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Up to 40% of methane produced in aquatic systems is oxidized before it is released into the atmosphere. Microbial oxidation of methane is an important sink and potentially an important pathway for the incorporation of detrital carbon into aquatic food webs. In this study, we tested the hypothesis that methane-derived carbon was an important carbon source for *Chironomus* larvae in a small arctic lake, but that utilization of methane-derived carbon by larvae differed with depth. We found that an order of magnitude more methane was produced at 5m than at 2m. PCR analysis of sediments, found a greater quantity of methanogen DNA at 5m than at 2m, while methanotroph DNA was less common in sediments except for surface sediments. Larval tubes showed a unique composition of methanogen and methanotroph communities when compared to surrounding sediments. A surprisingly larger abundance of methanogens was found in larval hindguts than larval foreguts of *Chironomus* from deeper sediments. The presence of methanogens and methanotrophs within the larval guts in addition to their depleted $\delta^{13}C$ signature is consistent with our hypothesis that methane-derived carbon is an important basal food resource in this small lake. Furthermore, the combination of biogeochemical and microbial approaches provides insight into functional differences among habitats for a ubiquitous benthic consumer.

EFFECTS OF METHANE PRODUCERS AND CONSUMERS ON THE DIET OF *CHIRONOMUS* LARVAE IN AN ARCTIC LAKE

By

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To my fiancé Philip, for his never-ending support throughout my academic career and for dealing with the many long stressful days of work in the pursuit of my degree

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Review of literature

Methane, in terms of concentration, is the second most effective greenhouse gas leading to temperature increases (Khalil 1999) and contributes about one third of the greenhouse effect due to trace gases (IPCC 2001). Methane is a gas of great interest because per molecule it absorbs infrared radiation significantly more than carbon dioxide (Lashof and Ahuja 1990). Caldwell *et al*. (2008) reported that methane is the most atmospherically abundant hydrocarbon with a twenty fold greater radiative effectiveness than carbon dioxide on a molecular basis.

The net release of methane to the atmosphere has been estimated at 500 Tg a year with a 1% annual increase (Caldwell *et al*. 2008). Methane concentration has dramatically increased in the last 100 years (Khalil and Rasmussen 1987) associated with increased population, industrialization and social development (Khalil 1999), as well as agricultural and industrial factors (Vitousek 1992). The rate of methane increase into the atmosphere exceeds that of carbon dioxide (Khalil 1999). In preindustrial times, methane concentrations were around 700 parts per billion. According to Khalil (1999), the current concentration is 1760 parts per billion, a 2.8 fold increase from preindustrial levels. In comparison, carbon dioxide has shown an increase of 1.4 times the preindustrial concentration.

Biogenic methane emissions from wetlands constitute a significant portion of atmospheric methane. Wetlands emit 145 Tg of methane annually, around one fourth of the total methane emissions (Whalen 2005). Methane is produced in aquatic sediments by methanogenic bacteria as a byproduct of anaerobic respiration (Werne *et al*. 2002) and diffuses upward to the atmosphere contributing to atmospheric methane concentrations (Whalen 2005).

Methanogens require anoxic environments and are important decomposers of organic material (Conrad 2005). Microbial production of methane is the result of degradation of organic matter in anoxic environments (Schulz and Conrad 1995). When organic material decomposes, hydrogen and carbon dioxide accumulate as oxygen and nitrate become depleted. The change in soil chemistry may fuel the hydrogenotrophic pathway of methanogenesis. In the hydrogenotrophic pathway, carbon dioxide and hydrogen are used as substrates that produce methane ($CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_20$). Another pathway, the acetoclastic pathway, uses acetate as the substrate ($CH_3COOH \rightarrow CH_4 + CO_2$). Acetate can build up in sediments from decomposition and fermentation byproducts leading this pathway to be the most common in biogenic methane production (Woltemate *et al.* 1984).

The fate of biogenic methane can be either release into the atmosphere or consumption by microbes in the sediment and water column. Methane oxidizing bacteria are most commonly found in areas near the sediment-water interface of aquatic systems where there are oxidizing conditions (Thauer & Shima 2006). Areas in sediment that contain both oxygen and methane are typically only a few millimeters deep, which

restricts methane oxidation to oxic / anoxic interfaces (Kajan and Frenzel 1999). Methane oxidizing bacteria, or methanotrophs, are the only known organisms that use methane as a direct carbon source (Eller *et al.* 2007). There are two types of methane oxidizing bacteria. The first type of bacteria belongs to the family Methylococcaceae and the second type belongs to family Methylocystaceae (Deines *et al.* 2007).

The consumption of methane, called methane oxidation, serves an important role in controlling methane release into the atmosphere (Caldwell *et al*. 2008). Up to 40% of methane produced in aquatic systems is oxidized before it can be released into the atmosphere (Whalen 2005). Microbial oxidation of methane is responsible for reducing marine derived methane by 2% of the global methane flux (Caldwell *et al*. 2008), and up to 20% of the net atmospheric methane flux (Valentine and Reeburgh 2000).

Oxidation of methane is also a way to recycle methane, and use methane-derived carbon in the food web. Microbes can fuel food webs with methane-derived carbon (Kohzu *et al.* 2004). Macroinvertebrate communities, in both terrestrial and aquatic habitats, obtain energy from carbon fixed by oxidizing methane through the consumption of methane oxidizing bacteria. Methanotrophic bacterial communities are very important in sustaining many species as important carbon and energy sources (Deines *et al.* 2007). One example, with strong experimental evidence for the incorporation of methane-derived carbon, is chironomid larvae. Isotopic carbon analysis has led to the conclusion that chironomid larvae assimilate biogenic methane-derived carbon from their food (Eller *et al.* 2007).

Isotopic fractionation has confirmed the incorporation of methane into the food web. $\delta^{13}C$ of biogenic methane is low due to the large isotopic fractionation associated with methanogenesis (Kohzu *et al.* 2004). Biogenic methane produced in anaerobic conditions can have a $\delta^{13}C$ value as low as -50 to -110 ‰ due to the isotopic fractionation during production by methanogenic bacteria (Jones *et al.* 2008). The depleted ^{13}C signature of methane-derived carbon results in a low $\delta^{13}C$ value for organisms that consume methanotrophs. It has been documented that some stream macroinvertebrates, like chironomids, obtain their organic matter from carbon that was originally methane gas (Kohzu *et al.* 2004). Chironomid species have been recorded with $\delta^{13}C$ values ranging from -40 to -75‰ (Eller *et al.* 2007). $\delta^{13}C$ values lower than -45‰ must come from biogenic methane, as it is the only known constituent sufficiently depleted in ^{13}C to be responsible for such a low value.

Chironomid larvae live in tubes constructed from silk and debris (Borror and White 1970). *Chironomus* tubes are U or J shaped and can stretch from the surface into anoxic sediment layers (Kajan and Frenzel 1999). It is believed that larval behavior within their tubes may promote growth and use of methane oxidizing bacteria on the tubes (Jones *et al.* 2008). *Chironomus* are known to use bioturbation and irrigating activities (Eller *et al.* 2005). Bioturbation is believed to help create conditions for growth of methane oxidizing bacteria and increasing rates of methane oxidation. Chironomid larval tubes and associated behavior are used to obtain oxygen and flush waste products, which allows for higher oxygen concentration than surrounding sediments (Stief *et al.*

2005). The microenvironment created by larvae is ideal for bacterial growth and for larvae to receive a constant supply of methane oxidizing bacteria (Eller *et al.* 2007).

