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Microbial methanogenesis, carried out by methanogens, and methane oxidation, carried out by methanotrophs, are integral parts of the carbon cycle and global climate change. These two processes have received attention in recent years in aquatic systems including major rivers, lakes, wetlands, and oceans. However, far less attention has been paid to flowing waters, especially low order streams, and to the potential impact of methane cycle processes in these environments. Streams are important to carbon cycling because as connectors to terrestrial ecosystems they are sites of organic matter processing, and important to biogeochemical cycling across the landscape. Twelve streams in the piedmont of North Carolina, consisting of three stream types: forested, urban unrestored, and urban restored, were sampled seasonally. Genomic DNA from seston, sediment, and epilithon samples was extracted and real-time PCR performed to analyze the abundance of methanogens and methanotrophs using group specific primers. Water samples were taken to measure methane concentration. There were significant differences in methanogen DNA abundance in late fall and methanotroph DNA abundance in late summer between stream types superimposed on a background of seasonal differences. The differences may be explained by the timing and magnitude of organic matter and inorganic matter inputs in forested versus urban streams. Urban unrestored streams receive a large pulse of input in late fall and little through the winter

because stormwater is piped in directly to the streams from impervious surfaces; in contrast forested streams receive organic input over time because it enters the stream through subsurface flow.

METHANOGENS AND METHANE OXIDIZING BACTERIA IN FORESTED, URBAN UNRESTORED, AND URBAN RESTORED STREAMS

by

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To all my friends and loved ones who have helped me through this endeavor. I would have not been able to achieve this dream and get through this without your endless support and love. Thank you.

APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Background

Methanogens and methanotrophs influence carbon cycling, greenhouse gasses, and global warming through methanogenesis and methane oxidation. Carbon dioxide is the most abundant greenhouse gas in the atmosphere: in 2005 the concentration of CO_2 in the atmosphere was 379 ppm (IPCC 2007); CO_2 rose to a global monthly mean of 399 ppm by June 2013 (NOAA). Methane is the most abundant hydrocarbon in the atmosphere and an important natural component in the global carbon cycle (Caldwell *et al.* 2008). It is deposited into the atmosphere at a rate of 500-600 Tg of methane per year from natural and anthropogenic sources (Conrad 2009, US EPA 2001, Reeburgh 2004). In 2011 the concentration of methane was 1816 ppb, increasing at a rate of 0.4% - 1% per year (US Dept of Energy 2013, NOAA 2013, IPCC 2007). Methane is twenty-one times more effective at trapping heat in the atmosphere than carbon dioxide (US EPA 2011, Smith *et al.* 2003). Methane contributes roughly 30% of all anthropogenic radiative forces to global climate change (Conrad 2009). This relatively high effectiveness and increasing concentration are of concern because of the effect increasing greenhouse gas effect on climate. Natural sources of methane include microbial processes linked to plants, aquatic sediments, termites, and gas hydrates

(Conrad 2009, Whalen 2005). The largest natural source of methane is wetlands (Whalen 2005). Anthropogenic sources of methane include landfills, reservoirs, agriculture, wastewater treatment plants, and biomass burning.

Methanogens and Methanogenesis

Methanogens produce methane through anaerobic decomposition for energy generation for growth. Methanogens are unique to *Archaea* and consist of three families, including twenty genera of methanogenic bacteria (Khalil 2000). Four of these genera have been found to inhabit freshwater sediments. They have large habitat diversity, but their distribution in the habitat depends on species optimum temperatures, pH, and salinity ranges (Jones *et al.* 1987, Khalil 2000). In general methanogens are found in higher temperatures, up to 40°C (104°F), and basic pH; however, there are species of methanogens that have been found in lower and higher temperatures and pH's (Khalil 2000). Methanogens are sensitive to the oxygen exposure (Whalen 2005).

Methanogenesis begins with complex organic matter, from the biomass of dead organisms and other carbon inputs, and consists of two main steps: fermentation (including syntrophic acetogenesis) and methanogenesis. In fermentation, organic matter is first decomposed by a number of species of non-methanogenic bacteria, and a small number of substrates, metabolites, are produced that methanogens are able to use (Khalil 2000) (Figure 1). These non-methanogenic bacteria are hydrolytic and

fermenting bacteria, H^+-reducing bacteria, and homoacetogenic bacteria. Methanogens are completely dependent on products of fermentation (Jones *et al.* 1995), which include hydrogen plus carbon dioxide, formate, acetate, methanol, methylamines, and methylsulfides (Jones *et al.* 1987, Khalil 2000). The substrates are then used in the last step, methanogenesis, where the final catabolic product is methane. Methane can be produced through a number of different pathways reflecting which substrate is used, species of methanogen, and perhaps environmental conditions (Figure 2).

Biogenic methane is produced predominantly through either acetoclastic methanogenesis or hydrogenotrophic methanogenesis (Whalen 2005). In freshwater sediments about thirty percent of methanogens are hydrogenotrophic using carbon dioxide and hydrogen as their substrates. Hydrogen is used as the electron donor to reduce carbon dioxide to produce methane and water. About seventy percent of methanogens are acetoclastic and use acetate as their substrate to produce carbon dioxide and methane. Only a small fraction of methanogens use formate, methanol, methylamines, or methylsulfides as substrates. All methanogenic pathways involve the cooperation of seven coenzymes, six of which are unique to methanogens and membrane-bound (Jones *et al.* 1987). An essential coenzyme in methane formation is MCR, methyl-coenzyme M reductase (Ermler *et al.* 1997). MCR is involved in the final step in methanogenesis and catalyzes the reduction of methyl-coenzyme M to produce methane. This process is one of the significant pathways for organic matter decomposition in anaerobic freshwater systems (Boon and Mitchell 1995).

Methanotrophs and Methane Oxidation

Methanotrophs oxidize methane to carbon dioxide. They are comprised of five genera of eubacteria, and two types (type I and type II) which have all been found in waters and aerobic microzones of sediments (Khalil 2000, Buriánková *et al*. 2012). Most methanotrophs obtain energy for growth through the oxidative pathway of methane, primarily through an intermediate, always releasing carbon dioxide and water as the product (Whalen 2005). These bacteria are predominately found where there is a supply of methane (Khalil 2000). Their habitats are varied, and like methanogens, their dispersal in the habitat depends on a variety of factors including optimum temperatures and pH levels. Methanotrophs have been shown to be able to deal with periods of anoxia.

Methane oxidation can occur both aerobically and anaerobically, although anaerobic methane oxidation has only been shown to occur in marine aquatic systems (Eller *et al.* 2005, Khalil 2000). Methane oxidation is controlled by temperature and pH (Whalen 2005). The optimum temperature for the oxidation of methane is between 25 and 35°C and the optimum pH is between 6.7 and 8.1, however it has been shown to occur at other temperatures and pH values (Bender and Conrad 1995). The stepwise pathway of methane oxidation goes from methane, to methanol, to formaldehyde, to formate, and finally to carbon dioxide as the final product, unless the intermediate formaldehyde carbon is used for growth (Whalen 2005). For every methane molecule

oxidized, one carbon dioxide molecule is produced. All methanotrophs have the enzyme methane monooxygenase (MMO), which catalyzes the initial step in the oxidative pathway. This enzyme is membrane-bound and requires oxygen. Every methanotroph genus has the particulate form of this enzyme, requiring much less oxygen; however, some also have the soluble form (Whalen 2005).

Methanogenesis and Methane Oxidation in Aquatic Ecosystems

Methanogenesis and methane oxidation balance the amount of methane released into the atmosphere. It is well known that methanogenesis and methane oxidation are important in most aquatic ecosystems, including freshwater, and they are important contributors to the global methane budget (Bastviken *et al.* 2011). Methanogenesis occurs in the anaerobic zones of aquatic systems, within the sediments and possibly in the anaerobic parts of microbial mats. Methane produced by methanogenesis can be released to the atmosphere where it can act as a greenhouse gas, or may be intercepted by methanotrophs (Figure 3). Methane oxidation occurs in aerobic habitats at the sediment water interface and in the water column of these aquatic systems (Khalil 2000). It is estimated that methanotrophs oxidize between 10 and 90 percent of biogenic methane before it is released into the atmosphere (Semrau *et al*. 2010). While methanogenesis and methane oxidation have been studied in lakes, wetlands, rivers, and marine systems, they have not been well studied in streams (Jones and Lennon 2009, Whalen 2005, Wilcock and Sorrell 2008). These processes are

important to evaluate in streams since streams drain most of the earth's land surface area. In the United States alone there are an estimated 3,533,205 miles of low and high order streams (US EPA 2009). Since streams are the connectors of terrestrial systems and a conduit for organic matter, they are potentially important in methane cycling.

Urban Stream Syndrome

Size, length, physical, and chemical and biological health vary widely among streams. In the US EPA's 2004 National Water Quality Inventory report to Congress a total of 44% of US streams that were assessed had been deemed impaired, and 3% of the streams assessed were good but threatened. Thus, an estimated of 1,660,606 miles of US streams are either impaired or threatened. Many of these streams are in urban areas.

Urban stream syndrome is defined by a list of symptoms that are often seen in streams within urban areas (Walsh *et al.* 2005), describing physical, chemical, and biological effects of urbanization on streams (Feminella and Walsh 2005, Finkenbine *et al.* 2001, Meyer *et al.* 2005, Paul and Meyer 2001, Walsh *et al.* 2005). One physical effect is a change in hydrology of urban streams due to the high amount of impervious surfaces in urban watersheds, which creates a large amount of surface runoff, leading to high flows that increase rapidly during rain events. Changes in geomorphology are another physical effect of urban land use on streams (Paul and Meyer 2001). The stream channel changes result in increases in width and depth due to bank erosion. This

erosion can be amplified by the removal of riparian zones. Humans also channelize urban streams resulting in the removal their natural bends and meanders. This can result in a lower water table around the streams, decreasing ground water recharge (Paul and Meyer 2001). There are also physical changes as a result of sediment supply into the streams. Urban streams are typically warmer than non-urban streams, another common physical change. Changes in water chemistry include an increase in nutrients, changes in organic matter input, and lower oxygen levels (Walsh *et al.* 2005). The lower oxygen levels are attributed to the increase in sedimentation. Biological changes in these streams include losses in fish and invertebrate communities, as well as changes in algal biomass, and dominance of pollution tolerant species (Walsh *et al.* 2005, Paul and Meyer 2001). Currently 75% of the United States population resides in urban areas with an expectation that urban areas will be even larger by 2030 (US Census Bureau 2001). With the increase of population, more streams are likely to exhibit these symptoms.

It is apparent that urban streams are different than forested streams. It is assumed that restoration of urban streams at least partially mitigates these symptoms depending on the type of restoration chosen. However the impacts of stream restoration are seldom reported, consequently our knowledge of its success is not extensive (Bernhardt *et al.* 2007). It is likely that even urban restored streams are still impaired. Restorations can be passive, letting the riparian zone regrow, or they can be active, using engineered structures. Structures include vanes to redirect the natural flow of the stream, bank stabilization structures, and step-pools to control the grade of

the stream (Bernhardt *et al*. 2007). The balance between methanogenesis and methane oxidation can be altered by many factors such as lower oxygen and increased organic matter often found in urban unrestored streams compared to non-urban streams (Walsh *et al.* 2005). It is likely that restoration affects these factors, which could change the amount of methane released from aquatic environments.

Current Research

Around the world streams are affected by urbanization that changes their physical and chemical characteristics, possibly changing the population of microbes in them. Together, methanogens and methanotrophs contribute to the global carbon budget through methanogenesis and methane oxidation. Many chemical, physical, and biological factors have been shown to affect both of these processes (Whalen 2005). These processes have been studied in wetlands, lakes, and rivers; but few studies have been done on low-order streams. Microbial populations can be studied a number of different ways. It has been shown that real-time polymerase chain reaction (PCR) is a suitable method for detecting methanogens and methanotrophs in water and aquatic sediments (Gentzel *et al.* 2012). In many studies it has been shown that methanogens and methanotrophs can be abundant in flowing aquatic systems and that these communities affect methane cycling processes (Buriánková 2012). As land use continues to change and urbanization increases, it will be important to study these processes further in flowing aquatic systems.

