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Herbivorous zooplankton feed on seston, including algal, detrital, and bacterial components. However, many studies have found that *Daphnia* $\delta^{13}C$ is more depleted than seston $\delta^{13}C$, and selective feeding on seston algal components has been hypothesized. We hypothesized that low zooplankton $\delta^{13}C$ was due to utilization of methane-derived carbon (MDC). Lake Brandt data indicated a strong seasonal effect on the $\delta^{13}C$ of *Daphnia* when compared to seston. During summer stratification, carbon signatures of *Daphnia* were more depleted than bulk seston, while during winter mixing *Daphnia* were more enriched. We conducted an experiment to assess utilization of MDC as a possible mechanism of depleted *Daphnia* $\delta^{13}C$. At low methane concentrations, methane $\delta^{13}C$ was more enriched than at high concentrations, indicating isotopic fractionation by methanotrophs. *Daphnia* $\delta^{13}C$ was slightly enriched compared to seston at low methane concentrations, but was depleted compared to seston at high methane concentrations, consistent with incorporation of MDC. An antibiotic appeared to limit methanotrophs in the water column resulting in enriched *Daphnia* $\delta^{13}C$. MDC contribution to the diet of *Daphnia* was estimated as almost 32% of assimilation. Our study provides strong evidence that assimilation of MDC resulted in depleted *Daphnia* $\delta^{13}C$ relative to seston. We suggest that this phenomenon is widespread in freshwater ecosystems, explaining the consistently light $\delta^{13}C$ of grazing zooplankton relative to bulk seston.

POTENTIAL ROLE OF METHANE-DERIVED CARBON AS A FOOD SOURCE FOR *DAPHNIA* IN A NORTH CAROLINA RESERVOIR

by

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This project is dedicated to the memory of Dr. W. John O'Brien, for his guidance, his stories, and his faith in me during so many long days.

APPROLVAL PAGE

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CHAPTER I

INTRODUCTION

Trophic dynamics of lakes have been of interest for decades. Lindeman (1942) described a lacustrine feeding cycle by assigning trophic levels to the biotic components of the ecosystem. The intricate relationships between producers and consumers were quantified in terms of energy transfer. Each discrete level depends on the preceding trophic level for energy (Lindeman 1942). Burns (1989) pointed out that these interactions are even more complicated because some species may be feeding at multiple trophic levels and/or simultaneously incorporating decomposers into their diet.

Stable isotope analysis has been used recently as a tool to characterize feeding behavior and trophic status of individual species in an ecosystem (Peterson and Fry 1987). The examination of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios, expressed in parts per thousand, as δ values, in biotic components of an ecosystem has been used for the classification of trophic levels and identification of food sources of individual species. Increases in δ values indicate retention of the heavier isotope, whereas, decreases indicate that the lighter isotope is abundant. To estimate the trophic level of a species, the $^{15}N/^{14}N$ ratio can be used. Animals metabolize and excrete the light isotope (^{14}N) faster than ^{15}N, making a species 2.2-3.4‰ more enriched than its food source (Fry 2006). However, with carbon isotopes fractionation is minimal, showing only 0-1‰ enrichment in ^{13}C in

the consumer, such that $\delta^{13}C$ is often used to determine food sources for consumers (Peterson and Fry 1987).

Many trophic studies of freshwater systems have used stable isotope analysis to construct aquatic food webs. In these studies seston or particulate organic mater (POM) in the water column is assumed to be the base of the food web and is thought to consist mainly of algae with both bacteria and detritus also present (Moss 1970). *Daphnia* and other herbivorous zooplankton feed on seston, or components of seston (del Giorgio and France 1996). Because "you are what you eat," with respect to carbon stable isotope analysis, we assume that the carbon signature of a consumer would match or be slightly enriched when compared to its food source. However, a paradox has been repeatedly recorded in many aquatic trophic analyses: the $\delta^{13}C$ values of seston from freshwater systems are consistently more enriched (+ 2-20‰) than the $\delta^{13}C$ values of *Daphnia* (del Giorgio and France 1996; Jones et al. 1999). It would be expected that since *Daphnia* are suspension feeders, the $\delta^{13}C$ values of *Daphnia* would be slightly enriched or the same as the seston, which does occur in the stable isotope signatures of herbivorous zooplankton in marine systems (del Giorgio and France 1996).