Chironomid larvae are important benthic fauna in lakes. As holometabolous non-biting midges, chironomids spend their larval and pupal stages submerged in aquatic sediments, where they dominate benthic lake communities (Jones *et al.* 2008). Chironomid feeding can be highly selective and variable. *Chironomus* are generally considered detritivores or grazers, but could also be surface deposit feeders. *Chironomus* are tolerant of low oxygen conditions and some pollution. Some chironomids are red in color due to hemoglobin, which helps in obtaining oxygen (Gullan and Cranston 2005).

Chironomid larvae play important roles in the benthic community and transfer of energy through trophic levels. Chironomids obtain carbon from methane-derived sources by the use of methane oxidizing bacteria (Eller *et al.* 2007). Furthermore, the methane-derived carbon will be transferred into the food web as chironomids are important prey items for both invertebrate and vertebrate predators. Also, after pupation, chironomids may eventually emerge and enter the terrestrial environment, thereby routing methane-derived carbon into the terrestrial food web (Kohzu *et al.* 2004). Thus, methane-derived carbon plays an important role in contributing to the carbon flux through food webs.

Methanotrophic bacteria are at the base of the complex cycling of methane and carbon through wetland systems as well as controlling the global carbon budget. Methanotrophs have been found in a wide variety of environments. Methanotrophs are obligate aerobic gram-negative bacteria that use methane as their only carbon and energy source (McDonald *et al* 2008). The main types of aerobic methane oxidizers occur in

Alphaproteobacteria as well as *Gammaproteobacteria* (Tavormina *et al.* 2008). The division of methanotrophic bacteria is based on several factors including cell morphology, metabolic pathways and phylogeny (McDonald *et al*. 2008). *Alphaproteobacteria* methanotrophs are type II methanotrophs, and *Gammaproteobacteria* methanotrophs are type I and type X methanotrophs (Osaka *et. al* 2008). Type I methanotrophs are more common in nutrient rich conditions, while type II tend to be more abundant in nutrient poor conditions (Osaka *et. al* 2008). Type I and type X methanotrophs use ribulose monophosphate pathway for assimilating formaldehyde, and type II methanotrophs use the serine pathway (Hua *et al.* 2007).

The oxidation of methane is catalyzed by either soluble or particulate forms of methane monooxygenase (Hanson and Hanson 1996). Under natural conditions all methanotrophic bacteria express a membrane bound enzyme, particulate methane monooxygenase (pMMO), which has narrow substrate specificity. Methanotrophic bacteria have been studied using the pMMO gene, *pmoA* (Tavormina *et al.* 2008). The *pmoA* gene encodes for the α-subunit of pMMO. The encoded protein is central to aerobic methanotrophy and is highly conserved within the bacterial domain (Hanson and Hanson 1996). *pmoA* a good marker for identifying methanotrophs because it is present in almost all known methanotrophic bacteria (Holmes *et al.* 1995). Studies have shown this gene is a good proxy for developing phylogenetic relationships to the genus level when combining 16S rRNA and *pmoA* primers to evaluate a sequence (Heyer *et al.* 2002; Kolb *et al.* 2003).

Methanogenic bacterial communities have also been studied in detail. Many archaea, such as methanogenic strains, possess a modified surface layer that has covalently linked glycan chains (Sleytr *et al* 1999). This modification is not found in many bacteria, such as methanotrophic bacteria. Four major orders in the methanogenic archaeal group have been studied: Methanococcales, Methanobacteriales, Methanomicrobiales and Methanosarcinales (Yu *et al.* 2005). Despite methanogens being a metabolically restricted group, they have extreme habitat diversity (Jones *et al.* 1987). Methanogenic species can be found in any habitat that has anaerobic biodegradation of organic compounds. Methanogens utilize compounds that are produced as end products of various degradations and fermentations (Jones *et al.* 1987). Such products could include hydrogen, formate, acetate, methanol, and methylamines.

A variety of methods including fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP) and PCR have been used to study and identify methanogens (Yu *et al.*2005; Wright and Pimm 2003). PCR has been used with great success and a variety of primers have been developed to detect and identify methanogens. A methanogenic specific gene, *mcrA*, encodes the α-subunit of methyl-coenzyme M reductase which helps in the formation of methane. This gene has been the target of several studies (listed within Juottonen *et al.* 2006). However, because methanogenic microbes have great phylogenetic diversity, it has been difficult to identify a single primer set to target all methanogens (Juottonen *et al.* 2006). Wright and Pimm (2003) evaluated conserved

regions of 16S genes from 82 methanogens to develop a methanogen specific primer set that was found to deliver methanogenic specific results.

Objectives

The objectives of this study were (1) to characterize the microbial community in and around *Chironomus* larvae focusing on the larval gut contents, larval tube and environmental sediments. (2) To evaluate assimilation of selected microbes by comparing microbial species found within the foregut to those found in the hindgut. (3) Confirm with isotopic analysis that *Chironomus* are consuming methane-derived carbon. (4) Determine the quantity of methane being produced within sediments of an arctic lake.

Hypotheses

(1) Microbial communities found around and within *Chironomus* larvae are distinct from the microbial community in the surrounding sediment. (2) Assimilation occurs in the larval foregut, altering the microbial community within the larval foregut when compared to the microbial community in the larval hindgut. (3) *Chironomus* in an arctic lake utilize methane-derived carbon, resulting in a depleted $\delta^{13}C$ signature. (4) There is more methanogenesis in deeper lake sediments compared to shallower sediments in an arctic lake, resulting in greater methane availability for methanotrophs and greater methane-derived carbon for *Chironomus*.

CHAPTER II

MATERIALS AND METHODS

Study site

The study site was located at the Toolik Lake Field Station (68°38'N, 149°43'W), just north of the Brooks Mountain Range in arctic Alaska. This study took place during the summer of 2009. The focus of my study was *Chironomus* larvae in Lake GTH 112. Lake GTH 112 (68°40'N, 149°14'W) has a surface area of 0.025 km^2, max depth of approximately 5.6m, and an average depth around of 2.1m. This oligotrophic lake is intermittently stratified and known for its production of methane (Hershey *et al.* 2006; unpublished data).

Sampling and lab methods

Samples were taken from 2m and 5m within GTH 112. Samples of larvae and larval tubes were collected using an Ekman dredge. Larval tubes were removed from the top of the dredge to minimize disturbance to the tubes. Tubes were gently picked off with forceps and placed into a glass scintillation vial filled with lake water. A total of 10 tubes were collected from each depth. Larval tubes were then separated and stored individually in 1mL of CTAB buffer for DNA extractions.

Sediment was sieved through a 450μm mesh net in the field to collect larvae for microbial sampling. Larvae and larger sediments were transported to the lab for sorting. *Chironomus* larvae were removed with forceps. Larvae were dissected under a dissecting microscope to remove and collect foregut and hindgut contents. A total of 10 larval foregut contents were collected. Each individual's foregut content was stored in a 1.5mL tube containing 1mL of CTAB buffer. This process was repeated for samples from both depths for foregut as well as hindgut contents. For each sample, either foreguts or hindguts from two larvae were pooled and preserved in 1mL of CTAB to ensure a large enough quantity of microbes for PCR analysis.

Three sediment cores from each depth (2m and 5m) were collected to sample for the microbial community distribution in the sediment. Samples were taken of the overlying water, the water/sediment interface (0mm), and the cores were sectioned to sample at 1mm, 2mm, 5mm, 10mm and 60mm down core. A small sample of sediment from each layer of each core was preserved in 1mL of CTAB for microbial processing. 10mL of overlying water in each core tube was filtered over a 25mm glass fiber filter (Whatman GFC) at the time of core sectioning. Filters were also stored in CTAB.