Objectives and Hypotheses

In my study I explore the extent to which landscape setting and urban stream restoration affect the microbes that control methanogenesis and methane oxidation. To explore this I have three objectives. My first objective in this study is to determine whether stream type affects methanogen communities. I hypothesize that methanogens are more abundant in urban unrestored streams compared to urban restored and forested streams due to increased sedimentation, creating a larger anoxic zone (Walsh 2005). My second objective in this study is to determine whether stream type also affects methanotroph communities. I hypothesize that methanotrophs are more abundant in urban unrestored streams compared to urban restored and forested streams in response to higher abundance of their carbon source provided by the expected higher abundance of methanogens (Pimenov *et al.* 2010). My third objective is to determine whether urbanization and stream restoration affects methane concentrations in low-order streams. I hypothesize that there is more methane concentration in urban unrestored streams compared to urban restored and forested streams in response to a larger abundance of methanogens.

CHAPTER II

METHODS

Study Site

Samples were taken from 12 streams in the piedmont of North Carolina (Figure 4, Table 1). The streams consist of three types: forested, urban unrestored, and urban restored streams. Streams were chosen based on physical similarity (Table 2). All four urban restored streams reestablished riparian zones by planting vegetation, stabilizing stream channels, stabilizing banks, and reforestation (City of Greensboro 2011). Specifically, Spring Valley Park had step pools constructed to accommodate the high amount of runoff, and Benbow Park had an aerial sewer that was relocated. All streams were sampled seasonally starting in the summer of 2011 until the spring of 2012. Sampling always occurred at least 24 hours after a rain event as to try to always sample at base flow rate in streams. Precipitation and air temperature data was acquired from weather underground (wunderground.com). The riffle sizes and pool sizes for each stream were measured during sampling periods. Within each stream, three riffles and three pools were selected for sampling. Water, sediment, and epilithon were sampled for DNA at each site in each stream. Separate water samples were taken for methane concentrations.

Sampling for DNA Analysis

Water samples were collected with wide mouth Nalgene bottles (250 mL) from each riffle and each pool chosen along each stream. The bottles were cleaned in a bleach solution and rinsed with deionized water before every collection. The water samples were pooled by collecting water from three spots within the riffles or pools in the Nalgene bottles at each location. Pooled water samples were taken to get an adequate representation of seston at each location. Samples were put on ice and brought to the University of North Carolina at Greensboro (UNCG). Once at UNCG, samples were filtered using 25 mm glass fiber filters (GFF) to collect seston. Before filtering, each bottle was mixed thoroughly by inverting and shaking. Each filter was placed in a pre-weighed 15 mL polyethylene Falcon tube containing 2 mL of cetyltrimethylammonium bromide buffer (CTAB) for DNA extraction.

A pooled epilithon sample was taken at each riffle and each pool chosen along the stream to get an adequate representation of epilithon from each location. Within each riffle and pool 3 rocks were collected for an epilithon sample. A known area of epilithon was scraped off each rock into a Nalgene basin using a sanitized toothbrush. The area was then rinsed with deionized (DI) water to wash off the remaining epilithon that was loosened by the toothbrush. If the bottom was not rocky, then sticks, or a combination of rocks and sticks, were used. Approximately six inches of epilithon was brushed off of the stick into the basin and then rinsed with DI water. All the sticks had

approximately the same diameters, one inch or less. This allowed for similar areas of epilithon. Once the epilithon had been collected into the basin, it was mixed well and filtered on site using a 25 mm glass fiber filter (GFF). The filter was then put into a pre-weighed 15 mL polyethylene Falcon tube containing 2 mL of CTAB buffer for DNA extraction.

A sediment sample was also taken at each riffle and each pool chosen along the stream. Three individual samples were taken with a 1.5 mL centrifuge tube within each site. These samples were pooled together into a weigh boat so that there would be an adequate representation of sediment at each location. The pooled sample was mixed thoroughly by swirling. Approximately 0.5 cm^3 of the sediment from the weigh boat was collected with a 1.5 mL centrifuge tube and placed into a pre-weighed 15 mL polyethylene Falcon tube containing 2 mL of CTAB that for DNA extraction.

Sampling for Methane Concentration

At each stream, in each riffle and each pool, a 3 mL water sample was collected using a 5 mL syringe with a needle size of 22 ½ gage and injected it into an Exetainer® or a serum vial that had previously been evacuated, filled with Nitrogen, and then fixed with 0.1 mL of 1 molar HCl. The HCl killed any microbes in the water to prevent the methane in the sample from being oxidized. Each 3 mL water sample was a pooled sample taken by drawing 1 mL of water from three different spots in the riffle or the pool. All the water samples in the Exetainers® or serum vials were brought back to

UNCG and stored upside down until measurement of methane concentration in the headspace at Dr. Stephen C. Whalen's lab at The University of North Carolina at Chapel Hill (UNC) could be taken.

DNA Analysis

I used a CTAB DNA extraction protocol that has been found to be successful for environmental DNA isolation (Schaefer 1997, Stewart 1993). After DNA isolation was completed, DNA concentration (ng/µL) from each sample was measured on a Nanodrop spectrophotometer to determine if dilution of the sample, to 5 ng/µL of genomic DNA, was needed for real-time PCR. Once all genomic DNA samples were measured and dilutions were performed, if needed, real-time PCR analyses were performed for the detection, amplification, and quantification of methanogens and methanotrophs.

For real-time PCR runs I used the Applied Biosystems StepOne™ real-time PCR System. Each 48 or 96 well plate contained 3 negative controls, triplicate DNA samples, and 3 or 4 triplicate concentrations of standards for positive control. Each well contained 10µL of Power SYBR® Green PCR master Mix (Life Technologies), 1µl of forward primer, 1µl of reverse primer, 8µl of sterile DI water, and 1µl of the genomic DNA sample. The PCR run method used was specific to methanogens and methanotrophs (Gentzel *et al.* 2012). The StepOne™ started by heating up the samples to 95 C° as an activation step, followed by forty cycles of 95 C° for 15 seconds, to 55 C°

for 30 seconds, to 72 C° for 60 seconds, and 78 C° for 15 seconds during which the fluorescence was measured. This was followed by a melt curve step.

Wright and Primm (2003) developed the methanogen specific PCR primer sets by assessing conserved regions of the MCR16S gene from eighty-two methanogens. The primer set that I used in each real-time PCR run to assess the genomic DNA for methanogens were Met86F and Met1340R (Table 3). With Met86F and Met1340R, I used a standard from genomic DNA of *Methanosarcina acetivorans* Strain C2A (ATCC® catalog item number 35395D-5). The primer set employed to assess the genomic DNA for methanotrophs was developed to amplify the pmoA gene, one of the genes that is involved with the particulate MMO (Costello and Lidstrom 1999). The primers developed were named A189gc and mb661 (Table 1). When using A189gc and mb661, I used a standard from genomic DNA of *Methylococcus capsulatus* Strain C2A (ATCC® catalog item number 35395D-5).

Methane Analysis

The Exetainers® and serum vials with water samples were brought to Dr. Stephen Whalen's lab at UNC to determine methane concentration using gas chromatography (GC). During storage and transport the samples were kept upside down as to minimize diffusion of gas through septa. Samples were analyzed on a Shimadzu Model GC8A Flame Ionization Detection gas chromatograph, using the following protocol (Whalen and Reeburgh 1988). A syringe was inserted into the

Exetainer® or serum vial containing the gas sample and was washed with the sample several times before a 5 mL aliquot was taken up in the syringe. Before the syringe was inserted into the chromatograph about 0.5 mL was evacuated from the syringe to remove any water contaminants that may have entered the needle when obtaining the aliquot. The needle was then wiped clean and the sample is injected into the gas chromatograph. The methane gas concentration was read at approximately 0.8 minutes retention time. The methane concentration (ppm) from the gas chromatograph was converted to correct for headspace volume and sample volume to obtain the total methane concentration (μg CH_4/L) in the sample (S. Whalen, University of North Carolina, School of Public Health, personal communication).

Statistical Analysis

I used IBM SPSS® statistical software for all my analysis (SPSS® Inc, Chicago, IL). A p-value of < 0.05 was considered a statistically significant difference. The Shapiro-Wilk test was first run on each dependent variable to test for normality. All seston, sediment, and epilithon data failed the Shapiro-Wilk test (all data $P > 0.05$), so log transformations were performed. When the data were log transformed, another Shapiro-Wilk test was run on the data. All log-transformed dependent variables were normally distributed (all data $P > 0.05$). I then ran two-way mixed ANOVA's on the methanogen and methanotroph log-transformed genomic DNA concentrations to evaluate if there were any significant differences between stream types, seasons, or an

interaction between seasons and stream types. Each two-way mixed ANOVA contained samples from pools and riffles from seston, sediment, or epilithon logged genomic DNA data from methanogens or methanotrophs as the dependent variable. Season and stream type were used as the fixed factors. If significant differences were seen for seasons and/or stream types, one-way repeated ANOVA's were run, with a Tukey post-hoc test to evaluate which seasonal or stream type differences were significant.

I ran a two-way ANOVA on the methane concentration to evaluate whether stream types and seasons have an effect on the concentration. The two-way ANOVA contained the methane concentration in parts per million (ppm) as the dependent variable. Season and stream type were used as the fixed factors. Before running the two-way ANOVA, the Shapiro-Wilk test was run on the data. All methane concentration data were considered normal ($P > 0.05$). If significant differences were seen for seasons and/or stream types, one-way ANOVA's were run, with a Tukey post-hoc test to evaluate which seasonal or stream type differences were significant.

CHAPTER III

RESULTS

Despite effort to identify streams with similar physical characteristics, they exhibited some variability (Table 2). The widths of riffles sampled ranges from 23.4 to 273.5 cm. The depths of riffles sampled ranges from 1.8 to 7.4 cm. The widths of pools sampled ranges from 112.8 to 371 cm. The depths of pools sampled ranges from 8.86 to 31.2 cm. The twelve streams varied slightly in sediment type from sandy to rocky sediments (cobbles and boulders in some streams). Two of the four forested streams were mostly sandy; whereas the other two forested streams contained rocky sediments (cobbles and boulders) in the riffles and some in the pools. The urban unrestored streams all contained a mixture of sandy and rocky sediments in the riffles and pools. The restored streams also all contained a mixture of sandy and rocky sediments.

Patterns of Methanogen Abundance

Average abundance of methanogen DNA from sediment samples in forested streams ranged from $1.42x10^3$ to $2.32x10^7$ pg/g with the smallest average abundance in early summer and the largest in spring. Samples in urban unrestored streams ranged from $6.92x10^3$ to $2.62x10^8$ pg/g with the smallest average abundance in early summer and the largest in late fall. Samples in urban restored streams ranged from $2.48x10^4$ to

$3.53x10^9$ pg/g with the smallest average abundance in early summer and the largest in late fall (Figure 5). A two-way mixed ANOVA run using yearly log methanogen data from sediments (pg/g) showed that there were significant differences between stream type ($p < 0.0001$) and seasons ($p < 0.0001$) (Table 4). When Tukey post-hoc tests were run with seasonal log methanogen DNA abundance data as the dependent variable and stream type as the factor it was found that urban restored streams were significantly higher in methanogen abundance from sediments than urban unrestored streams and forested streams (urban unrestored streams $p=0.022$; forested streams $p=0.001$) in early summer. In late fall it was found that forested streams were significantly lower in methanogen abundance from sediments than urban unrestored streams and urban restored streams (urban unrestored streams $p=0.016$; urban restored streams $p=0.011$). No significant differences were found between stream types in late summer or spring. When Tukey post-hoc tests were run with stream type log methanogen DNA abundance data as the dependent variable and seasons as the factor it was found that in forested streams, early summer was significantly lower in methanogen abundance from sediments than the spring ($p < 0.0001$), late summer was significantly lower in methanogen abundance from sediments than the spring ($p=0.002$), and late fall was significantly lower in methanogen abundance from sediments than the spring ($p < 0.0001$). In urban unrestored streams it was found that early summer was significantly lower in methanogen abundance in sediments than late fall and spring (late fall $p < 0.0001$, spring $p < 0.0001$). In urban restored streams it was found that early summer

was significantly lower in methanogen abundance in sediments than late fall and spring (late fall p=0.003, spring p=0.005).