Jones et al. (1999) found that herbivorous zooplankton, including several species of *Daphnia* were generally depleted in ^{13}C when compared to seston. The *Daphnia* spp. were 3-17‰ more depleted than the seston of the associated lake (Jones et al. 1999). Twenty-eight temperate lakes yielded the same trend in $\delta^{13}C$ depletion of herbivorous zooplankton (del Giorgio and France 1996). Algal cells, bacteria, and detrital content have all been assumed to contribute to the diet of *Daphnia* (Edmondson 1957;

Brendelberger 1991; Salonen and Hammar 1986; Hessen et al. 1990). Yet, this seems inconsistent with the phenomenon of *Daphnia* being considerably depleted in ^{13}C when compared to seston.

There are three potential explanations for the paradox. The first involves selective assimilation of an isotopically light fraction of the seston. The second explanation is differential metabolic fractionation by *Daphnia*, resulting in a more depleted $\delta^{13}C$ value than their food. However, based on extensive research of stable isotope analysis by Fry (2006), it has been accepted that all consumers only fractionate their carbon source 0-1‰ toward the heavier isotope (Peterson and Fry 1987). The third potential explanation is that the *Daphnia* in freshwater systems are incorporating an isotopically light carbon source that is not represented in the measures of $\delta^{13}C$ of seston.

Historically, Brooks and Dodson (1965) described *Daphnia* spp. as "food collectors", suggesting an inability to selectively discriminate between food sizes. They noted that the size of the incorporated food was pre-determined by the dimensions of the filtering structures used by individual zooplankton (Brooks an Dodson 1965). Food particles ranging from 1-15 μm can be captured by all herbivorous zooplankton species, while larger cladocerans and copepods may capture particles up to 50 μm (Brooks and Dodson 1965). Burns (1968) found a correlation between the carapace length of cladocerans and the maximum size of the food particle that can be ingested during an experiment using plastic beads. The resulting correlation is $y = 22x + 4.87$, where x is the carapace length in millimeters and y is diameter of largest bead that could be incorporated by the zooplankton (Burns 1968). Other feeding experiments have found

that the filtering mesh of some cladocerans can capture smaller food particles, such as bacteria, as small as 0.05 μm but the size of the smallest particle captured is not related to carapace length (Peterson et al. 1978). Note that particles < 0.7 μm can pass through the glass fiber filters typically used to collect seston for stable isotope analysis.

Although these herbivores cannot modify the range of their food sizes, they can select which food particles to reject during feeding (DeMott 1982). Cladocerans perform suspension feeding, in which their thoracic legs create a water current within the carapace, bringing in water and suspended particles. Here, the limbs filter the particles and transport them up the ventral food groove toward the mouth where they are formed into a food bolus, or mass, which is then ingested (Watts and Petri 1981; Gophen and Geller 1984). At the mouth, *Daphnia* and *Bosmina* may reject an unsuitable bolus before ingestion using the postabdominal claw (DeMott 1982). The shape, size, and quality of the food are usually the factors that determine which food is rejected (Quiblier-Llobéras et al. 1996). However, because this mass of food may contain many kinds of particulates, the assumption of selective rejection is arguable.

Selectivity of cladocerans feeding has been highly debated. DeMott (1982) studied the feeding selectivities of *Daphnia* and *Bosmina* with ^{14}C-labeled algae and ^{3}H-labeled bacteria. He found that while *Daphnia* may be able to reject certain food particles with the postabdominal claw, it was a relatively nonselective suspension feeder, incorporating both the algae and bacteria regardless of their relative abundance (DeMott 1982). However, *Bosmina* preferentially selected the algal cells over the bacteria cells even when the algae were in low abundance, suggesting that *Bosmina* may have

physiological or behavioral mechanisms that make it a more efficient selective feeder than *Daphnia* (DeMott 1982). Another study explored cladoceran and rotifer selectivity, measured by algal cell ingestion rates with and without clay particles. Kirk (1991) found that although the presence of clay particles decreased the algal ingestion rates of cladocerans, cladocerans and rotifers showed significant selectivity of algal cells over clay particles ($p < 0.05$). Within the cladocerans, algal selectivity had a positive correlation with body size, resulting in greater selectivity for algal cells in larger cladocerans (Kirk 1991).

