October, which included data from both 2014 and 2015 sampling dates and all locations, there were notable correlations between soil moisture and copy number with respect to soil dry weight, which may suggest that at this time soil moisture became a limiting parameter. There were no observed correlations between potential denitrification values and functional gene copy numbers over either normalization method.

Spatial analyses similarly yielded no significant relationships except for in the flooding zone, which considered data from both SM1 and SM2 in analysis, and can potentially be considered a hot spot for denitrification. Strong correlations as determined by R^2 values were obtained in analyzing soil moisture and functional gene copy numbers over both normalization methods, except for in the case of *nirS*. Additionally, potential denitrification values correlated well with functional gene copy numbers, though

normalized to soil dry weight only. Once again, *nirS* was the exception to the strong correlation found with all other genes studied.

Discriminant analyses demonstrated that samples taken across all dates tended to display functional gene profiles that were distinctly different between in channel, flooding zone, and non-flooding zone locations. The same clustering results were observed for temporal analyses, with the 2014 samples taken across all locations grouping closely based upon month of sampling.

With respect to abiotic parameters, potential denitrification values and soil moisture values demonstrated a strong correlation (R^2=0.67). Additionally, there was found to be no correlation between air temperature and either soil moisture or potential denitrification values. Dissolved oxygen from water sampling showed a slight correlation with potential denitrification measurements taken from in channel measurements.

Overall, while some trends could be established, no overall conclusions could be made with respect to all data. To further investigate the controls over denitrification, as well as to identify hot spots of activity, such as in the flooding zone, studies should include mRNA or enzyme analysis.

Chapter 3. Future Directions

In exploring the relationship between the genetic basis for denitrification, measured by associated functional genes such as *nirK* and *nosZ* amongst others, and potential denitrification, it is of utmost importance to have accurate concentrations of both genes and currently there are issues that arise with respect to both parameters. The measurement of genetic copy numbers relies almost fully on the primers available, though handling and the application in qPCR to a lesser degree. Also, potential denitrification measured with the acetylene block method is known to produce results of varying reliability. With inaccuracies in both genetic measurements and with the values meant to describe denitrification rates, it may be impossible to identify relationships and expand upon our understanding, even if relationships do exist.

Metagenomic approaches may prove to be the best way to get around the lack of reliable and accurate primer sets for these functional groups of microbes (Correa-Galeote and Tortosa 2013). High throughput DNA sequencing of environmental samples allows for the identification of new sequences, especially from that of those microbes that are currently unculturable. With new sequences available, primers can be more finely tuned to include as many permutations of denitrification functional genes as possible so that the most accurate measurements can be made.

Of notable exclusion from most studies that attempt to characterize denitrification rates are other non-bacterial microbes, such as archaea and fungi, both of which are known to possess denitrifying genes. In fact, it is well known that fungal biomass is often many times greater than that of bacterial biomass in a system (Ruzicka et al. 2000), suggesting that these organisms are likely quite impactful to their respective ecosystems. With the exclusion of such a ubiquitous group of microorganisms from the conversation it seems logical to assume that no truly useful relationship can be established between the genetic bases for denitrification and measured rates. To ensure understanding of the process of denitrification in real settings, it is of utmost important to include all denitrifying microorganisms in the conversation and in study, otherwise the results are at best inaccurate, and at worst unrealistic in application over broader contexts.

The acetylene block/inhibition method is a well-established and frequently implemented method for determining potential denitrification values (Yoshinari and Knowles 1976), often due to the expense involved as well as the ability for a large number of samples to be processed. However, there are issues with the process that should be considered when interpreting the results of the test. Issues include, but are not limited to, underestimates due to gaseous loss in the form of nitric oxide (Bollmann and Conrad 1997), soil sample properties leading to diffusion difficulties of acetylene gas (Oehler, Bordenave, and Durand 2007), as well as nitrification being blocked (Groffman et al. 2006). Though these limitations present problems for interpretation and certainly extrapolation, it is thought that within experiments the issues should be consistent among samples and therefore give reliable results relative to other samples (Orr et al. 2007). The problem remains, however, that the measurements themselves may be unreliable or inconsistent and therefore hinder the ability to observe relationships that are, in fact, present.

Genetic methods involving DNA extraction from soil samples and subsequent qPCR are powerful tools, but can be expanded upon by extending beyond just DNA to RNA and even protein studies. While DNA measurements are able to describe what exists in a given sample, it is unable to tell us anything about activity and function within the environment. For example, it is possible to extract intact DNA from an organism that is no longer living and is therefore not metabolically active, as well as the extraction of DNA that exists extracellularly in the soil. Additionally, there are circumstances in which a gene is possessed by an organism but is not currently used, or the situation in which an organism possesses multiple copies of a single gene. In both situations DNA cannot be used as a proxy for activity, which is what is most important when comparing to process rates, such as that of denitrification rates.

In order to get an accurate picture of activity within a sample, it would be preferable to use mRNA measurements, or perhaps even proteins. The quantification of mRNA, using RNAseq or RT-qPCR, can elaborate on which genes are actively being transcribed for use by the cell, and provides a more accurate picture of activity than that of DNA alone. There are issues which make the use of mRNA more complex than simply extracting the genetic material and running qPCR protocols as with DNA, such as the

existence of primers and more importantly, the lack of stability of mRNA after extraction. Degradation begins almost immediately and requires the samples to be processed directly after extraction, which can prove difficult. Additionally, the processing is much more expensive and requires an experienced hand, making the method costly for researchers. However, if reliable results can be obtained, the use of mRNA would prove invaluable in further elaborating on what DNA alone cannot explain.

In an effort to expand upon the field sampling done for the project, several laboratory experiments were done at Saint Anthony Falls Laboratory's Outdoor Stream Lab (OSL), which will be the focus of future analyses and study mean to supplement the SMC study. The experiments included controlled flooding events of the OSL and sampling analogous to that of which was done in the field, including at time points just before flooding, directly after flooding, and several days after flooding events. Additionally, basin flooding experiments were done to more closely mimic the banks under flooding conditions. The data that results from such experiments will help provide insight into the SMC results with respect to applicability over various locations and times, as well as provide further context for previous results.

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