Average methanogen DNA abundance from epilithon samples in forested streams ranged from 30.78 to $1.59x10^6$ pg/cm^2 with the smallest average abundance in early summer and the largest amount in spring. Samples from urban unrestored streams ranged from 127.34 to $6.14x10^6$ pg/cm^2 with the smallest average abundance in early summer and the largest in late fall. Samples from urban restored streams ranged from 702.12 to $1.79x10^6$ pg/cm^2 with the smallest average abundance in early summer and the largest in late fall (Figure 6). A two-way mixed ANOVA run using yearly log methanogen DNA abundance data from epilithon (pg/cm^2) showed that there were no significant differences between stream types, but did show that there are significant differences between seasons ($p < 0.0001$) (Table 5). When Tukey post-hoc tests were run with seasonal log methanogen DNA abundance data as the dependent variable and stream type as the factor it was found that forested streams were significantly lower in methanogen abundance from epilithon than urban unrestored streams and urban restored streams (urban unrestored streams p=0.018; urban restored streams p=0.035) in late fall, but there were no significant difference between stream types in other seasons. When Tukey post-hoc tests were run with stream type log methanogen DNA abundance data as the dependent variable and seasons as the factor it was found that in forested streams spring was significantly higher in methanogen abundance from epilithon than all other seasons (early summer $p < 0.0001$; late summer p=0.004; late

fall p<0.0001). In urban unrestored streams early summer was significantly lower in methanogen abundance from epilithon than late fall and spring (late fall p=0.006; spring p=0.009). In urban restored streams early summer was significantly lower in methanogen abundance from epilithon than late fall and spring (late fall p=0.036; spring p=0.047).

Average methanogen DNA abundance from seston samples in forested streams ranged from 4.68 to 2.20×10^4 pg/mL with the smallest average abundance in early summer and the largest in spring. Samples in urban unrestored streams ranged from 12.60 to 1.00×10^5 pg/mL with the smallest average abundance in early summer and the largest in late fall. Samples in urban restored streams ranged from 42.19 to 8.30×10^4 pg/mL with the smallest average abundance in late summer and the largest in late fall (Figure 7). A two-way mixed ANOVA using log methanogen DNA abundance data from seston (pg/mL) did not show significant differences between stream type, but showed significant differences between seasons ($p < 0.0001$) (Table 6). When Tukey post-hoc tests were run with seasonal log methanogen DNA abundance data as the dependent variable and stream type as the fixed factor it was found restored streams have greater seston methanogen abundance than forested streams in early summer (p=0.047), but no other significant differences were found in seasons between stream types. When Tukey post-hoc tests were run with stream type logged methanogen DNA abundance data as the dependent variable and seasons as the fixed factor it was found that seston methanogen abundance in forested streams was significantly higher in spring than in

any other season (early summer $p < 0.0001$; late summer $p=0.008$; late fall $p=0.008$). In urban unrestored streams a lower abundance of seston methanogens was found in early summer than in late fall and spring (late fall $p=0.003$, spring $p=0.001$), and late summer had a lower abundance of methanogens from seston than spring ($p=0.039$). In urban restored streams methanogen abundance was not significantly different between seasons.

Patterns of Methanotroph Abundance

Average abundance of sediment methanotroph DNA from forested streams ranged from $1.51x10^3$ to $1.85x10^4$ pg/g with the smallest average abundance in late summer and the largest in spring. Samples from urban unrestored streams ranged from $2.84x10^3$ to $1.74x10^4$ pg/g with the smallest average abundance in early summer and the largest in late summer. Samples from urban restored streams ranged from $5.03x10^3$ to $3.89x10^4$ pg/g with the smallest average abundance in early summer and the largest in spring (Figure 8). A two-way mixed ANOVA run using yearly log methanotroph DNA abundance data from sediments (pg/g) showed that there are significant differences between stream types ($p=0.001$) and seasons ($p < 0.0001$) (Table 7). When Tukey post-hoc tests were run with seasonal log methanotroph DNA abundance data as the dependent variable and stream type as the factor it was found that forested streams were significantly lower in sediment methanotroph abundance than urban unrestored streams and urban restored streams (urban unrestored streams $p < 0.0001$; urban

restored streams $p < 0.0001$) in late summer. When Tukey post-hoc tests were run with stream type log methanotroph DNA abundance data as the dependent variable and season as the factor it was found that in forested streams the spring was higher in methanotroph abundance from sediments than early summer and late summer (early summer p=0.001; late summer p=0.003). In urban unrestored streams late summer was significantly higher in sediment methanotroph abundance than early summer and late fall (early summer p=0.002; late fall p=0.039), and spring was significantly higher in methanotroph abundance from sediments than early summer (p=0.032). In urban restored streams early summer was significantly lower in methanotroph abundance than late summer and spring (late summer p=0.007; spring p=0.002).

Average methanotroph DNA abundance from epilithon samples from forested streams ranged from 17.97 to 1.27×10^3 pg/cm^2 with the smallest average abundance in early summer and the largest in spring. Samples from urban unrestored streams ranged from 79.17 to 1.74×10^4 pg/cm^2 with the smallest average abundance in early summer and the largest in late summer. Samples from urban restored streams ranged from 451.21 to 3.17×10^4 pg/cm^2 with the smallest average abundance in early summer and the largest in late summer (Figure 9). A two-way mixed ANOVA run using yearly log methanotroph DNA abundance data from epilithon (pg/cm^2) did not show that there were any significant differences between stream types, but did show that there are significant differences between seasons (p=0.001) (Table 8). When Tukey post-hoc tests were run with seasonal logged methanotroph DNA abundance data as the dependent

variable and stream type as the factor it was found that there were no significant differences between stream types in specific seasons. When Tukey post-hoc tests were run with stream type log methanotroph DNA abundance data as the dependent variable and season as the factor is was found that in urban unrestored streams early summer was significantly lower in methanotroph abundance from epilithon than late summer ($p=0.005$). There were no significant differences between seasons in forested streams and urban restored streams.

Average methanotroph DNA abundance from seston samples from forested streams ranged from 0.11 to 4.10 pg/mL with the smallest average abundance in late summer and the largest in spring. Samples from urban unrestored streams ranged from 0.73 to 3.35 pg/mL with the smallest average abundance in late fall and the largest in spring. Samples from urban restored streams ranged from 0.61 to 6.95 pg/mL with the smallest average abundance in late fall and the largest in late summer (Figure 10). A two-way mixed ANOVA run using yearly log methanotroph DNA abundance data from seston (pg/mL) showed that there were significant differences between stream types ($p=0.008$) and between seasons ($p=0.002$) (Table 9). When Tukey post-hoc tests were run with seasonal log methanotroph DNA abundance data as the dependent variable and stream type as the factor it was found that urban restored streams are significantly higher in methanotroph abundance from seston than forested streams and urban unrestored streams (forested streams $p < 0.0001$; urban restored streams $p=0.018$) in late summer. No other significant differences between stream types in seasons were

found. When Tukey post-hoc tests were run with stream type log methanotroph DNA abundance data as the dependent variable and seasons as the factor it was found that in forested streams late summer was significantly lower in methanotroph abundance from seston than spring ($p=0.002$). In urban restored streams late summer was significantly higher in methanotroph abundance from seston than late fall ($p=0.044$). There were no significant differences among seasons in urban unrestored streams.

Patterns of Methane Concentration

Average methane concentration in samples from forested streams ranged from 4.90 to 288.64 μg CH_4/L with the smallest average concentration in late summer and the largest in spring. Average methane concentration in samples from urban unrestored streams ranged from 3.97 to 363.16 μg CH_4/L with the smallest average concentration in late fall and the largest in spring. Average methane concentration in samples from urban restored streams ranged from 7.17 to 396.91 μg CH_4/L with the smallest measured amount in late fall and the largest in spring (Figure 11). The highest methane concentration for all stream types was in spring. A two-way ANOVA run using yearly methane concentration data (μg CH_4/L) showed that there were no significant differences between stream types and there were significant differences between seasons ($p < 0.0001$) (Table 10). When Tukey post-hoc tests were run with seasonal methane concentration as the dependent variable and stream type as the factor it was found that in late summer urban restored streams are significantly higher in methane

concentration than urban unrestored streams and forested streams (urban unrestored streams p=0.008; forested streams p=0.009). In late fall forested streams are significantly higher in methane concentration than urban unrestored streams (p=0.003). There were no significant differences between stream types in early summer or the spring. When Tukey post-hoc tests were run with stream type methane concentration as the dependent variable and seasons as the factor it was found that in forested streams spring was significantly higher in methane concentration than all other seasons (early summer $p < 0.0001$; late summer $p < 0.0001$; late fall $p < 0.0001$). In urban unrestored streams spring was significantly higher in methane concentration than all other seasons (early summer $p < 0.0001$; late summer $p < 0.0001$; late fall $p < 0.0001$). In urban restored streams spring was significantly higher in methane concentration than all other seasons (early summer $p < 0.0001$; late summer $p < 0.0001$; late fall $p < 0.0001$).

CHAPTER IV

DISCUSSION

This study found significant seasonal differences in the abundance of methanogens and methanotrophs, and methane concentration, and some significant differences in these variables among stream types. Seasonal differences in abundance of methanogens, methanotrophs, and methane concentration are to be expected in temperate zone streams. However, significant interactions between seasons and stream types suggest that methane cycle processes that are different between stream types mediate seasonal differences.

Methanogen DNA abundance in sediments showed significant differences among stream types (Fig 5). In general, sediments are where highest methanogen abundances should be expected because methanogens are strict anaerobes and anaerobic zones are likely to be more prevalent in sediments than in epilithon or the water column. In forested streams the highest abundance of methanogen DNA occurred in the spring, but in urban unrestored and restored streams the highest abundance was found in late fall. The difference in timing of peak methanogen DNA abundance may reflect differences in the timing and magnitude of organic inputs in forested versus urban streams (Paul and Meyer 2001). Timing of organic matter inputs are controlled by hydrology, land use patterns that control organic matter delivery, and

groundwater influences. Leaf drop, in fall (late October and November in central NC) provides a large pulse of organic matter, which is an important source of organic matter in stream ecosystems (Gulis and Suberkropp 2003, Meyer 1980). The magnitude of organic input in forested streams is likely much larger than that of urban streams because there is more tree canopy. Further, the timing of organic input into forested streams is also spread out over a longer time period. When the leaves first fall, much of the decomposition, including methanogenesis, occurs on the forest floor and remaining organic matter then moves into the streams from upslope during rain events through subsurface flow throughout winter and spring (Swanson 1982, Aitkenhead-Peterson 2003). The infiltration of rainwater into soils in the forest slows the rate of runoff leading to a less "flashy" flow during rain events compared to urban streams that have higher percentages of impervious surfaces. Less runoff in forested streams allows the organic input to be retained longer in the forest streams, making it available to methanogens for an extended period following leaf fall. Methanogens react by increasing abundance over time (Conrad and Klose 2006), which is consistent with highest methanogen DNA abundance in the spring. In contrast, urban streams have flashy flow patterns because storm water is piped in through storm drains, large amount of imperious surface, and lack of forested buffer (Bernhardt *et al*. 2007, Walsh *et al*. 2005). Thus, during late fall rain events particulate and dissolved organic matter loading occurs in a large pulse in urban streams. When I sampled in late fall, soon after the leaf drop, temperatures were moderate, favorable for development of methanogen

populations, consistent with highest methanogen DNA abundance in the late fall in both urban stream types. The organic matter derived from fall leaf drop has a lower retention time in urban streams because of the flashiness of the streams (Walsh *et al.* 2005). Therefore, organic matter in urban streams is lower in the spring, consistent with lower measured methanogen DNA abundance observed in spring.