Sediment and gut content samples were processed to extract and amplify DNA using the CTAB DNA extraction protocol (Schaefer 1997). Sample DNA was quantified and checked for purity using a Thermo Scientific Nanodrop Spectrophotometer ®. Once the DNA was purified and quantified, all samples were assayed by real time PCR. Primers included methanogenic and methanotrophic specific primers (Table 1). The primer pair for methanotrophs targeted enzyme genes specific for the essential metabolic

pathway of methanotrophy. The primer pair for methanogens targeted 16s rRNA specific for methanogens. Copy numbers of ribosomal RNA in archaea is 1 (Klappenbach *et al*. 2001). If the methane monooxygenase copy number is similar to the ribosomal RNA numbers in archaea, the targets are good proxies for biomass. However, enzyme copy numbers are usually higher than ribosomal copy numbers. Initial PCR reactions were run on Cepheid Smart Cycler®1 following published and modified protocols to test for conditions that would optimize results. Following several test runs, primer sets A189/mb661 and Met86/Met1340 were determined to deliver consistent results. Samples were also tested to determine appropriate dilutions of extracted DNA to obtain the best results. Once primer sets, conditions and necessary dilutions were determined, all subsequent PCR runs were done using an Applied Biosystems StepOne™ real – time PCR System using 48 well plates. Each reaction consisted of 10µl of Power Sybr® Green PCR Master Mix, 1µl of each 10µM forward and reverse primer, 8µl of sterile DI water, and 1µl of template. Each plate included 3 negative controls, samples run in triplicate and 3 concentrations of standards also run in triplicate. When using primer set A189/mb661, the standard was genomic DNA from *Methylococcus capsulatus* (ATCC® catalog item number 19069D - 5). When using primer set Met86/Met1340, the standard was genomic DNA from *Methanosarcina acetivorans* Strain C2A (ATCC® catalog item number 35395D - 5). Standards were set up in dilution series of 0.5ng μl^{-1}DNA, 0.05ng μl^{-1}DNA and 0.005ng μl^{-1} DNA. The PCR run consisted of: 1) an initial activation step of 95°C for 0:15 minutes; 2) 40 cycles of: 95°C for 0:15 seconds; 55°C for 0:30 seconds; 72°C for

1:00 minute; and 80°C for 0:15 seconds during which fluorescence was measured, and 3) a melt curve.

After the real time PCR was completed, melt curves were inspected visually to ensure sample peaks were close to or identical to that of the standards. Some variation was expected in sample melt curves as samples consist of a community rather than a single species like the standard. I also made sure the results had a normalized standard curve and that sample Ct values were similar. Results from assays indicating pipette error or sample inhibition were removed from further analysis. PCR results (ng DNA μl of extract) were converted to ng DNA $gram^{-1}$ sediment for comparison.

For isotopic analysis, *Chironomus* larvae were collected into a microcentrifuge tube and dried in a 60°C oven for 3 days. Dried larvae were homogenized and crushed with a mortar and pestal. Subsamples (1mg) were analyzed for $\delta^{13}C$ by the Stable Isotope Laboratory at the University of California - Davis.

Core samples were also taken in GTH 112 for methane production analyses. Six cores were taken at each depth (2m and 5m) and placed in an incubation chamber at 8°C. Stir bars were placed in the cores and overlying water was stirred at 1 rpm, a rate that did not disturb the sediment-water interface. Oxic condition readings were measured beginning 12 hours after incubation, and at 24 hours and 36 hours. 3 mL samples were taken from the overlying water of each core using a 5 mL syringe and a cannula. Each water sample was injected into 30-cc serum vial fixed with 0.1mL of 1 molar HCl. Head space water in the core tube was replaced using appropriate lake water. Methane concentrations were read using a gas chromatograph.

Data analysis tools

Methanogenesis core data were analyzed to determine rate of net methane production in Lake GTH 112, and differences between depths were compared with an independent sample t-test. These data were used to compare the amount of methane available for carbon uptake, or methane oxidation that could be utilized by larvae at the two depths. Carbon isotope samples of larvae were used to confirm that methane-derived carbon was incorporated into *Chironomus* larvae and to compare larvae from 2m and 5m. The isotopic signatures from larval samples at 2m were compared to those from 5m with an independent sample t-test. PCR results were analyzed for the concentrations of either methanogen or methanotroph DNA in each location down core, between depths. For methanogen and methanotroph DNA concentration, separate two way ANOVAs were used to investigate effect of lake depth and down core depths, and the interaction between lake depth x down core depth. In the event of no significant interaction, independent sample t-tests were used to make further comparisons of methanogens and methanotrophs at various locations down core, at both depths. In cases of significant interaction, ANOVA's were used to compare methanogens and methanotrophs down core, at each depth. Analyses with independent samples t-test were used on larval foregut and hindgut for 5m samples, for both methanogens and methanotrophs. Analyses of the microbial community in *Chironomus* gut contents were used to evaluate whether larvae are feeding from their sediment tubes and if the larvae selectively assimilate methanogens or methanotrophs.

CHAPTER III

RESULTS

Methanogenesis

The mean production of methane at 2m in Lake GTH 112 was 23 ± 16 μmol CH_4 / m^2 / day (Figure 1). Significantly more methane, 846 ± 507 μmol CH_4 / m^2 / day, was produced at 5m (*t*=2.1, df=10, p=0.003).

Isotope

Isotopic analysis of *Chironomus* larval $\delta^{13}C$ found that larvae have a depleted signature compared to photosynthetic food sources. Larvae at 5m had a significantly lower $\delta^{13}C$ value (-35.59 ± 0.10 ‰) than those at 2m (-35.04 ± 0.06 ‰) (*t*=4.7, df=4, p=0.009: Figure 2). Isotopic analysis of *Chironomus* larval $\delta^{15}N$ were also determined, but will not be discussed here (see Appendix C).

Sediment analyses

Concentrations of methanogen and methanotroph DNA were, on average, greater in the 5m cores than in the 2m cores (Table 2). Methanotroph DNA concentrations were found to depend on lake depth (2m and 5m) and depth down core (interaction of depth x depth down core: $F_{6, 141}$=10.75, p=<0.001). Methanogen DNA concentrations were also

found to depend on lake depth (2m and 5m) and depth down core (interaction of depth x depth down core: $F_{6, 135}$=11.25, p=< 0.001).

Down core patterns in methanogen and methanotroph DNA concentrations differed between 2m and 5m (Figure 3). In the 2m cores, there was a significant difference between concentration of methanotroph DNA in the sediments down core ($F_{2, 62}$=5.08, p=<0.001: Figure 3a), but not a significant difference between concentration of methanogen DNA in sediments down core ($F_{2, 62}$=2.10, p=0.066). In the 5m cores, there was a significant difference between concentration of methanotroph DNA in sediments down core ($F_{2, 79}$=16.09, p=<0.001: Figure 3b), and for concentration of methanogen DNA in sediments down core ($F_{2, 73}$=17.50, p=<0.001).