Further selectivity studies have been used to categorize the types of algae and bacteria that are ingested most efficiently by cladocerans. Edmondson (1957) presents a detailed summary of suitable and unsuitable algal food sources for *Daphnia*. He presents conditions that may limit food ingestion including cell size and shape associated with filtering mesh size and the ability of the *Daphnia* to digest the algal cell based on the thickness of its cell wall (Edmondson 1957). Knisely and Geller (1986) also found that *Daphnia* preferentially graze on algal sources based on different cell characteristics. The size of ingested algal cells was based on the size of the feeding apparatus, where extremely small particles were not retained on the filtering appendages and large cells were unable to enter the carapace or the mouth opening (Knisely and Geller 1986). They also found that *Daphnia* ingested more flagellated algal cells than coccoid cells and suggested that flagellated cells may be caught in the filtering appendages more efficiently (Knisely and Geller 1986). Furthermore, Brendelberger (1991) found that bacterial cell ingestion was also highly correlated with the filter mesh size of the feeding appendages

of *Daphnia*. Based on these studies, active selectivity toward depleted carbon food sources seems unlikely suggesting that ingestion of a light carbon source is passive.

Two hypotheses have been offered to explain the depleted $\delta^{13}C$ values of *Daphnia*. Hamilton et al. (1992) found that microalgae were considerably depleted in ^{13}C compared to bulk POM and suggested that these microalgae were the major food source for macroinvertebrates. Alternatively, the highly depleted carbon source being assimilated by *Daphnia* may be methane-derived carbon, which is known to have a very depleted $\delta^{13}C$ value (Bunn and Boon 1993; Kankaala et al. 2006). Bunn and Boon (1993) used stable isotope analysis to determine the major carbon source for three flood-plain lakes. They measured the carbon signatures of emergent, floating, and submerged aquatic plants including bulk POM and found that the $\delta^{13}C$ signatures of zooplankton, including *Daphnia*, were more depleted than any potential food source that was measured within the lakes. They concluded, like many other studies, that zooplankton must have been utilizing a highly depleted food source, perhaps in addition to algae and detritus (Bunn and Boon 1993). A reasonable sink of highly depleted carbon resides in methane-derived carbon and ingestion of methane-derived carbon may explain the depleted carbon signatures of the zooplankton.

Methanogenic bacteria produce methane using an anaerobic microbial pathway, while methanotrophic bacteria oxidize the methane produced (Whalen 2005). Biogenic methane has a $\delta^{13}C$ value of -80 to -52‰ in freshwater habitats and if these methane-associated prokaryotes are abundant in the habitat, they may be fed upon by zooplankton, resulting in a more depleted carbon signature (Bunn and Boon 1993). Kankaala et al.

(2006) conducted an investigation of this hypothesis by enriching *Daphnia* cultures with biogenic methane and by performing an *in situ* study in a polyhumic lake. They concluded that the decrease in the $\delta^{13}C$ signature of the *Daphnia* could not be attributed to selective algal feeding, but instead was due to the incorporation of methanotrophic bacteria into the diet of the zooplankton (Kankaala et al. 2006).

Using stable isotope analysis, we investigated utilization of methane-derived carbon and metabolic fractionation of food sources in *Daphnia* from a temperate reservoir, and estimated the relative impact of methane-derived carbon on their $\delta^{13}C$ signature.

The overall goal of our research was to evaluate how different carbon sources in a reservoir impact the $\delta^{13}C$ signature of *Daphnia*. We hypothesized that the depleted carbon signature of *Daphnia* relative to seston is due to the assimilation of methane-derived carbon. Our first objective was to identify the natural $\delta^{13}C$ signatures of *Daphnia spp.*, seston, and methane in Lake Brandt. Our second objective was to establish the methane concentration and abundance of methanotrophic and methanogenic bacteria in the reservoir. Our third objective was to determine the carbon isotope signature of *Daphnia magna*, when only algae are available as a carbon source. We predicted that the $\delta^{13}C$ signature of *Daphnia* to be 0-1‰ more enriched than the $\delta^{13}C$ signature of the algae. Finally, our last objective was to identify the relative impact of methanogenic and methanotrophic bacteria on the carbon signature of *Daphnia*. We predicted that the presence of methanogenic and methanotrophic bacteria would result in a $\delta^{13}C$ signature of *Daphnia* that is more depleted than the $\delta^{13}C$ signature of the seston.

CHAPTER II

METHODS

Lake Brandt, an 816-acre municipal reservoir in northern Greensboro, NC (36.18764 N, -79.81226 W), was sampled for this study. The reservoir is classified as temperate monomictic, with thermal stratification occurring during the summer. Complete mixing of the water column occurs during winter and spring, directly after fall turnover. Lake Brandt drains 151.3 km^2 of land and is almost completely surrounded by a forested buffer. Water samples, sediment samples, and zooplankton were collected from the lacustrine section of the reservoir.