Methanogen DNA abundance in epilithon in general showed similar differences among stream types to those seen in sediment methanogen DNA abundance; where the highest abundance of methanogen DNA abundance in epilithon in forested streams was in spring, and there was a high abundance of methanogen DNA abundance in epilithon in both urban streams in late fall and spring (Fig 6). However, in epilithon samples, methanogen DNA abundance in restored streams was not greater than forested and unrestored methanogen DNA abundance in early summer as it was in sediments. Methanogen DNA abundance in epilithon and sediments cannot be compared directly because the units are not the same. It is not surprising for the patterns seen to be similar because epilithon could be exposed to the same differences in the timing and magnitude of organic inputs in forested streams versus both urban streams. Epilithon methanogens could respond to the particulate and dissolved organic input from surface and subsurface flows throughout late fall and spring in forested streams, and the large pulse of organic matter input in urban streams in late fall and spring.

Methanogen DNA in seston showed similar pattern of relative abundance, as did the sediments and epilithon, although fewer of the contrasts, including stream type,

were statistically significant. During baseflow in small streams, seston is derived from upstream organic matter inputs and material that is eroded from the benthic fine particulate organic matter and epilithon compartments (Vannote *et al.* 1985). Assuming that upstream contributions were in equilibrium with resuspension and settling, seston methanogen DNA should reflect patterns seen in sediments and epilithon. However, since methanogens are anaerobes (Yuan *et al*. 2009), they are likely to occur in anaerobic microsites within the sediment and epilithic matrix, and thus may be less susceptible to entrainment into the seston than more surficial components of these compartments that are more readily eroded (Rezanka and Hershey 2003), which is consistent with the somewhat weaker observed pattern.

Methanotroph DNA abundance in sediment showed significant differences among stream types in late summer. Forested stream methanotroph DNA abundance was significantly lower than methanotroph DNA abundance in the late summer in both urban stream types (Fig 8). High methanotroph abundance would be expected in sediments because the sediment surface is generally well oxygenated with a supply of methane from the ground water and in-stream methanogenesis, and electron acceptors for methane oxidation. Flow regime and nutrient inputs from runoff may be affecting the differences between stream types in late summer. In urban areas it is common to fertilize lawns, parks, and golf courses during the growing seasons, especially during late summer/fall and spring. High rainfall during late summer 2011 produced runoff that would have washed nutrients from fertilizers into the urban streams. The combination

of nutrients from fertilizers may be assimilated and used for growth in methanotrophs, increasing their abundance. Although nitrogen in the form of ammonium, binds to the methane monooxygenase enzyme, inhibiting methane oxidation (Macinelli 1995), the combination of nitrogen in the form of urea, potassium chloride, phosphate, and crop residues have been shown to increase methanotroph abundance (Zheng 2008), consistent with the increase of methanotroph DNA abundance in late summer in both urban stream types. Further, when oxygen is unavailable, nitrate, sulfate, phosphates, and oxidized forms of iron and manganese from fertilizers can act as alternative electron acceptors (Mueller and Helsel 2013, Lopes *et al.* 2011). In contrast to urban streams, the amount of inorganic input and fertilizers into forested streams is different because infiltration in the forest buffer zone slows the input into the streams (Castillo 2012). Forests are also not fertilized, resulting in less nutrient availability and lower concentrations of electron acceptors for methanotrophs, consistent with lower late summer methanotroph DNA abundance in forested streams.

Methanotroph DNA abundance in epilithon showed no significant differences among stream types, which was in contrast to the expectation that methanotroph DNA abundance in epilithon would be similar to methanotroph DNA abundance in sediment (Fig 9). While epilithon methanotrophs are likely exposed to the same nutrients and electron acceptor input as sediment methanotrophs, they are not exposed to the same supply of methane. Sediment methanotrophs are exposed to methane from methanogensis in sediments, methane from ground water, and methane in the water

column. In contrast, epilithon methanotrophs are only exposed to methane in the water column and possibly methane from methanogensis in microsites in epilithon. The decrease in methane exposure could explain the lack of significant differences between stream types in methanotroph DNA abundance in epilithon.

Methanotroph DNA abundance in seston showed significant differences among stream types in the late summer, where methanotroph DNA abundance in urban restored streams were significantly higher than methanotroph DNA abundance in urban unrestored and forested streams (Fig 10). It was expected that the same significant difference seen in sediment methanotrophs would be seen in seston methanotrophs because the majority of the methanotrophs in seston have been eroded from the stream bottom, even though some resident populations can exist in deep pools (Blumenberg *et al.* 2007). However, seston methanotrophs are only exposed to methane in the water column. As methane concentrations change, methanotroph abundances change as well (Hanson and Hanson 1996). Late summer methane concentrations in urban restored streams were significantly higher than the methane concentrations in urban unrestored and forested streams. The significant difference in methane concentration in late summer is consistent with the significant difference seen in late summer for methantroph DNA abundance in seston. It is likely that the difference in methantroph DNA abundance in late summer is mediated by methane exposure while the amount of nutrients and electron acceptors plays a role as well.

There have been some studies that have quantified methanogen and methanotroph abundance. For example, Frey *et al*. (2011) reported methanogen abundance in forest soils targeting the number of methyl coenzyme M reductase (MCR) and found $3.1 \times 10^5 - 2.1 \times 10^7$ target gene copies/g soil. In China wetlands methanogen abundance was reported to be between $1.07 - 8.29 \times 10^9$ cells/g soil (Liu *et al.* 2011). Methanotroph abundance in freshwater lake sediments targeting pmoA was reported to be $3.6 - 7.4 \times 10^8$ cells/ g dry weight (Costello *et al.* 2002), and in meadow and flooded rice field soils was reported to be about $1 - 5 \times 10^6$ pmoA molecules/g of fresh soil. This study was designed to provide methanogen and methanotroph abundance comparisons among sample and stream types. Although rough conversion of my abundance data to cell numbers yields values within or near the ranges of those reported in other studies, they are not reported because direct comparisons are problematic since the gene copy numbers vary widely among methanogen and methanotroph species (Hildenbrand *et al* 2011, Stolyar *et al.* 1999), and the sample basis (wet weight of soil versus dry weight) also varies among studies.

Methane concentration showed no significant differences among stream types; however, methane concentration in spring was significantly higher than methane concentrations in all other seasons across stream types (Fig 11). Wilcock and Sorrell (2008) found methane concentrations in three low-gradient streams in New Zealand ranged from 1.44 to 481.6 µg CH_4/L, which is comparable with the concentrations found in this study. Methane is introduced into flowing waters from upstream, ground water,

lateral entry from riparian zones and soils, and from the streambed through methanogenesis (Jones and Mulholland 1998a, Jones and Mulholland 1998b, Daniel and Harned 1998). Methane is oxidized in streams through methane oxidation performed by methanotrophs. Methanogen and methanotroph communities in lowland streams affect the concentration of methane (Buriánková *et al*. 2012). Consequently, any seasonal changes in these two communities will affect the methane concentration in streams. In this study, the overall patterns of abundance in methanogen and methanotroph communities did not coincide with the patterns of methane concentration measured in the water column. Thus, the changes in community are most likely not the largest contributor to seasonal changes in methane.

Seasonal methane concentrations are more likely controlled by rainfall and stream flow rates. Riparian zones, groundwater, and other terrestrial zones surrounding the streams are primary sources of methane in stream water (Jones and Mulholland 1998a, Jones and Mulholland 1998b, Daniel and Harned 1998). The riparian zones and groundwater interact with the flow path of the stream releasing methane. Groundwater recharge contributes 33.6 - 60.7% of the volume of stream waters in Guilford County in the Piedmont of North Carolina (Daniel and Harned 1998). Seasonal rainfall was highest in spring 2012 during sampling, 9.33 inches, which would have increased groundwater recharge and runoff input into the streams. The increased groundwater recharge would increase the amount of methane released into the stream water. The subsurface discharge from terrestrial zones around the streams could also

contribute to the methane concentration in stream water (Jones and Mulholland 1998b). Jones and Mulholland found average groundwater and subsurface methane concentrations in a forest stream ranged from 158.9 to 549.2 μg CH_4/L. These concentrations are comparable to methane concentrations seen in this study, especially in the spring. The groundwater recharge and subsurface discharge may explain the significantly high methane concentration in spring throughout the stream types, however many things could be contributing to this increase.

This study found differences among stream types in methanogen and methanotroph DNA abundances, but not in methane concentration. Seasonal differences were also seen in all parameters as expected. Methane concentration differed seasonally with-in streams, but not among streams. These findings are consistent with the idea that land use changes impact methanogen and methanotroph communities in streams, but not in the way it was expected. The contrast in the timing and magnitude of particulate and dissolved organic matter input (Paul and Meyer 2001), inorganic input, and hydrology in forested streams versus both urban stream types seem to be the driving forces influencing the significant differences in methanogen DNA abundance in late fall and the differences in methanotroph DNA abundance in early summer among stream types. Further research is needed to better understand the quantitative as well as qualitative relationships between the seasonal differences in organic matter loading in these stream types and the responses of their methanogen and methanotroph communities.

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APPENDIX A

TABLES AND FIGURES

Table 1.

Latitude and Longitude for 12 Study Sites.

Stream	Latitude	Longitude
Forested		
Talbots	35.6364981787	-79.9043422133
Biermans	36.1985095135	-79.9872176491
Burnettes	36.1871004551	-79.9614752012
Strawberry	36.2086704993	-79.8638314034
Urban Unrestored		
Peabody Park Golf Course	36.0727539010	-79.8121042490
Rolling Park	36.0356522935	-79.8331422202
College Park	36.0716955613	-79.8154233682
Shannon Hills	36.0118042693	-79.8191463384
Urban Restored		
Arboretum	36.0698289237	-79.8416063464
Brown Bark Park	36.0963467793	-79.8514840276
Benbow Park	36.0963467793	-79.8514840276
Spring Valley Park	36.0366246956	-79.8118444607

Table 2.

Physical Differences in Streams.

Stream	Avg Pool Depth (cm)	Avg Pool Width (cm)	Avg Riffle Depth (cm)	Avg Riffle Width (cm)	Sediment Type
Forested					
Talbots	8.86	115.5	1.8	23.4	Mainly Rocky (some sandy parts)
Biermans	9.6	120.1	2.8	59.5	Sandy
Burnettes	13.45	112.8	5.49	84.25	Mixed (rocky riffles, sandy pools)
Strawberry	13.6	181.1	3.8	115.8	Sandy
Urban Unrestored					
Peabody Park Golf Course	19.8	371	2.4	258.3	Mainly Sandy (some rocky parts)
Rolling Park	19.55	369.6	4.8	273.5	Mixed (rocky riffles, sandy pools)
College Park	11.28	208.3	4.8	195	Mixed (rocky riffles, sandy pools)
Shannon Hills	12.8	332.5	3.85	230.9	Mainly Sandy (some rocky parts)
Urban Restored					
Arboretum	24.4	301	7.4	207.7	Mixed (rocky riffles, sandy pools)
Brown Bark Park	20.3	197.5	4.28	136.3	Mainly Sandy (some rocky parts)
Benbow Park	17.8	224.16	4.3	161.7	Mixed (rocky riffles, sandy pools)
Spring Valley Park	31.2	318.6	7.1	140.3	Mixed (rocky riffles, sandy pools)

Table 3.