The methanogen pattern of DNA concentration in the 2m cores (Figure 3a) did not follow the same pattern of concentration as in the 5m cores (Figure 3b). The mean concentration of methanogen DNA at 1mm into the 2m cores was greater than the sediment-water interface (mean difference: 4.2 ng DNA gram^{-1} sediment; *t*=1.6, df=15, p=0.05), but less than the small peak in abundance at 2mm down core (Figure 3a). The quantity of methanogen DNA at 5mm declined from the value found at 2mm (mean difference: 6.3 ng DNA gram^{-1} sediment; *t*=2.1, df=18, p=0.03: Figure 3a). In the deeper parts of the 2m cores, the concentration of methanogen DNA continuously increased from 10mm – 60mm (Figure 3a). The 5m cores had the highest concentration of methanogen DNA from 1 – 2mm down core (Figure 3b). Methanogen quantities then declined at 5mm down core (mean difference from 2mm to 5mm: 559.7 ng DNA gram^{-1}

sediment; t=3.7, df=22, p=<0.001) and remained low at 10mm and 60mm down core in the 5m cores (Figure 3b).

The pattern of methanotroph DNA concentration in the 2m cores also did not follow the same pattern as methanotroph DNA concentration in the 5m cores (Figure 3). The 2m cores had uniformly low methanotroph DNA concentration in the overlying water and the sediment-water interface (Figure3a). Methanotroph DNA concentration was relatively high at 1mm down core in the 2m cores (mean difference from sediment-water interface to 1mm: 37.3 ng DNA gram^{-1} sediment; t=3.2, df=16, p=0.003), followed by a decline in mean concentration at 2mm and 5mm down core (mean difference from 1mm to 2mm: 34.8 ng DNA gram^{-1}sediment; t=3.6, df=19, p=0.001: Figure 3a). Deeper into the cores, the mean concentration of methanotroph DNA again increased at 10mm through to 60mm (mean difference from 5mm to 10mm: 19.1 ng DNA gram^{-1} sediment; t=2.1, df=20, p=0.024: Figure 3a). Overlying water at 5m had about 10 times more methanotroph DNA mL^{-1} water than 2m cores; and the sediment-water interface at 5m had about 5 times more methanotroph DNA gram^{-1} sediment than the 2m cores (Figure 3b). Methanotroph DNA concentration was greatest from 1 – 2mm down core in the 5m cores. Following this large peak, there was a sharp decline in methanotroph DNA concentration found deeper in the sediment (mean difference from 2mm to 5mm: 294.5 ng DNA gram^{-1} sediment; t=4.1, df=24, p=<0.001: Figure 3b). The methanotroph DNA concentration did not change with increasing depths from 5mm to 60mm in the 5m cores (mean difference from 5mm to 10mm: t=0.1, df=22, p=0.466; mean difference from 10mm to 60mm: t=0.4, df=16, p=0.366; Figure 3b).

Larval tube and gut analyses

Chironomus larval tubes had microbial compositions that were different than surrounding sediments, and varied between 2m and 5m (Figures 3 and 4). *Chironomus* larval tubes sampled from 5m were found to have a greater concentration of methanotroph DNA (t=2.6, df=46, p=0.003) and methanogen DNA (t=2.5, df=46, p=0.003) than *Chironomus* larval tubes sampled from 2m (Figure 4). Larval tubes from 5m had approximately 10 times more methanotroph DNA concentration and about 5 times more methanogen DNA concentration than 2m larval tubes (Figure 4). *Chironomus* larval tubes had significantly greater methanogen DNA concentration (t=4.7, df=139, p=<0.001) and methanotroph DNA concentration (t=4.2, df=136, p=<0.001) than larval gut contents (Table 3).

Gut contents from 2m larvae had the same concentrations of methanotroph DNA in the foregut and the hindgut (Table 3, Figure 5a). The concentrations of methanotroph DNA in 5m larval foreguts are comparable to 2m larval foregut concentrations (Table 3, Figure 5a). There was a significantly greater concentration of methanotroph DNA in *Chironomus* larval hindgut than in the larval foregut in 5m samples (t=2.9, df=43, p=0.006; Figure 5a). The concentration of methanogen DNA in the 2m larval hindgut was similar to the quantity in the foregut (Table 3, Figure 5b). The 5m gut samples had a greater concentration of methanogen DNA in the larval hindgut than in the larval foregut (t=3.5, df=45, p=0.001).

CHAPTER IV

DISCUSSION

This study shows generally consistent patterns of methanogenesis; distributions of methanogen and methanotroph DNA in sediment, on *Chironomus* larval tubes and presence of these microbes in larval gut contents; and with δ^{13}Cvalues of *Chironomus* at two depths in an arctic lake. Microbes on *Chironomus* larval tubes suggest that differing down core depths in sediments, or that specialized larval behavior contribute to the unique microbial components on larval tubes. Although presence of methanogens and methanotrophs in gut contents is consistent with the hypothesis that larvae assimilate methane-derived carbon, there was an unexpected pattern of more methanogen and methanotroph DNA in the hindguts of larvae from 5m compared to foreguts of those larvae.

The methanogenesis results from this study show that there was significantly more methane produced at 5m than at 2m. The depth difference in rates of methanogenesis from this study is comparable to previous studies of methanogenesis with respect to depth in Lake Mendota, WI, USA (Zeikus and Winfrey 1976). The authors noted that the deeper depths were aerobic and had more ammonia than the shallower lake sediments. Lake GTH 112 has very high rates of methanogenesis compared to Lake Batata and Lake Mussura in the floodplain of the Trombetas River (Conrad *et al.* 2010). In a review by St Louis *et al.* (2000) the global average of

methanogenesis in natural lakes averaged only 9 mg / m^2 / day (144 μmol / m^2 / day), suggesting this study lake to be producing methane at rates up to 10 times the average natural lake.

In general, arctic tundra wetlands emit between 20 to 40 Tg of methane a year, contributing to 8% of total methane emissions (Cao et al., 1996; Christensen et al., 1999). Other studies of arctic wetland methane emissions have shown great variability in methane emission from a variety of different sources (Ganzert 2007, Kling *et al.* 1992; Whalen and Reeburgh 1992). This could be a concern for overall methane concentration and its effect on global warming. It has been suggested that the arctic is warming more rapidly than the rest of the globe (Hansen *et al.* 2005). This could lead to longer summer seasons in the arctic, resulting in more methane emissions. Studies have shown that warmer temperatures have increased rates of methanogenesis (Ganzert 2007; McKenzie *et al.* 1998).

This study extends previous findings by suggesting depth differences in methane production can be attributed to greater concentration of methanogen DNA at the deeper site. During the summer season when these samples were obtained, the lake water profile showed 5m to be hypoxic (0.9mg / L). Given the low dissolved oxygen above the sediment water interface at 5m, more methanogens were expected in sediments at 5m than at 2m since these microbes require anoxic conditions (Conrad 2005). At 2m, the water profile showed that dissolved oxygen was approximately 13 times higher than at 5m. Oxygen penetrates deeper into sediments when dissolved oxygen is higher in the overlying water (Kajan and Frenzel 1999). Thus, deeper oxygen penetration is a likely

explanation for the lower methanogen abundance at 2m, and the lower rate of methanogenesis.

Down core peaks of methanogen or methanotroph DNA concentrations suggest sediment depths of favorable conditions for those respective microbes. Higher concentrations of methanogen DNA were found in sediments than methanotroph DNA. If the copy numbers of 16S rRNA for methanogens and methane monooxygenase genes for methanotrophs are similar, then this implies there were more methanogens than methanotrophs in these samples. Methanogen DNA showed peak concentrations nearer to the sediment water interface at 5m than at 2m. Since sediments were likely anaerobic at 5m, and methanogens can be found in any moist anaerobic habitat with suitable substrates (Jones *et al.* 1987), it is likely that large quantities of degradation and fermentation byproducts had accumulated at this depth. The quantity of methanogens found in the sediments at 2m increased with increasing depth of sediment, which also suggests accumulation of substrates derived from aerobic metabolism in overlying sediments as well as a greater depth of anoxia compared to conditions at 5m (Schulz and Conrad 1995). Therefore, the strong difference in methanogen abundance and distribution at 2m compared to 5m could be reflective of the presence of oxygen at 2m.