Sampling and techniques

Sampling from Lake Brandt was done in the lacustrine section of the reservoir on three winter sampling dates from October to November of 2009. During each sampling time, two 1.5 m tows using a 153 μm zooplankton net were taken. Using a horizontal Van Dorn, 8 L of water were collected from 1.5 m and placed into 4 L cubitainers. In the lab, 100 *Daphnia* were counted and picked from the net sample. They were put into 4x6 tin capsules and placed in a drying oven at 60°C for 24 hours. To establish the $\delta^{13}C$ of the seston, 300 mL of the water was filtered on grade 40 Millipore glass fiber filters (0.7 μm pore size) and put into tin foil wrappers and allowed to dry for 24 hours in the drying oven. Both *Daphnia* and the filters were then packaged and sent to the Marine Biological Laboratory in Woods Hole, Massachusetts for carbon stable isotope analysis. A 10 mL

syringe was used to extract 7 mL of water from a 1.5 m and a 4 m Van Dorn sample for analyses of methane concentration and methane $\delta^{13}C$ in the reservoir. The 7 mL water samples were injected into helium-evacuated 12 mL exetainers that had been injected with 0.1 mL of 1N HCL to arrest microbial activity. After injection, the water samples were shaken vigorously and stored upside down. In lab, samples were refrigerated until further use. One sample was sent to UC Davis Stable Isotope Facility in Davis, California for analysis of $\delta^{13}C$ of methane. The other was taken to UNC-Chapel Hill and analyzed for methane concentration (ppm) using gas chromatography.

To measure the concentration of methanotroph and methanogen DNA in the reservoir, an additional water sample was taken from a 1.5 m Van Dorn sample and placed into a 250 mL Nalgene sample bottle. In lab, 100 mL was filtered on a 25 mm Whatman glass microfiber filter (0.7 μm pore size). The filter was preserved in a 15 mL capped polypropylene test tube with 2 mL of 2x CTAB buffer until DNA extraction for real-time PCR occurred. Using an Eckman grab, a sediment sample was taken to determine methanotroph and methanogen DNA concentration. Oxic sediment from the sediment-water interface was placed into a 15 mL polypropylene test tube, while anoxic sediment from 12 cm was placed into a different test tube. Both were labeled, capped, and frozen until DNA extraction occurred.

Genomic DNA was extracted from both water (100 mL) and sediment (0.25 g) samples using the CTAB extraction method (Schaefer 1997). The abundance of DNA (ng/μL) within each sample was measured using a Thermo Scientific NanoDrop

Spectrometer®. Samples were then diluted to 1:10, 1:100, and 1:1000 aliquots, depending on the amount of DNA present.

Real-Time PCR was run on an Applied Biosystems StepOne Real-Time PCR System® using SYBR® Green, a fluorogenic dye that binds to double stranded DNA. To measure the concentration of methanotroph DNA, the forward primer A189 (5'-GGNGACTGGGACTTCTGG-3') and reverse primer mb661 (5'-CCGGMGCAACGTCYTTACC-3') were used with DNA standards (0.5, 0.05, and 0.005 ng/µL) derived from *Methylococcus capsulatus*. This primer targets the pmoA enzyme gene that is essential for methane oxidation in all methanotrophic bacteria (Holmes et al. 1995), and we used this as an index of methanotroph abundance. Methanogen DNA concentration was measured in collected samples using the forward primer Met86 (5'-GCTCAGTAACACGTGG-3') and reverse primer Met1340 (5'-GGTGTGTGCAAGGAG-3') (Wright and Pimm 2003), with *Methanosarcina acetivorans* genomic DNA (0.5, 0.05, and 0.005 ng/µL) for positive control standards in each run. We used the methanogen DNA as an index of methanogen abundance. Optimizations for each primer set were adapted from Gentzel (2010).