Methanogen and Methanotroph Specific Primers used in Real-time PCR.

Primer	Sequence (5' – 3')	Target genus or gene	Reference
Met86F	GCTCAGTAACACGTGG	*M. mobile* 16S rRNA	Wright and
Met1340R	CGGTGTGTGCAAGGAG	*M. mobile* 16S rRNA	Primm 2003
A189gc	GGNGACTGGGACTTCTGG	*pmoA*	Costello and
Mb661	CCGGMGCAACGTCYTTACC	*pmoA*	Lidstrom 1999

Table 4.

Summary of ANOVA Analyses for Methanogen Genomic DNA from Sediment Samples. Forested = F; Urban Unrestored = U; Urban Restored = R; Early Summer = ES; Late Summer = LS; Late Fall = LF; Spring = Spr. The F-ratio, n, df, and p-values refer to the ANOVA's. The right column refers to the significant pairwise contrasts from Tukey's test.

Analysis	F-ratio	n	df	P	Significant Tukey's pairwise contrasts
MET Sediment 2-way Mixed ANOVA					
Stream Type	10.046	3	2	0.0001	
Season	28.416	4	3	0.0001	
Repeated Measures: ES MET Sediment	9.176	3	2	0.001	R > F = U
Repeated Measures: LS MET Sediment	1.304	3	2	0.291	ns
Repeated Measures: LF MET Sediment	6.205	3	2	0.006	U = R > F
Repeated Measures: Spr MET Sediment	0.233	3	2	0.794	ns
Repeated Measures: Forested MET Sediment	17.545	4	3	0.0001	Spr > ES = LS = LF
Repeated Measures: Unrestored MET Sediment	12.615	4	3	0.0001	LF = Spr > ES
Repeated Measures: Restored MET Sediment	6.51	4	3	0.001	LF = Spr > ES

Table 5.

Summary of ANOVA Analyses for Methanogen Genomic DNA from Epilithon Samples. Forested = F; Urban Unrestored = U; Urban Restored = R; Early Summer = ES; Late Summer = LS; Late Fall = LF; Spring = Spr. The F-ratio, n, df, and p-values refer to the ANOVA's. The right column refers to the significant pairwise contrasts from Tukey's test.

Analysis	F-ratio	n	df	P	Significant Tukey's pairwise contrasts
MET Epilithon 2-way Mixed ANOVA					
Stream Type	1.971	3	2	0.146	
Season	16.165	4	3	0.0001	
Repeated Measures: ES MET Epilithon	0.413	3	2	0.671	ns
Repeated Measures: LS MET Epilithon	0.34	3	2	0.716	ns
Repeated Measures: LF MET Epilithon	5.003	3	2	0.014	U = R > F
Repeated Measures: Spr MET Epilithon	0.34	3	2	0.715	ns
Repeated Measures: Forested MET Epilithon	16.556	4	3	0.0001	Spr > ES = LS = LF
Repeated Measures: Unrestored MET Epilithon	6.118	4	3	0.002	LF = Spr > ES
Repeated Measures: Restored MET Epilithon	4.614	4	3	0.011	LF = Spr > ES

Table 6.

Summary of ANOVA Analyses for Methanogen Genomic DNA from Seston Samples. Forested = F; Urban Unrestored = U; Urban Restored = R; Early Summer = ES; Late Summer = LS; Late Fall = LF; Spring = Spr. The F-ratio, n, df, and p-values refer to the ANOVA's. The right column refers to the significant pairwise contrasts from Tukey's test.

Analysis	F-ratio	n	df	P	Significant Tukey's pairwise contrasts
MET Seston 2-way Mixed ANOVA					
Stream Type	2.425	3	2	0.093	
Season	17.965	4	3	0.0001	
Repeated Measures: ES MET Seston	3.658	3	2	0.039	R > F
Repeated Measures: LS MET Seston	0.382	3	2	0.688	ns
Repeated Measures: LF MET Seston	1.964	3	2	0.158	ns
Repeated Measures: Spr MET Seston	1.964	3	2	0.158	ns
Repeated Measures: Forested MET Seston	12.073	4	3	0.0001	Spr > ES = LS = LF
Repeated Measures: Unrestored MET Seston	7.852	4	3	0.0001	LF > ES; Spr > ES = LS
Repeated Measures: Restored MET Seston	4.195	4	3	0.012	ns

Table 7.

Summary of ANOVA Analyses for Methanotroph Genomic DNA from Sediment Samples. Forested = F; Urban Unrestored = U; Urban Restored = R; Early Summer = ES; Late Summer = LS; Late Fall = LF; Spring = Spr. The F-ratio, n, df, and p-values refer to the ANOVA's. The right column refers to the significant pairwise contrasts from Tukey's test.

Analysis	F-ratio	n	df	P	Significant Tukey's pairwise contrasts
MOB Sediment 2-way Mixed ANOVA					
Stream Type	7.946	3	2	0.001	
Season	14.282	4	3	0.0001	
Repeated Measures: ES MOB Sediment	0.697	3	2	0.506	ns
Repeated Measures: LS MOB Sediment	18.236	3	2	0.0001	U = R > F
Repeated Measures: LF MOB Sediment	1.161	3	2	0.336	ns
Repeated Measures: Spr MOB Sediment	0.448	3	2	0.644	ns
Repeated Measures: Forested MOB Sediment	7.416	4	3	0.001	Spr > ES = LS
Repeated Measures: Unrestored MOB Sediment	6.75	4	3	0.002	LS > ES = LF; Spr > ES
Repeated Measures: Restored MOB Sediment	6.495	4	3	0.001	LS = Spr > ES

Table 8.

Summary of ANOVA Analyses for Methanotroph Genomic DNA from Epilithon Samples. Forested = F; Urban Unrestored = U; Urban Restored = R; Early Summer = ES; Late Summer = LS; Late Fall = LF; Spring = Spr. The F-ratio, n, df, and p-values refer to the ANOVA's. The right column refers to the significant pairwise contrasts from Tukey's test.

Analysis	F-ratio	n	df	P	Significant Tukey's pairwise contrasts
MOB Epilithon 2-way Mixed ANOVA					
Stream Type	0.473	3	2	0.625	
Season	6.684	4	3	0.001	
Repeated Measures: ES MOB Epilithon	0.199	3	2	0.821	ns
Repeated Measures: LS MOB Epilithon	3.752	3	2	0.05	ns
Repeated Measures: LF MOB Epilithon	0.879	3	2	0.448	ns
Repeated Measures: Spr MOB Epilithon	0.603	3	2	0.558	ns
Repeated Measures: Forested MOB Epilithon	1.976	4	3	0.156	ns
Repeated Measures: Unrestored MOB Epilithon	4.701	4	3	0.009	LS > ES
Repeated Measures: Restored MOB Epilithon	3.022	4	3	0.05	ns

Table 9.

Summary of ANOVA Analyses for Methanotroph Genomic DNA from Seston Samples. Forested = F; Urban Unrestored = U; Urban Restored = R; Early Summer = ES; Late Summer = LS; Late Fall = LF; Spring = Spr. The F-ratio, n, df, and p-values refer to the ANOVA's. The right column refers to the significant pairwise contrasts from Tukey's test.

Analysis	F-ratio	n	df	P	Significant Tukey's pairwise contrasts
MOB Seston 2-way Mixed ANOVA					
Stream Type	5.047	3	2	0.008	
Season	5.399	4	3	0.002	
Repeated Measures: ES MOB Seston	0.961	3	2	0.394	ns
Repeated Measures: LS MOB Seston	16.744	3	2	0.0001	R > F = U
Repeated Measures: LF MOB Seston	0.043	3	2	0.958	ns
Repeated Measures: Spri MOB Seston	0.406	3	2	0.671	ns
Repeated Measures: Forested MOB Seston	5.549	4	3	0.003	Spr > LS
Repeated Measures: Unrestored MOB Seston	1.997	4	3	0.139	ns
Repeated Measures: Restored MOB Seston	3.277	4	3	0.034	LS > LF

Table 10.

Summary of ANOVA Analyses for CH_4 Concentration. Forested = F; Urban Unrestored = U; Urban Restored = R; Early Summer = ES; Late Summer = LS; Late Fall = LF; Spring = Spr. The F-ratio, n, df, and p-values refer to the ANOVA's. The right column refers to the significant pairwise contrasts from Tukey's test.

Analysis	F-ratio	n	df	P	Significant Tukey's pairwise contrasts
CH_4 concentration 2-way ANOVA					
Stream Type	0.501	3	2	0.606	ns
Season	46.292	4	3	0.0001	
1-way ANOVA: ES CH_4 ppm	2.01	3	2	0.144	ns
1-way ANOVA: LS CH_4ppm	6.76	3	2	0.002	R > U = F
1-way ANOVA: LF CH_4ppm	6.299	3	2	0.003	F > U
1-way ANOVA: Spr CH_4 ppm	0.355	3	2	0.703	ns
1-way ANOVA: Forested CH_4 ppm	11.766	4	3	0.0001	Spr > ES = LS = LF
1-way ANOVA: Unrestored CH_4 ppm	15.2	4	3	0.0001	Spr > ES = LS = LF
1-way ANOVA: Restored CH_4 ppm	20.408	4	3	0.0001	Spr > ES = LS = LF

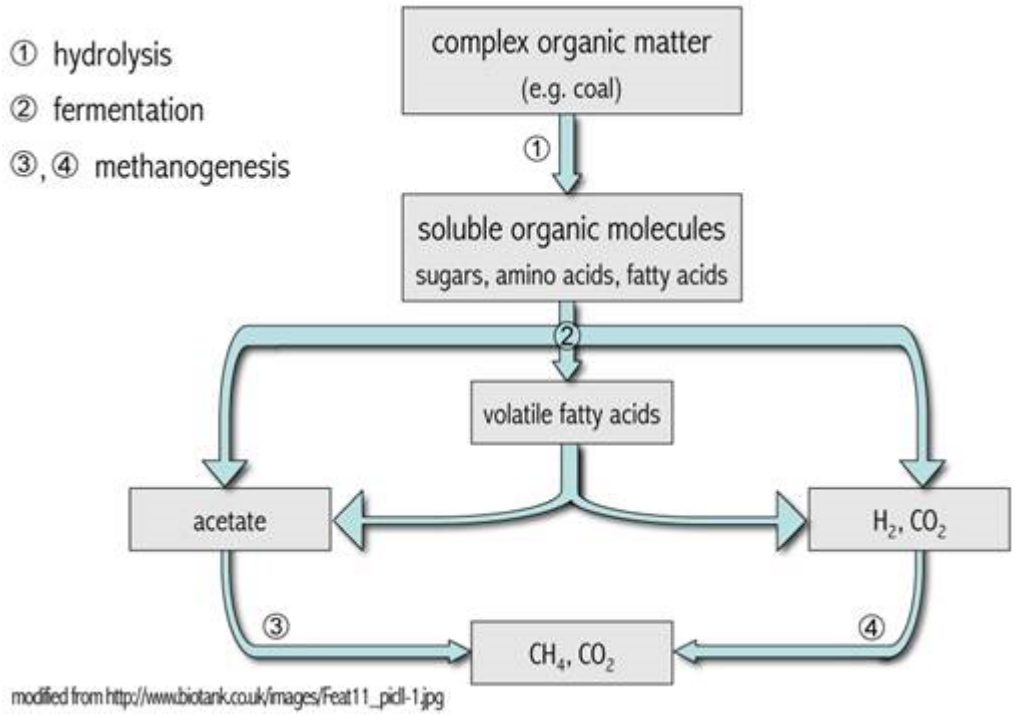

Figure 1.