Methane oxidation is an important component of methane cycling. Methane oxidation directly consumes methane, and thus reduces the amount of methane released to the atmosphere (Caldwell *et al.* 2008; Valentine and Reeburg 2000; Whalen 2005). Because methanotrophs are aerobes, methanogens and methanotrophs are not expected to be spatially coincident (Thauer & Shima 2006; Jones *et al.* 1987). Dissolved oxygen was

low at 5m and concentrations of methanotroph DNA was greatest 1 – 2mm into sediments, similar to findings by Kajan and Frenzel (1999), suggesting that this sediment layer contained both oxygen and methane. The sediment profile at 5m suggests methanotrophs are present at all depths of sediment up to 60mm, but in much lower quantities further down core.

The occurrence of methane oxidation at 2m is consistent with expected patterns, but also suggests an important role for bioturbation and /or physical mixing at this depth. The greatest methane production in the 2m cores occurs in depths greater than 5mm; whereas greatest concentration of methanotrophs occurs at only 1mm down core, where sediments were likely to be well oxygenated. This is consistent with the expected pattern that methane diffuses upward from the deeper sediment before being oxidized (Whalen 2005; Valentine and Reeburgh 2000). However, an additional peak in methanotroph DNA occurs from 10 – 60mm down core, where anaerobic conditions would be anticipated (Whalen, personal communication). However, *Chironomus* tubes likely extend deeper into sediments at 2m than at 5m due to greater dissolved oxygen penetration, and may provide microsites for methanotrophs (Jones *et al*. 2008; Kajan and Frenzel 1999). Furthermore, turbulence in the epilimnion is also likely to provide greater down core mixing of dissolved oxygen, which also could lead to development of oxygenated microsites in deeper sediments.

The process of methanogenesis leads to carbon isotope fractionation resulting in depleted $\delta^{13}C$ values (Kohzu *et al.* 2004). The signature of biogenic methane also depends on the pathway of methane formation (Alperin *et al.* 1992). The two major

pathways of methane production, hydrogenotrophic and acetoclastic, may yield methane with different isotopic signatures. Reports of $\delta^{13}C$ values for biogenic methane range from -40‰ to as low as - 110‰ (Kohzu *et al.* 2004; Jones *et al.* 2008). Previous studies in Lake GTH 112 have shown that the $\delta^{13}C$ of methane was approximately – 42‰ (Hershey, unpublished data), which is consistent with methane produced predominately from acetate fermentation (Alperin 1992). However, there is great variability in the amount of fractionation that is associated with methane oxidation (Venkiteswaran and Schiff 2005; Whiticar 1999; Wolteman *et al*. 1984; Whiticar and Faber 1985) which makes it difficult to attribute methanogenesis pathways to discrete $\delta^{13}C$ values of organism or water column methane, or compute mixing models to estimate the percentage of methane derived carbon from consumer $\delta^{13}C$ values.

Organisms that use methane-derived carbon will also have a low $\delta^{13}C$ signature. In general carbon has only a 0.5 – 1 ‰ enrichment that occurs between an animal and its food (Peterson and Fry 1987). The *Chironomus* larvae in this study have a depleted carbon signature, which is consistent with the incorporation of methane-derived carbon. The *Chironomus* larvae $\delta^{13}C$ signatures in this study were similar to those found in Deines *et al.* (2009) suggesting the incorporation of acetate-dependent methane-derived carbon. The isotopic signature of larvae from 2m suggests that these larvae use less methane-derived carbon than *Chironomus* larvae at 5m, however the difference was small. Non-methane-derived carbon sources have $\delta^{13}C$ values ranging from -26 to -32‰ (Hershey *et al.* 2006), compared to -35.0‰ and -35.6‰ for 2m and 5m, respectively, reported here. This is consistent with the conclusion of Hershey *et al.* (2006) who studied

invertebrate $\delta^{13}C$ values in GTH 112 as well as other arctic lakes. The present study also provides supporting evidence based on methanogen and methanotroph DNA in sediments, on tubes, and in gut contents.

Chironomus larvae are reliant on their sediment tubes for protection and possibly even in obtaining food (Hershey 1987). Most chironomid larvae are generally confined to the surface layers of soft sediments but some may penetrate deeper (Pinder 1986). Additionally, the location within the sediment inhabited by the larvae reflects primary feeding habits (Deines *et al* 2009). For larvae that primarily live and remain in the tube they construct, larval behavior, mainly bioturbation, could promote conditions favorable for respiration and obtaining food (Hershey 1987; Jones *et al.* 2008; Eller *et al.* 2007; Pinder 1986; Johnson 1989). Deines *et al.* (2007) suggested that the bioturbation activity of chironomids creates a microhabitat suitable for the growth of methane oxidizing bacteria on the larval tubes, even in anaerobic sediment. This in turn would provide larvae with a constant supply of ^{13}C depleted carbon food source. The bioturbation behavior and resulting growth of methanotrophic bacteria on the larval tubes has been considered 'microbial gardening' (Deines *et al.* 2007). The concept of 'microbial gardening' (Deines *et al.* 2007, 2009) may explain the large concentration of methanotroph DNA on 5m *Chironomus* larval tubes.

Methanogen and methanotroph DNA concentrations on larval tubes were distinct with respect to depth and suggestive that the larval tubes may be positioned differently within the sediments at the two depths. The lower concentration of methanogen DNA found on larval tubes compared to methanotroph DNA suggests tubes to be distinctive

from nearby sediments, as suggested in Stief *et al.* (2005). The methanotroph DNA concentration found on larval tubes suggests the presence of oxic conditions. The larval tubes from 2m have concentrations of methanotroph DNA that are similar to sediment depths ranging from 2 - 5mm into the 2m cores; 5m larval tubes have concentrations most similar to down core sediment deeper than 5mm in the 5m cores. While these similarities are evident, the actual location of *Chironomus* within the sediments is not certain, though these similarities suggest larvae are occupying different sediment depths at 2m than 5m. Further studies are needed to evaluate the location of tubes within the sediment profile, and the ecological implication of tube vertical distribution.

Modes of feeding that have been suggested or demonstrated for *Chironomus* include ingestion of surficial sediments and associated organic particles, and directly scraping particles off the larval tube (Johnson1989; Pinder 1986). Grazing on tube algae by the chironomid *Orthocladius* has been directly observed by Hershey *et al.* (1988). Furthermore, food sources include plants, algae, heterotrophic microbes and detritus (Oliver 1971). It has been documented that larval feeding can impact the tube flora (Hershey *et al.* 1988; Pringle 1985). The microbial components found on the larval tube combined with PCR results from gut contents in this study adds to previous findings that chironomid larvae consume methane oxidizing bacteria (Deines *et al.* 2007; Eller *et al.* 2007). It is assumed that *Chironomus* larvae ingested the methanotrophic biomass and acquire their low $\delta\ ^{13}C$ from the 'microbial garden' they have developed on their larval tubes (Deines *et al.* 2007; Eller *et al.* 2007). However, gut content analyses from this study found a higher concentration of methanogens in larval foreguts than

methanotrophs, especially at 5m (again, assuming similar gene copy numbers of respective coding enzymes for methanogens and methanotrophs). Also, the methanogen DNA concentration was lower on larval tubes, which could occur if microbes were selectively grazed upon (Johnson 1989). Eller *et al.* (2007) found methanogenic archaea were not an important component of chironomid diets. While this study is not inconsistent with previous studies on chironomid consumption and assimilation of methanotrophic bacteria, it also suggests the need for further investigation on the role of methanogenic archaea in chironomid diets.