Each reaction well on the 48-well PCR plate received 20 µL of a master mix made up of 500 µl of Power SYBR® green mix (Applied Biosystems), 400 µl of sterile water, 50 µl of forward primer, and 50 µl of reverse primer. 1 µl of sample genomic DNA was then introduced into wells to assay for field and laboratory samples. Other wells received 1 µl of the DNA standard as a positive control and to construct a standard curve, while 1 µl of sterile water was introduced into additional wells as a negative control. Samples,

standards, and negative controls were run in triplicate. The real-time PCR protocol began with initial denaturation at 95°C for 15 minutes, followed by forty cycles of: 15 seconds at 95°C, 30 seconds at 55°C, 1 minute at 72°C, and 15 seconds at 80°C. Quantification of DNA abundance was calculated using the standard curve and given in the well table read-out provided by the Applied Biosystems StepOne Real-Time PCR System®.

Laboratory experiments

Laboratory experiments were conducted to examine metabolic fractionation of *Daphnia* and the effects of prokaryotic presence on the $\delta^{13}C$ of *Daphnia*. To assess metabolic fractionation, *Daphnia* were introduced into treatment tanks of filtered water with only algae added as the available food source. In the second treatment, unfiltered water from Lake Brandt was used to examine the effect of non-methanotrophic and non-methanogenic bacteria on the carbon signature of *Daphnia*. Finally, treatment tanks with both sediment and unfiltered water established whether methanotrophs and methanogens are responsible for the depleted carbon signature of *Daphnia*.

Three replicates of each treatment were constructed in lab. Nine glass 10 L tanks were divided in half and fit with a plexi-glass tops that prevented contamination. Each top was outfitted with one hole on each side of the divided tank. The hole allowed tubing to be submerged into the treatment water and served as a stirring apparatus to jostle the water twice daily in order to ensure particle suspension. A bacterial filter was placed on the outflow of the tube to eliminate contamination.

For treatment one, approximately 15 L of lake water was filtered on grade 40 Millipore glass fiber filters (0.7 μm pore size) to remove suspended particles and

introduced into each side of the divided tank. Fifty mL of a *Chlorella* culture from Carolina Biological Supply Company was added to each side and allowed to grow in the water for about two-weeks until substantial algal growth was visible. The tank was sealed off from any contamination with the fitted top. After substantial algal growth was established, the experimental side of the tank was spiked with 100 μg/mL of ampicillin (1.325 g), a broad spectrum antibiotic that suppresses many gram-negative and gram-positive bacteria including methanotrophic bacteria (Campbell et al. 2004; Schnell and King 1995; Carolina Biological Supply Company personal communication). Twenty-four hours after spiking, directly before introduction of *Daphnia*, two water samples were taken from both sides of the tank and examined for methane concentration and the $\delta^{13}C$ signature of any methane present in the water column, using the techniques described previously.

During the 24-hour period after the addition of antibiotic, 75 *Daphnia magna* from Carolina Biological Supply Company were separated from bulk samples and washed of external and internal bacteria to avoid introduction of excess microbes. For this wash process, the *Daphnia* were placed into sterile, filtered lake water without available food sources for 24 hours. During the first 12 hours, they were transferred to uncontaminated water every 2 hours. In the last 12 hours, transfer occurred every six hours. This process allowed the *Daphnia* to clear their guts and expel previous ingested food particles and bacteria (Gophen and Gold 1981). After this 24-hour period, 75 washed *Daphnia* were introduced into each side of the tank and allowed to feed on the *Chlorella* for four days. This four-day period ensured adequate tissue turnover that

allowed the *Daphnia* to obtain a carbon signature of the targeted food source (Chopelet et al. 2008).

After the four days, the experiment was terminated. *Daphnia* were sieved from the tank, placed in tin capsules, and allowed to dry for 24 hours. Additionally, 300 mL of water from each side of each tank was filtered on grade 40 Millipore glass fiber filters (0.7 μm pore size) and dried for 24 hours. Both the filters and *Daphnia* were analyzed for carbon and nitrogen ($\delta^{15}N$ not discussed, see Table 10 for data) stable isotopes at the Marine Biological Laboratory in Woods Hole, Massachusetts. Three water samples were taken to measure methane concentration, the $\delta^{13}C$ signature of methane, and the concentration of methanotroph and methanogen DNA in each side of each tank, following the same procedures noted previously.

The second and third treatments were set-up simultaneously directly after completion of the first treatment. For the second treatment, unfiltered water from Lake Brandt was introduced into the tanks, without the addition of *Chlorella*. The same variables were measured and processes were followed, including antibiotic spiking in the experimental side of each tank. This treatment distinguished the effect of *Daphnia* feeding on the natural seston from the effect of feeding on *Chlorella* only (treatment one), while also separating the effect of methanotrophs and methanogens on the $\delta^{13}C$ signature of *Daphnia magna*.