The Process of Fermentation and Major Pathways of Methanogenisis. 1 and 2: the use of non-methanogenic bacteria in the hydrolysis of complex organic matter to soluble organic matter, and the fermentation of soluble organic matter to volatile fatty acids. 3 and 4: the use of the products of fermentation in methanogenesis, by methanogens. (From: http://ese.mines.edu/research_projects/biogenic_methane.html)

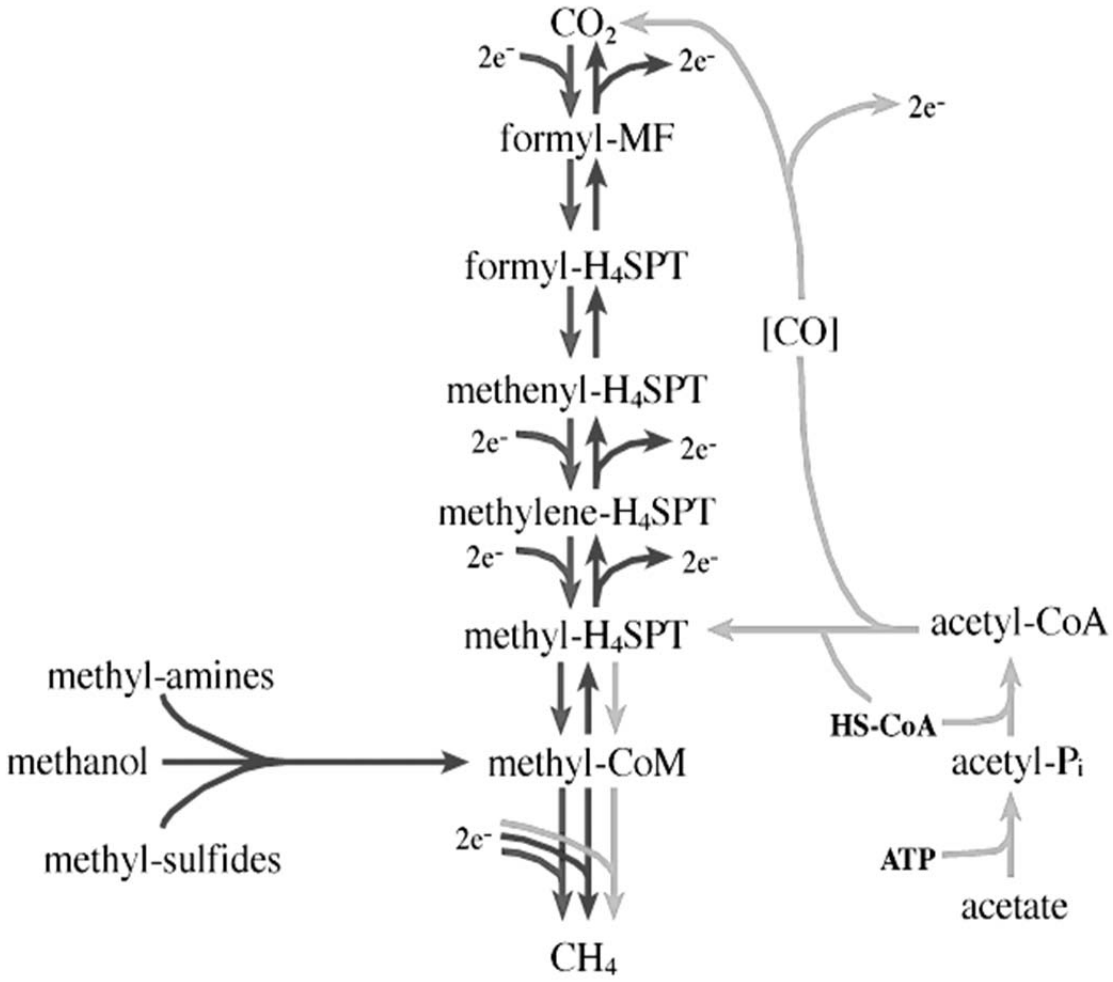

Figure 2.

The Pathway of Methane Formation by Methanogens. Methanogens use the substrates from fermentation: methyl-amines, methanol, methyl-sulfides, acetate, formate, methylsulfides, or hydrogen and carbon dioxide, to produce methane. (From: http://www.uni-frankfurt.de/fb/fb15/institute/inst-3-mol-biowiss/AK-Rother/research.html)

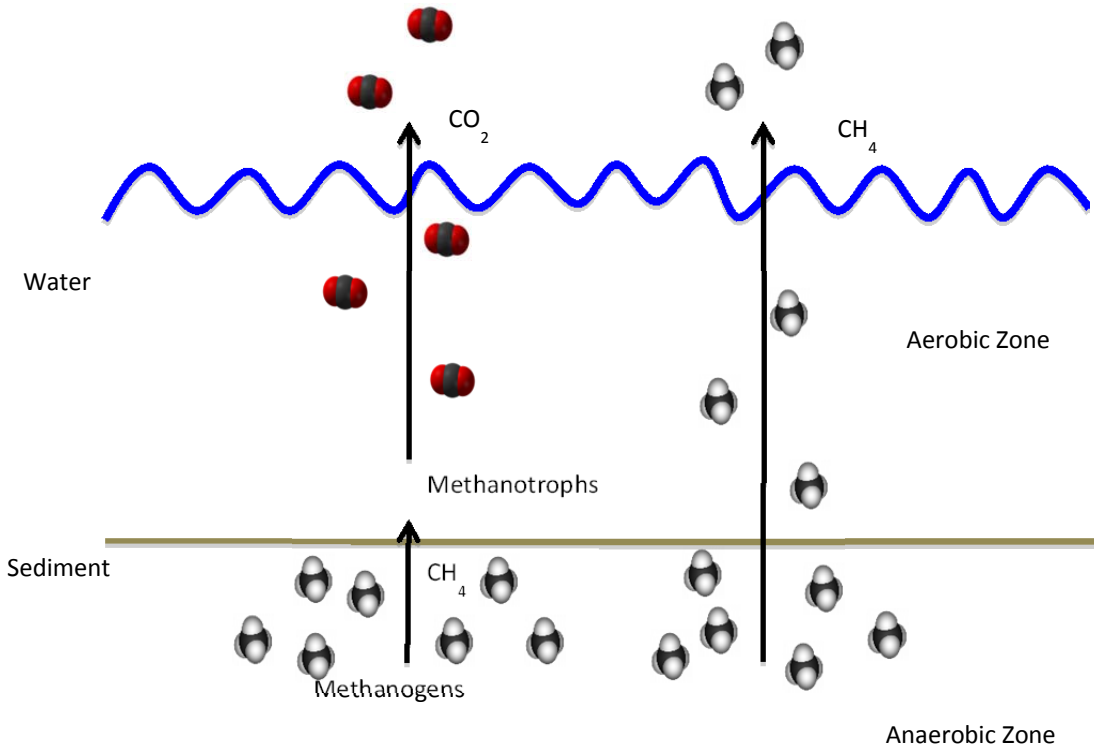

Figure 3.

Diagram of Methanogenesis and Methane Oxidation in an Aquatic System.

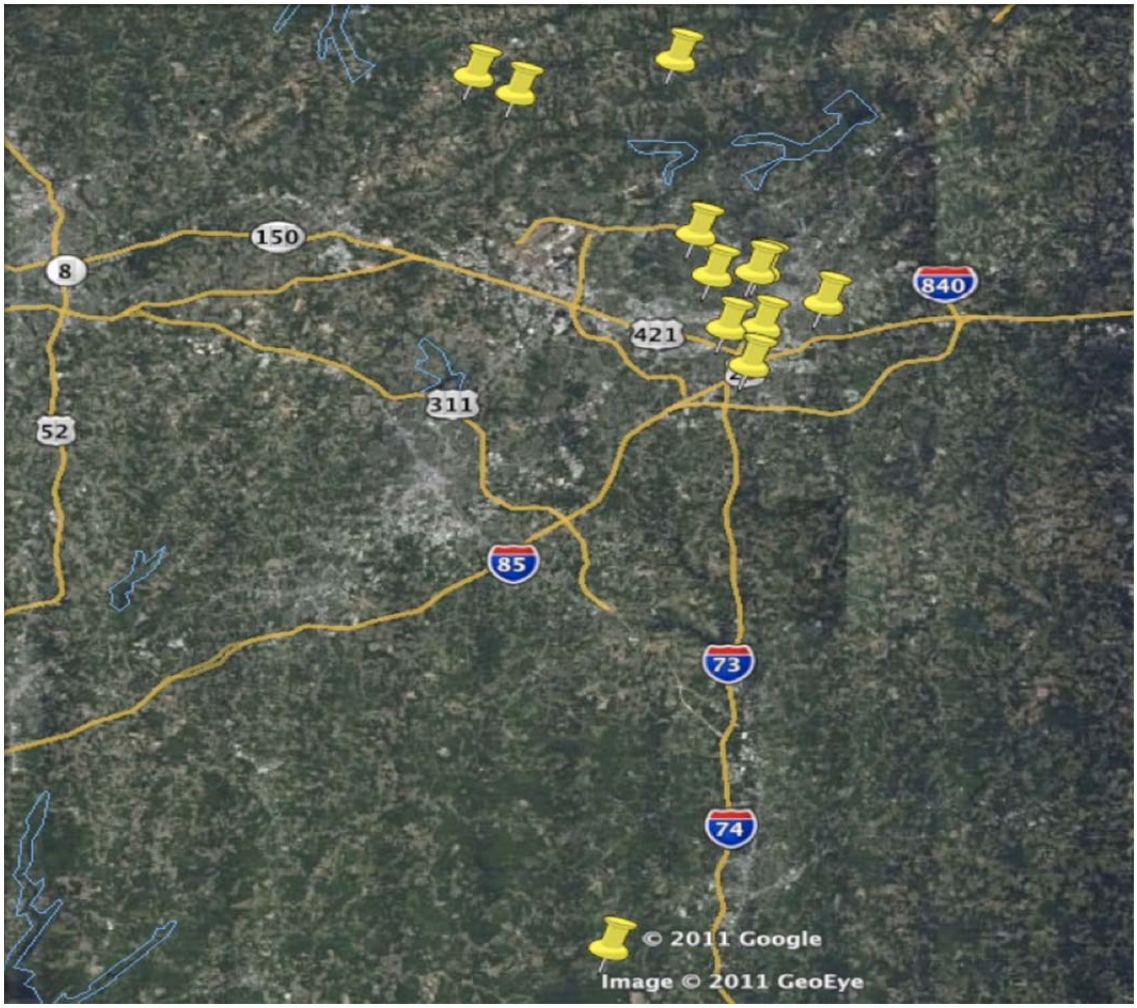

Figure 4.

Map Showing the Location of the 12 Study Sites Used.