An unexpected finding in this study was the higher concentration of methanogen DNA in *Chironomus* hindgut than in foregut contents. Since, in insects, assimilation and absorption occur in the midgut, (Breznak 1982) the mechanism contributing to significantly greater concentration of methanogen DNA in the larval hindgut than in the foregut is unclear. It is known that *Chironomus* larval guts are completely anoxic (Deines *et al.* 2007) which would make them a suitable location for methanogenic microbial growth. One possible suggestion is that *Chironomus*, like termites, harbor methanogens in the hindgut (Gomathi *et al.* 2009; Ohkuma *et al.* 1999; Breznak 1982). However, previous studies have found no evidence of a symbiotic relationship between chironomid larvae and methanogenic archaea by fluorescence *in situ* hybridization analyses of larval tissue thin sections in *Chironomus plumosus* (Eller *et al.* 2007; Deines *et al.* 2007). In the study by Eller *et al.* (2007), analysis for a symbiotic relationship was done using Eubacteria and Archaea probes, different from the probes used in this study which were methanogen and methanotroph specific. The presence of methanotroph and archaea DNA

found in chironomid guts from this study is consistent with Eller *et al.* (2007), however this study provides further evidence for methanogen DNA being present in chironomid larval guts. The results from this study suggests that a symbiotic relationship is possible, and justifies further investigation of the role of methanogens within the digestive track of *Chironomus* larvae, that is independent of a nutritional role of methane-derived carbon for C*hironomus*.

In addition to there being increased methanogen DNA concentration in *Chironomus* larval hindguts, there was also an increased concentration of methanotroph DNA in the 5m larval hindguts when compared to the foreguts, also indicating methanotroph activity within the gut. However, methanotrophs can only be active in areas where there are both methane and oxygen present (Thauer & Shima 2006; Kajan and Frenzel 1999) implying these microbes are obtaining oxygen. *Chironomus* larvae are known to have hemoglobin which helps in obtaining and storing oxygen (Gullan and Cranston 2005). Previous studies in termites have determined methane oxidation does not occur in the hindguts (Pester *et al.* 2007). However, termites do not have hemoglobin. The large concentration of methanotroph DNA in 5m larval hindguts suggests the possibility of methanotrophic bacteria to be able to obtain oxygen through diffusion across *Chironomus* gut wall supporting oxidation of methane.

CHAPTER V

CONCLUSIONS

Results of this study consistently show that methane-derived carbon is important to *Chironomus* at 5m, but less so at 2m. The presence of methanotrophs appears to play a significant role in this lake, and the resulting methane-derived carbon is an important carbon source for *Chironomus* larvae. The importance of bacterial components in *Chironomus* diets has been shown to differ between lakes and seasons (Grey and Deines 2005) although assimilation of select components is difficult to determine (Doi *et al.* 2006). This study has shown the importance of methanotrophic bacteria in the diets of *Chironomus* larvae in a shallow arctic oligotrophic lake, especially in deeper sediment. The unexpected discovery of larger concentrations of methanogen and methanotroph microbes in the hindguts of *Chironomus* suggests the need for further investigation; the hindgut is unlikely to have a role in larval nutrition, but data presented here suggests the possibility of microbial gut symbionts. Additionally, the results of this study indicate rates of methanogenesis are substantial in this small arctic lake and that greater depths produce greater quantities of methane. This lake and other arctic wetland systems that produce large amounts of methane may be significant contributors of methane to the atmosphere. Methane production and the use of methane-derived carbon in the food web are processes not well studied in many lakes, but reports that methane-derived carbon is important in aquatic food webs are appearing at an increasing rate. The findings

of this study can be foundations for future research on the mechanism leading to the utilization of methane-derived carbon in aquatic ecosystems. The location of this lake in the arctic makes it a very susceptible to the effects of global climate change processes. Understanding the cycling of methane, a greenhouse gas, in this lake could have future implications for methane and carbon cycling. Based on the unique findings within *Chironomus* larvae gut contents, it is evident that the utilization of methane-derived carbon and its associated microbes is an important carbon source but needs further investigation.

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APPENDIX A: TABLES

Table 1. PCR primers used in initial PCR testing and their respective references.

Primer Pair	Sequence	T_m (°C)	Target	Reference
S-P-March S-D-Arch	GYGCAGCAGGCGCGAAA GGACTACVSGGGTATCTAAT	61.5	Methanogen	Sawayama *et al.* 2004
Met83F Met 86 F Met 1340R	ACKGCTCAGTAACAC GCTCAGTAACACGTGG GGTGTGTGCAAGGAG	46.3	Methanogen	Wright and Pimm 2003
mmoX1 mmoX2	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT GGCTCGACCTTGAACTTGGAGCCATACTCG	71.6	Methanotroph	Miguez *et al.* 1997
*pmo*Afor *pmo*Arev	TTCTGGGGNTGGACNTAYTTYCC TCNACCATNCMHATYTARTCNGG	58.7	Methanotroph	Steinkamp, Zimmer and Papen 2001
A189 mb661	GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	56.2	Methanotroph	Costello and Lindstrom 1999

Primer pairs listed with forward primer on top of reverse primer. (Met83F and Met 86F are both forward primers for Met 1340R reverse primer).

Table 2. 2m and 5m methanogen and methanotroph microbial mean DNA concentrations in sediment cores

A

Type Microbe	Mean DNA concentration (ng DNA gram^{-1} sediment)
Methanogen	27. 03 ± 8.39
Methanotroph	13.48 ± 8.32

B

Type Microbe	Mean DNA concentration (ng DNA gram^{-1} sediment)
Methanogen	130.50 ± 18.90
Methanotroph	81.42 ± 18.07

2m (A) and 5m (B) methanogen and methanotroph mean DNA concentrations in sediment cores (ng DNA gram^{-1} sediment). All data are given in mean vales ± standard errors. All down core sectioning were pooled together to obtain general means for 2m and 5m sediment cores. Sample sizes: n = 69 for 2m samples. Sample sizes: n = 80 for methanogen, n = 86 for methanotroph for 5m samples.

Table 3. Concentrations of methanotroph and methanogen DNA in larval guts and tubes for 2m and 5m depths

Location	Depth	Methanotroph DNA concentration (ng DNA gram^{-1} sediment or gut)	Methanogen DNA concentration (ng DNA gram^{-1} sediment or gut)
	2m		
Foregut		0.02 ± 0.01	0.04 ± 0.01
Hindgut		0.02 ± 0.01	0.06 ± 0.02
Tube		3.82 ± 0.81	2.88 ± 0.81
	5m		
Foregut		0.04 ± 0.01	0.12 ± 0.04
Hindgut		0.32 ± 0.09	0.76 ± 0.17
Tube		31.44 ± 10.78	16.30 ± 5.22

All data are given as means ± standard error. Sample sizes for gut components were ≥ 91, sample sizes for tubes was 96. DNA concentrations are ng DNA gram^{-1} sediment or gut.