To examine the effects of methanotrophs and methanogens, 5 cm of sediment from Lake Brandt were introduced into each side of the tank followed by 15 L of unfiltered lake water containing algae, detritus, and bacteria. The tank was allowed to sit

10-12 days to allow the sediment to become anoxic and methane production to occur. The tank was sealed and vented as in the other treatments. After this waiting period, the experimental side of the tank received 100 μg/mL of ampicillin (1.325 g) to inhibit methane uptake by methanotrophic bacteria (Schnell and King 1995). In the control treatment, we expected methanotrophic and non-methanotrophic bacteria to proliferate without the addition of antibiotic. The antibiotic was expected to inhibit the growth of methanotrophic bacteria in the experimental treatments.

Twenty-four hours after the addition of antibiotic, water samples were taken to determine the methane concentration and the methane $\delta^{13}C$ signature in the water column. Seventy-five washed *Daphnia magna* were then introduced into each side of the tank, as described above. After 4 days, *Daphnia* were extracted and 300 mL of water from each treatment was filtered on grade 40 Millipore glass fiber filters (0.7 μm pore size) and both were packaged, dried, and sent for stable isotope analysis. After *Daphnia* removal, three additional water samples from each tank were analyzed for methane concentration, the methane $\delta^{13}C$ signature, and the concentration of methanotroph and methanogen DNA. Oxic sediment samples were extracted from the sediment-water interface and anoxic sediment samples were taken from 4-5 cm and analyzed using real-time PCR to determine the concentration of methanotroph and methanogen DNA.

Data analysis

For Lake Brandt data, normality and homogeneity were examined. The isotopic shifts between *Daphnia* and seston in Lake Brandt were calculated for winter sampling dates and previously unpublished summer sampling dates. An independent samples t-test

was used to compare the mean isotopic shifts between seasons. A two-way ANOVA and subsequent independent samples t-tests were used to assess the differences in methane concentration and $\delta^{13}C$ of methane between sampling depths (1.5 m and 4 m). Mean sediment methanotroph and methanogen DNA values were compared in oxic and anoxic sediment using independent samples t-tests.

For experimental data, normality and homogeneity of each measured variable was also assessed. Unequal variances between the three treatment groups were a serious issue with most of the data. To solve this problem, natural log transformation was first used. If this did not solve the problem, means of measured variables in filtered water and unfiltered water treatments were examined with an independent samples t-test and these treatments were pooled if no difference was found (S. N. Gupta, UNCG Department of Mathematics and Statistics, personal communication). If homogeneity was still lacking, independent samples t-tests were run between pairs of individual treatments depending on antibiotic presence. Resulting p-values were compared to a Bonferroni corrected rejection criteria of < 0.017. A non-parametric permutation test was used to assess the mean difference between treatment groups for the methane concentration difference variable due to non-normality and unequal variances following transformation. A significant difference was found, therefore, consecutive permutation tests were run to compare means between pairs of treatment groups. Resulting p-values were compared to the Bonferroni corrected p-value of 0.017 (Ramsey and Schafer 2002).

Normality and homogeneity of variance occurred for one variable, the $\delta^{13}C$ of seston. After natural log transformation, the final methane concentration values and the

concentration of water column methanogen DNA values became homogenous. A two-way ANOVA was then used to assess the effect of treatment, antibiotic presence, and their interaction on these variables. The two-way ANOVAs for both $\delta^{13}C$ of seston and final methane concentration yielded significant main effects and a significant interaction. Therefore, one-way ANOVAs and Tukey's test were run separately on values with and without antibiotic inoculation to assess differences between treatment groups. For methanogen DNA concentration, only treatment was found as a significant predictor. A one-way ANOVA and Tukey's test were then run to determine differences among treatments.

When transformation did not resolve homogeneity, pooling of filtered and unfiltered water values occurred if no significant difference in means existed between these treatments. Pooling occurred on two variables: the isotopic difference between *Daphnia* and seston and the natural log of the concentration of methanotroph DNA within the water column. A two-way ANOVA was then used to assess the effect of treatment, antibiotic presence, and their interaction on all measured variables. Both pooled two-way ANOVAs yielded significant main effects and interactions. Therefore, independent samples t-tests were run separately on values with and without antibiotic inoculation to examine the difference between pooled water treatments and the sediment treatment.