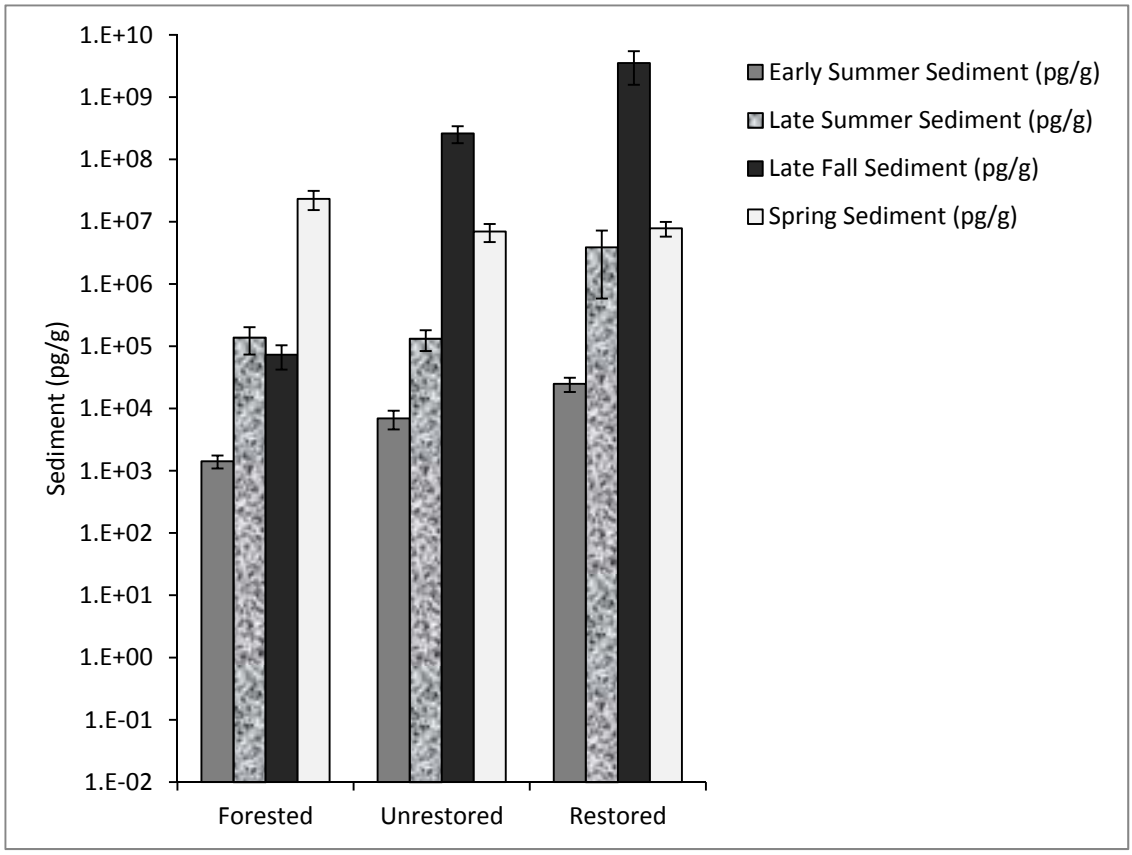

Figure 5.

Seasonal Average Methanogen data from Sediments (pg/g) across Stream Types. Values are means of 65-72 replicates ±SE. In early summer, late fall, and the spring, 72 replicates were taken. In late summer 65 replicates were taken.

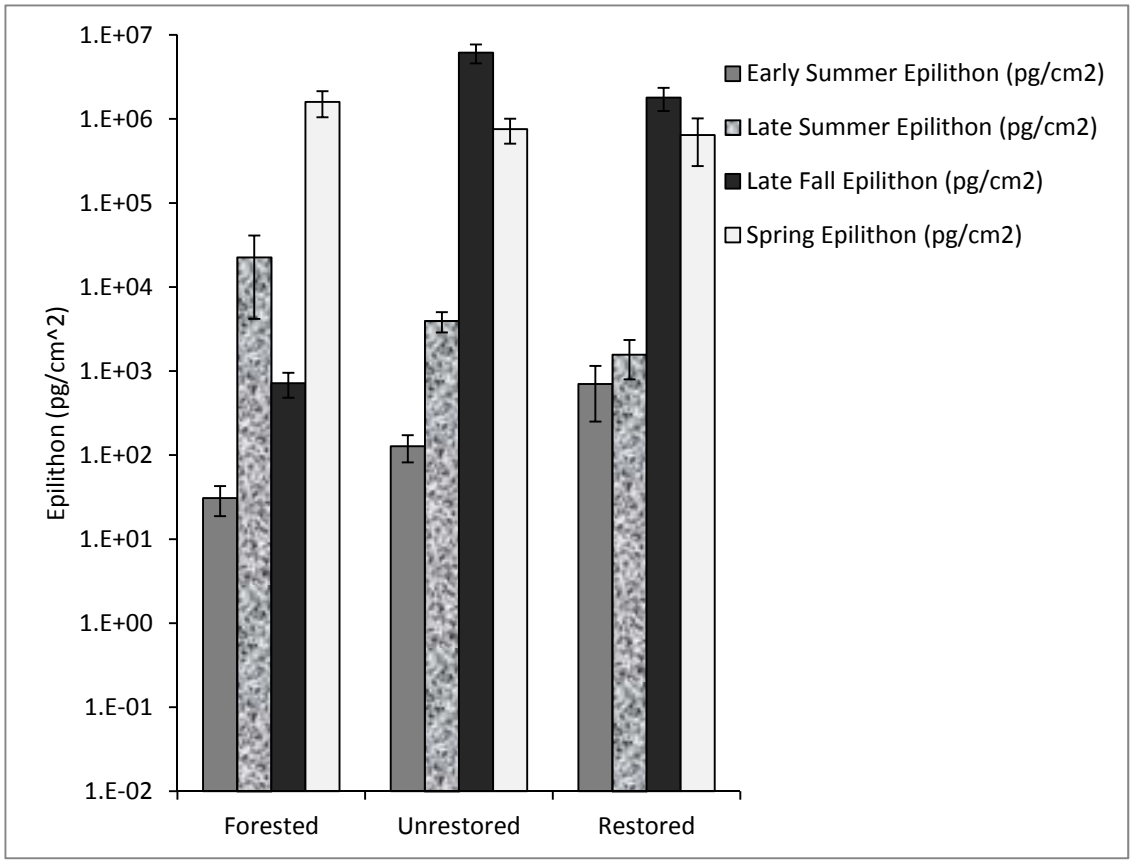

Figure 6.

Seasonal Average Methanogen data from Epilithon (pg/cm^2) across Stream Types. Values are means of 66-72 replicates ±SE. In early summer, late fall, and the spring, 72 replicates were taken. In late summer 66 replicates were taken.

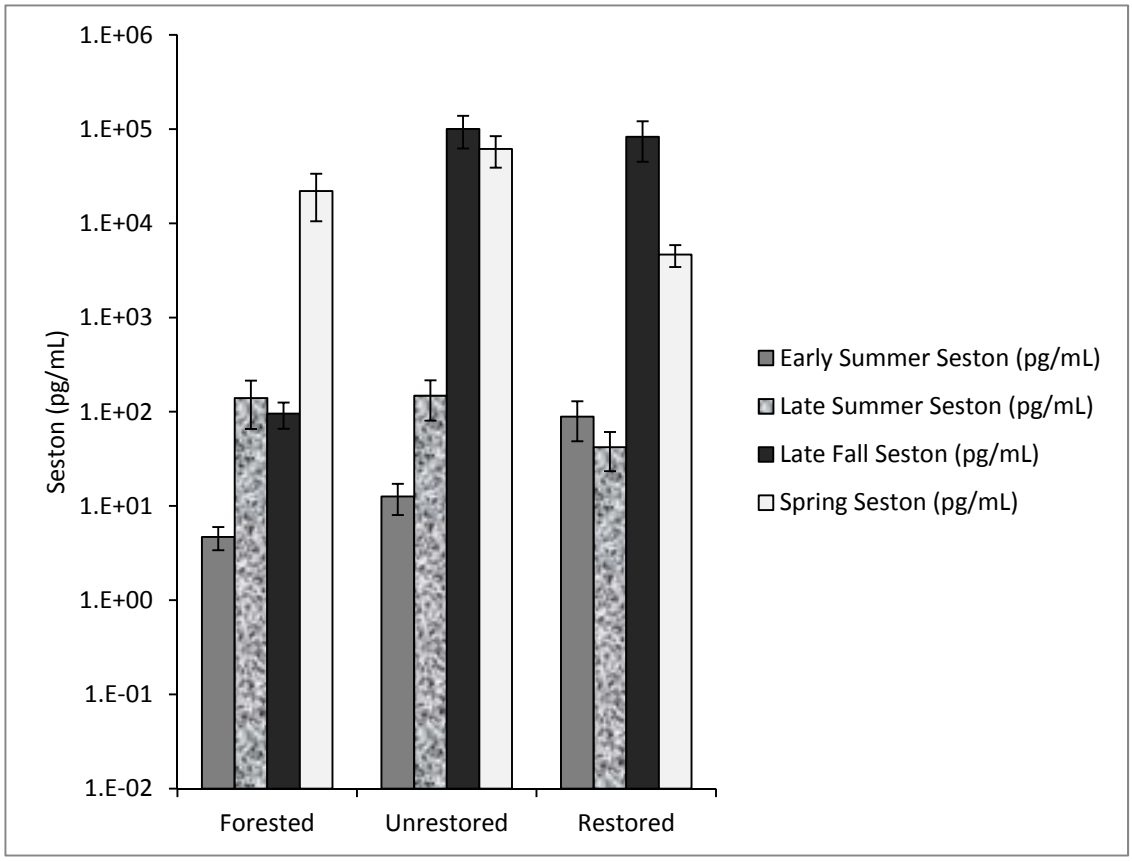

Figure 7.

Seasonal Average Methanogen data from Seston (pg/mL) across Stream Types. Values are means of 66-72 replicates ±SE. In early summer and the spring, 72 replicates were taken. In late summer 66-72 replicates were taken. In late fall 69-72 replicates were taken.

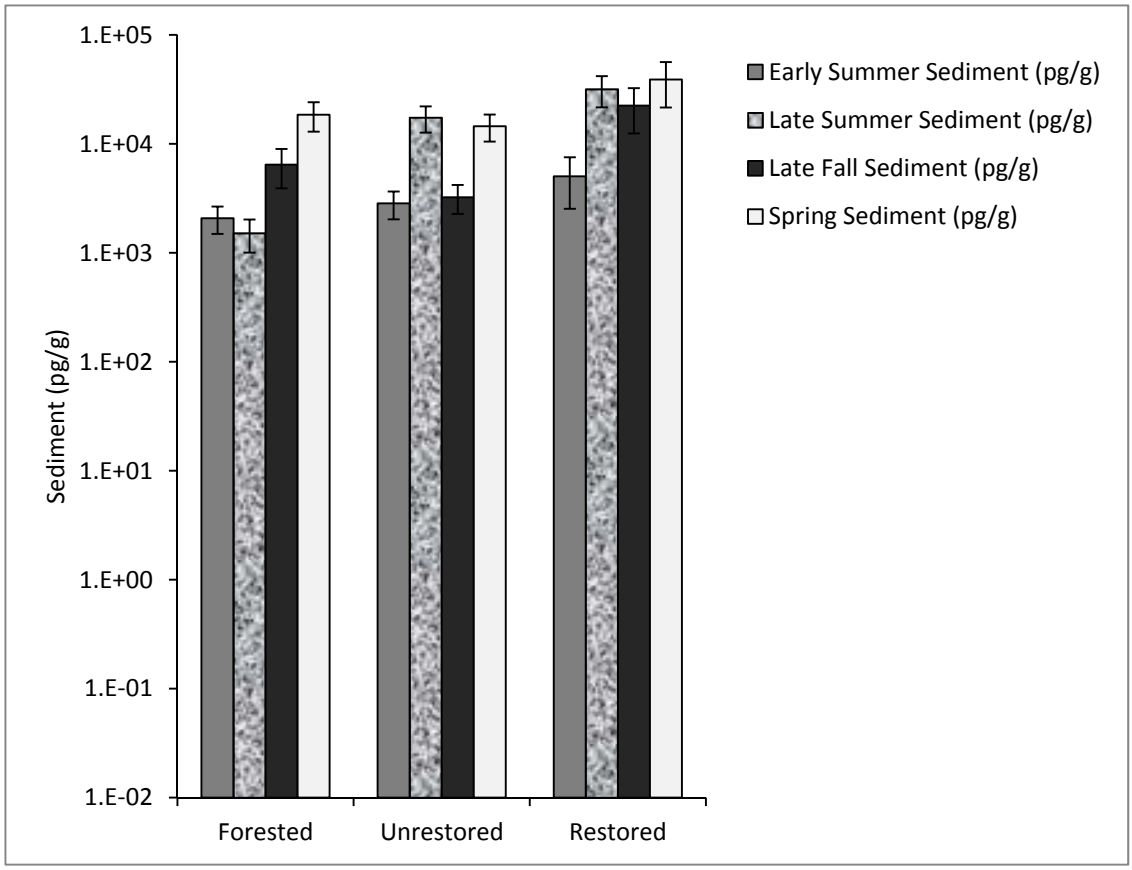

Figure 8.