APPENDIX B: FIGURES

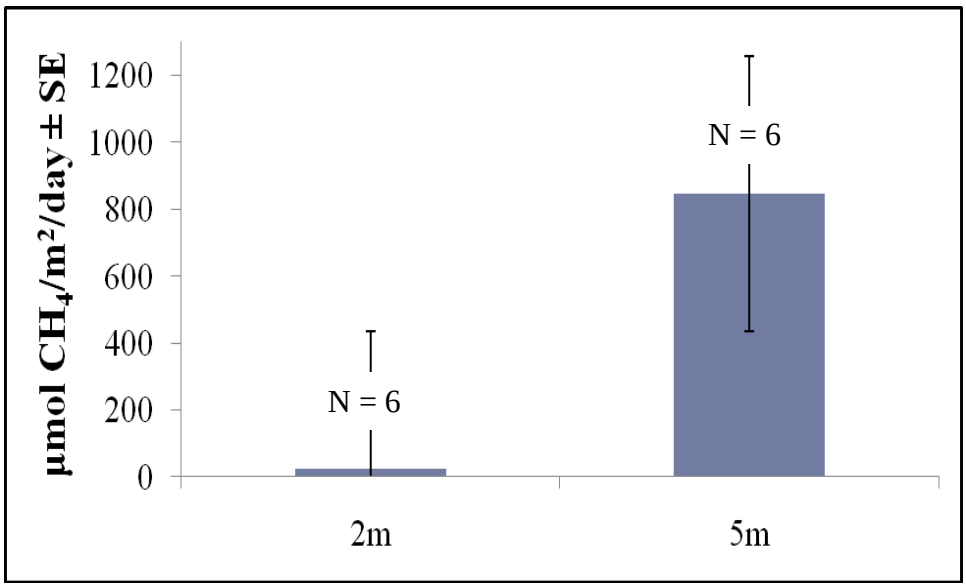


Figure 1: Quantity of µmol CH_4 / meter^2 / day in Lake GTH 112 at 2m and 5m. Sample sizes for both depths were 6. Bars represent mean net production, with standard error lines.

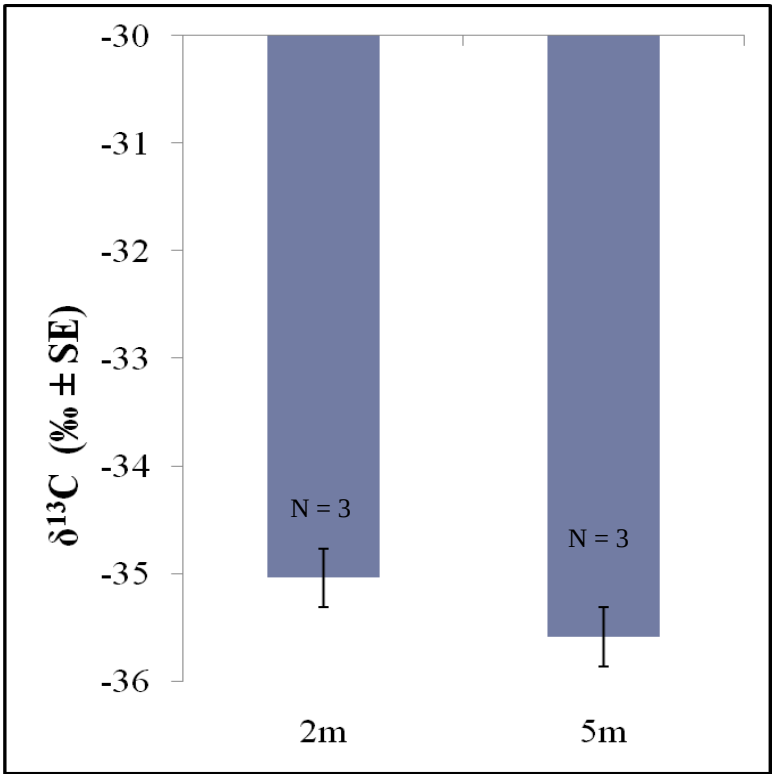


Figure 2. $\delta^{13}C$ of *Chironomus* larvae in Lake GTH 112 at 2m and 5m. Samples sizes for both depths were 3. Bars represent mean values, with standard error lines.

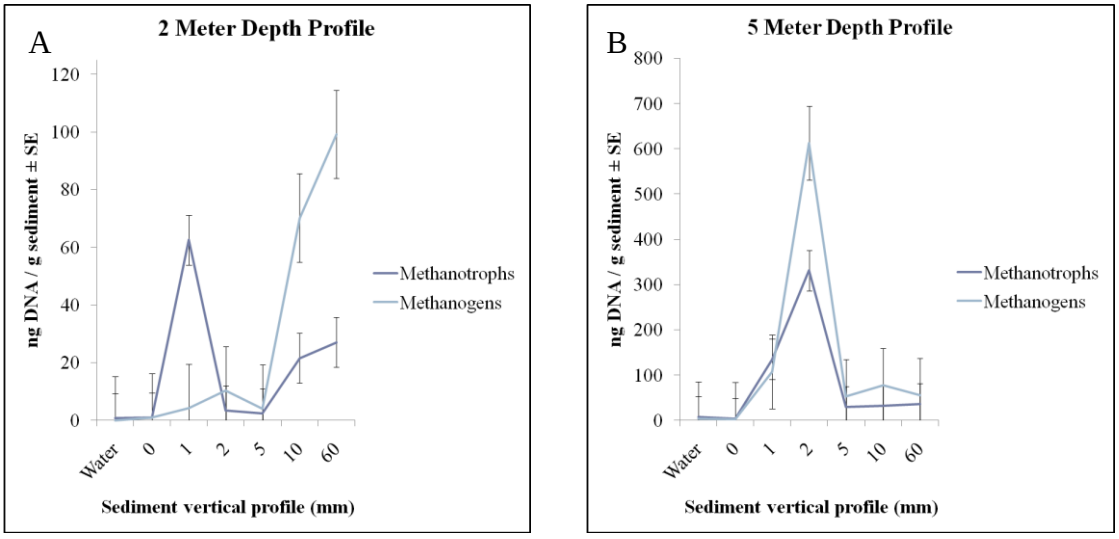


Figure 3. Sediment profile of methanogen and methanotroph DNA concentrations at (A) 2m and (B) 5m. Trend lines are based on down core sectioning means for each depth with standard error lines. Note: the y-axis scales on A and B are different.

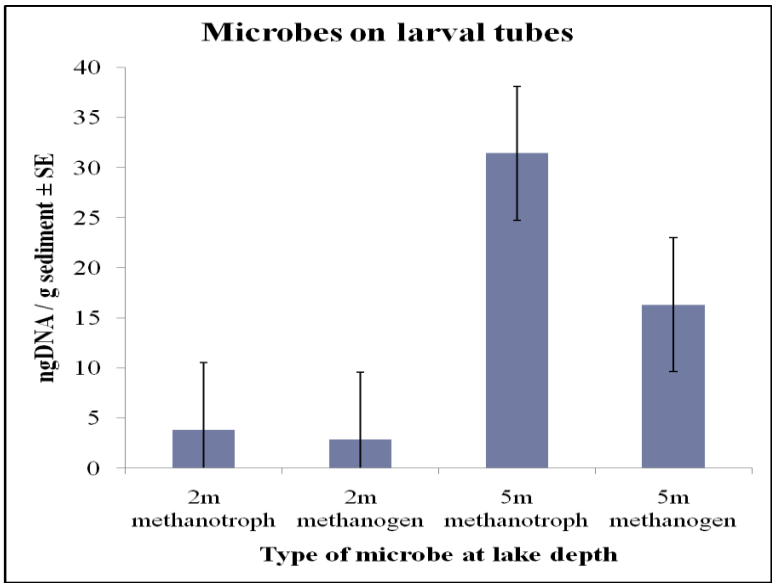


Figure 4. Concentration of methanogen and methanotroph DNA on larval tubes in GHT 112. Bars represent mean quantities, with standard error lines.