Seasonal Average Methanotroph data from Sediments (pg/g) across Stream Types. Values are means of 66-72 replicates ±SE. In early summer, late fall, and the spring, 72 replicates were taken. In late summer 66 replicates were taken.

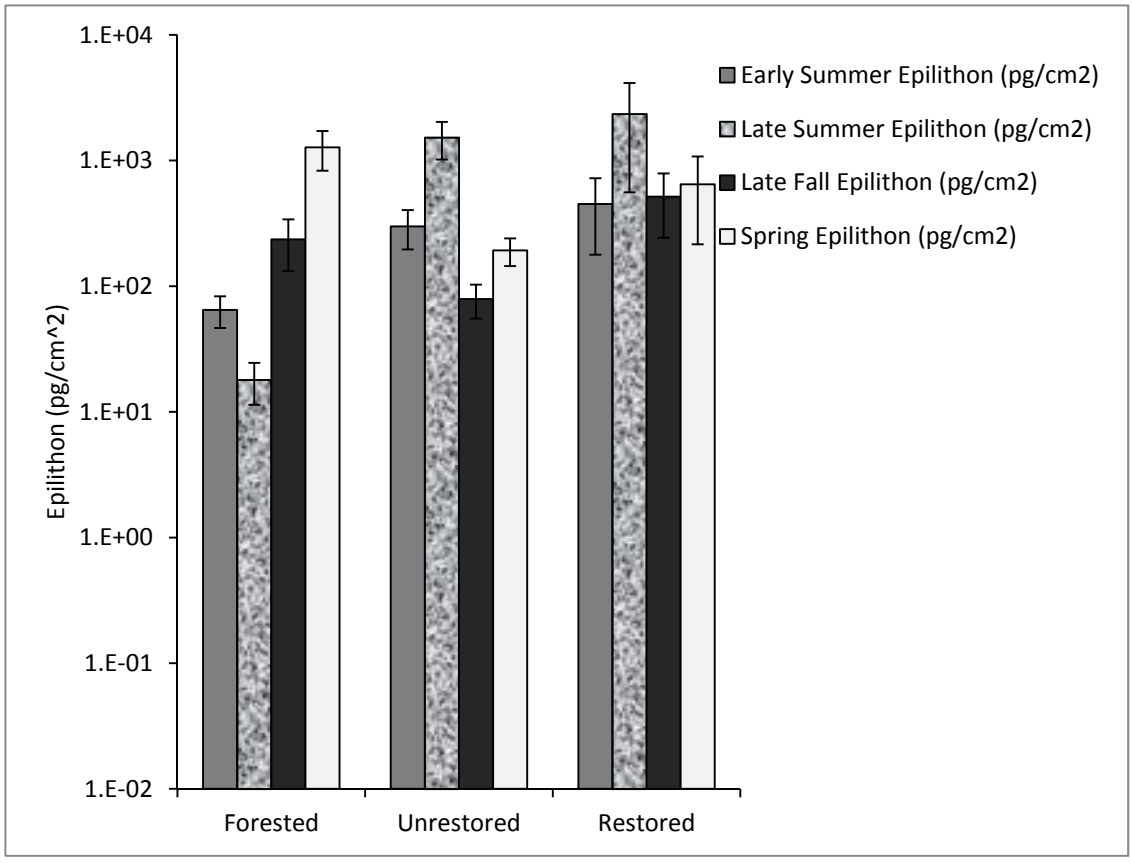

Figure 9.

Seasonal Average Methanotroph data from Epilithon (pg/cm^2) across Stream Types. Values are means of 66-72 replicates ±SE. In early summer, late fall, and the spring, 72 replicates were taken. In late summer 66 replicates were taken.

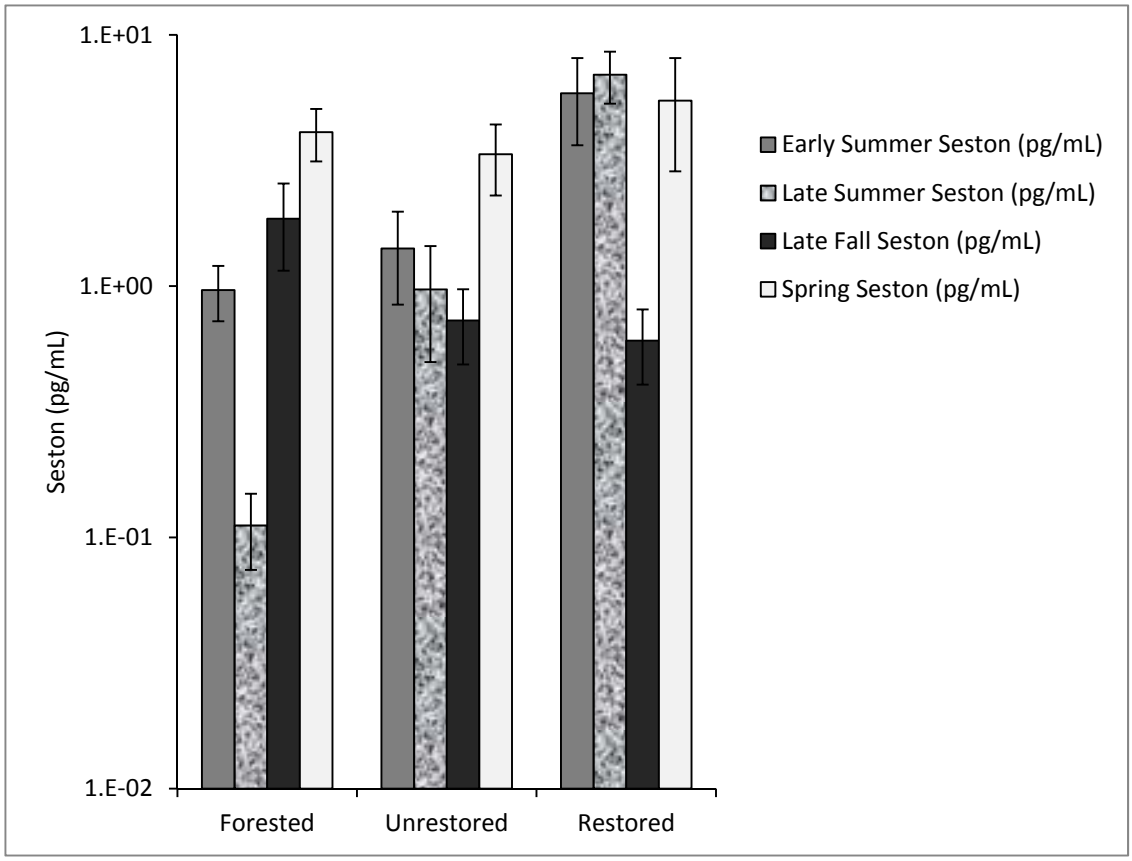

Figure 10.

Seasonal Average Methanotroph data from Seston (pg/mL) across Stream Types. Values are means of 66-72 replicates ±SE. In early summer, late fall, and the spring, 72 replicates were taken. In late summer 66 replicates were taken.

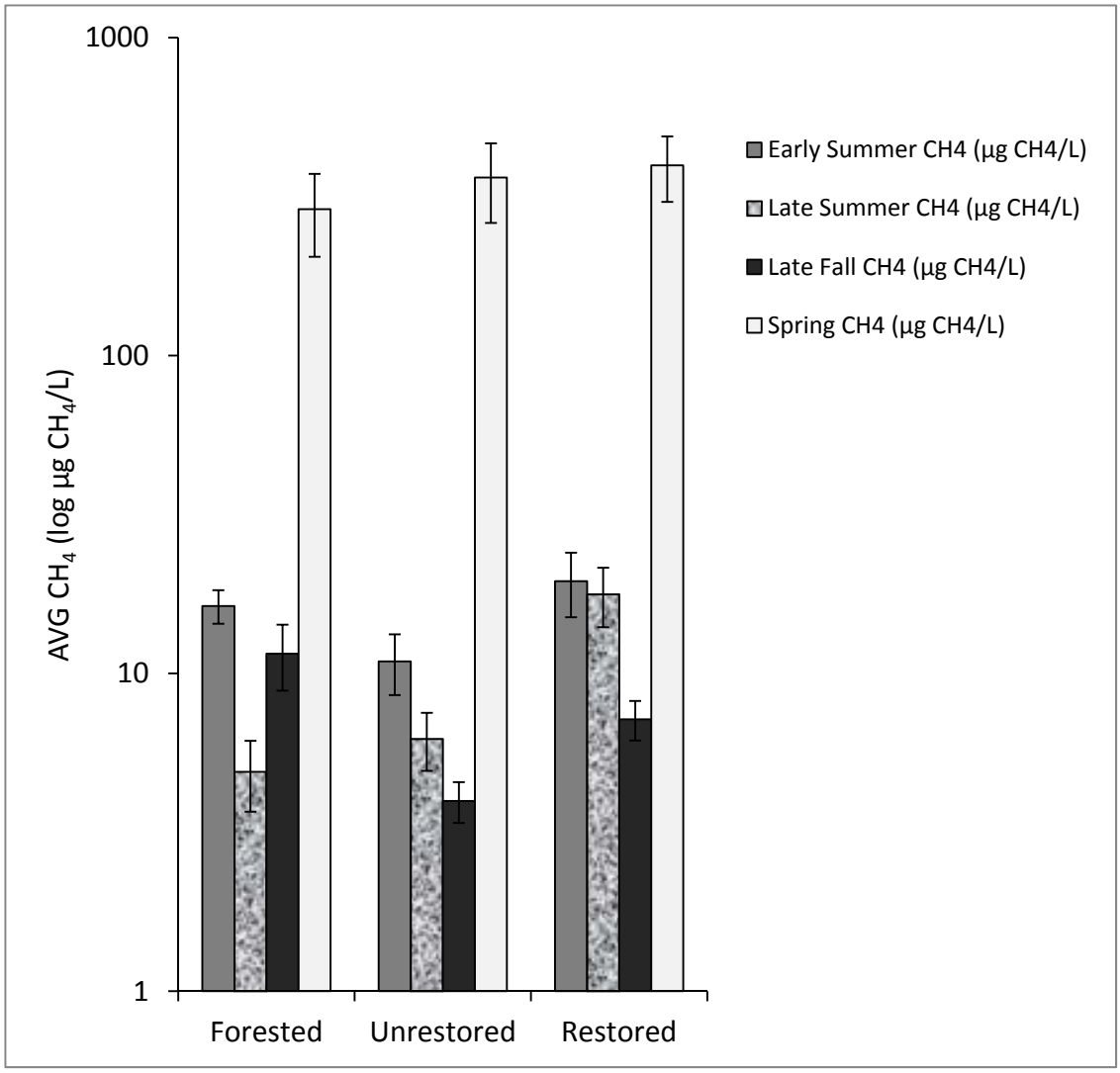

Figure 11.

Seasonal Average Methane Concentration (ppm) across Stream Types. Values are means of 13-23 replicates ±SE. Forested streams in early summer have 23 replicates, in late summer there are 13 replicates, in late fall there are 16 replicates, and in spring there are 16 replicates. Unrestored streams in early summer have 17 replicates, in late summer there are 20 replicates, in late fall there are 22 replicates, and in the spring there are 16 replicates. Restored streams in early summer have 16 replicates, in late summer there are 22 replicates, in late fall there are 19 replicates, and in the spring there are 16 replicates.