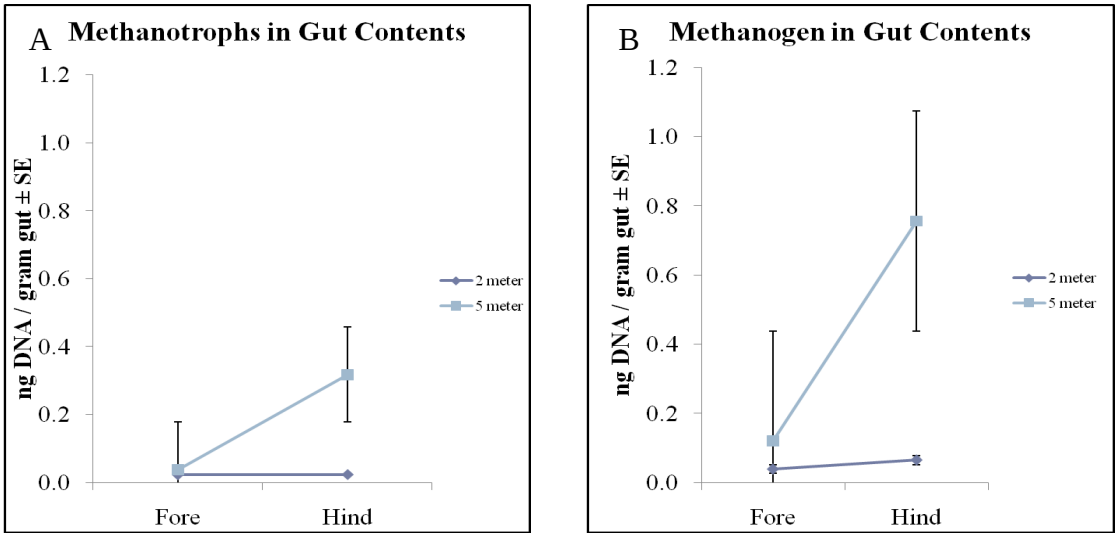
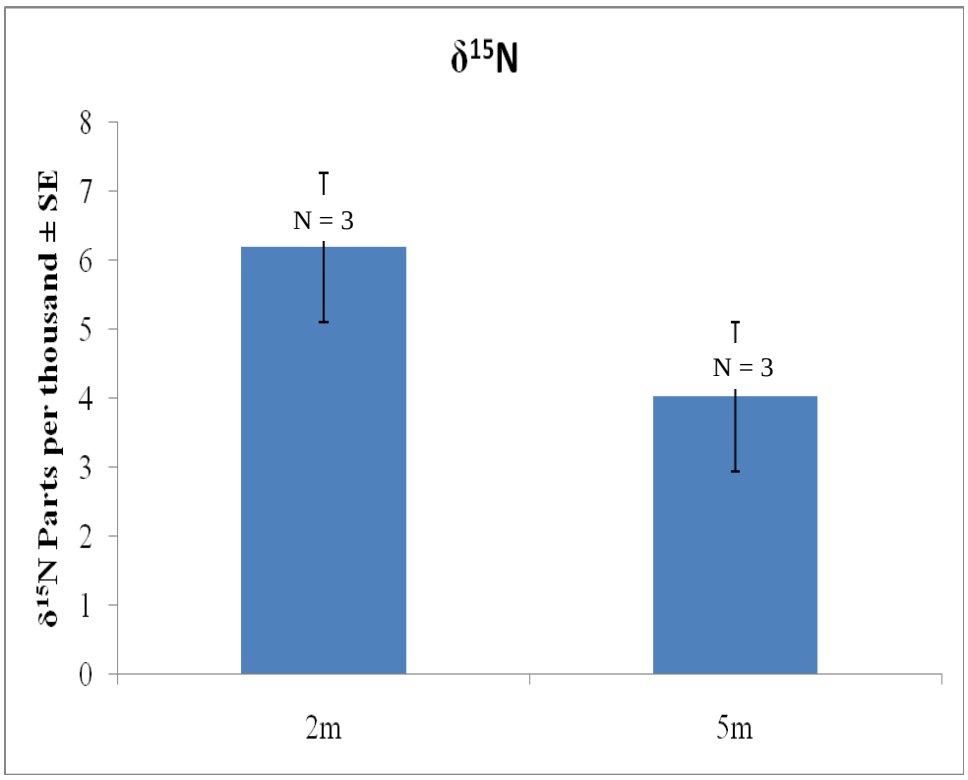


Figure 5. Concentration of select microbe DNA in larval gut contents at 2m and 5m for (A) methanotrophs and (B) methanogens. Trend lines are based on mean quantity in each location, with standard error lines.

APPENDIX C: $\delta^{15}N$ *CHIRONOMUS* LARVAE



$\delta^{15}N$ of *Chironomus* larvae in Lake GTH 112 at 2m and 5m. Samples sizes for both depths were 3. Bars represent mean values, with standard error lines.

APPENDIX D: STATISTICAL OUTPUT

ANOVA output for sediment analyses in order they appear in results

Tests of Between-Subjects Effects: Methanotrophs

Dependent Variable:ngDNA_gSediment

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.516E6	13	116628.395	16.462	.000
Intercept	321657.229	1	321657.229	45.402	.000
DownCore	454354.507	6	75725.751	10.689	.000
Depth	164841.456	1	164841.456	23.267	.000
DownCore * Depth	456904.419	6	76150.736	10.749	.000
Error	998933.206	141	7084.633		
Total	3182769.579	155			
Corrected Total	2515102.335	154			

a. R Squared = .603 (Adjusted R Squared = .566)

Tests of Between-Subjects Effects: Methanogens

Dependent Variable:ngDNA_gSediment

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.066E6	13	389707.050	15.246	.000
Intercept	842083.611	1	842083.611	32.945	.000
DownCore	1501159.523	6	250193.254	9.788	.000
Depth	363337.671	1	363337.671	14.215	.000
DownCore * Depth	1725545.068	6	287590.845	11.251	.000
Error	3450659.809	135	25560.443		
Total	1.035E7	149			
Corrected Total	8516851.455	148			

a. R Squared = .595 (Adjusted R Squared = .556)

ANOVA: 2m methanotrophs

ngDNA_gSediment

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14651.269	6	2441.878	5.081	.000
Within Groups	29798.664	62	480.624		
Total	44449.933	68			

ANOVA: 2m methanogens

ngDNA_gSediment

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	108325.882	6	18054.314	2.100	.066
Within Groups	533031.883	62	8597.288		
Total	641357.764	68			

ANOVA: 5m methanotrophs

ngDNA_gSediment

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1184429.002	6	197404.834	16.092	.000
Within Groups	969134.542	79	12267.526		
Total	2153563.544	85			

ANOVA: 5m methanogens

ngDNA_gSediment

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4196620.596	6	699436.766	17.500	.000
Within Groups	2917627.926	73	39967.506		
Total	7114248.522	79			