Sediment methanotroph and methanogen DNA concentrations in the sediment treatment were analyzed using a two-way ANOVA to examine the effects of oxygen availability and antibiotic presence and the interaction of these factors.

Neither transformation nor pooling of treatments resolved the problem of unequal variances with the carbon signature of *Daphnia*. Therefore, a Bonferroni corrected individual significance rate was calculated for the data ($p = .017$). Independent samples t-tests were then run separately between treatments based on antibiotic presence and assessed using this significance level.

The unequal variances of the methane concentration difference variable could not be solved by transformation and, in addition, distribution was non-normal. Therefore, non-parametric permutation tests were used to evaluate the difference between treatments, as described above. SPSS and SAS statistical packages were used to analyze data. Reported significant relationships have a p-value < 0.05, excluding $\delta^{13}C$ of *Daphnia* and the difference of initial and final methane concentrations, where Bonferroni corrected rejection criteria of 0.017 was used. All mean values are reported with ± one standard error.

A two-source mixing model was used to estimate the relative contribution of methane to the diet of *Daphnia magna* using the following equation: $p_{methane} = (\delta^{13}C_{Daphnia} - \delta^{13}C_{seston} - f_1 - f_2)/(\delta^{13}C_{methane} - \delta^{13}C_{seston})$, where $p_{methane}$ is the proportion of methane in the diet of *Daphnia*, f_1 is the fractionation value of *Daphnia,* and f_2 is the fractionation value of methanotrophs (Bunn and Boon 1993). The average carbon signatures for *Daphnia* and methane in the sediment treatment without antibiotic addition were used. The $\delta^{13}C_{seston}$ was calculated from the unfiltered water treatment with antibiotic spiking because this value reflects the carbon signature of natural seston without a significant methane contribution. Normal fractionation between food sources and *Daphnia* (1‰)

was assumed as suggested by Peterson and Fry (1987). The mean fractionation value for methanotrophs was calculated from the difference between the final methane $\delta^{13}C$ values with and without antibiotic in the sediment treatment. Antibiotic spiking halted methane oxidation, therefore, the difference between $\delta^{13}C$ of methane in antibiotic and control tanks reflected isotopic fractionation by methanotrophs. The fractionation factor calculated was -0.38‰. However, because of a wide range of reported methanotrophic fractionation in the literature, $p_{methane}$ was also estimated using hypothetical fractionation values for methanotrophs (f_2) of 1‰, 16‰, and 30‰ (Whiticar 1999).

CHAPTER III

RESULTS

Methane concentration

In Lake Brandt, mean methane concentrations were low (<8.5 ppm). A significant interaction between sampling date and depth was found to effect the methane concentration in Lake Brandt (Two-way ANOVA, $R^2 = 0.966$). Methane concentration did not differ between depths on the first 2 sampling dates (Independent samples t-test, $t = -0.326$, $df = 4$, $p = 0.761$; $t = 2.455$, $df = 4$, $p = 0.070$, respectively, Table 1). However, methane concentration in Lake Brandt was significantly higher at 4 m than at 1.5 m on the November 9th sampling date (Independent samples t-test, $t = -10.352$, $df = 4$, $p < 0.001$, Table 1).

The mean difference in methane concentration before addition and after extraction of *Daphnia* was significantly different between filtered and unfiltered water and highly suggestive of a difference between water treatments and the sediment treatment (Pair-wise permutation test, filtered water-unfiltered water, $p = 0.001$, unfiltered water-sediment, $p = 0.021$, filtered water-sediment, $p = 0.021$, Table 2). Average methane increased by 435.32 ppm in the sediment treatments, while the concentration was near zero in water treatments (<1.3 ppm). Treatments with sediment had significantly more final methane (ppm) than those with only water, with and without antibiotic (One-way ANOVA, $p < 0.001$, 0.001; Tukey's test, filtered water-unfiltered water, $p = 0.905$, 0.919,

unfiltered water-sediment, $p < 0.001$, 0.001, filtered water-sediment, $p < 0.001$, 0.001, respectively, Table 3). Ampicillin failed to limit methane production in filtered and unfiltered water treatments (Independent samples t-test, $t = 0.262$, $df = 4$, $p = 0.807$, $t = 0.098$, $df = 4$, $p = 0.927$, respectively). However, final methane concentration was significantly higher in the sediment treatment when antibiotic spiking occurred (Independent samples t-test, $t = -4.999$, $df = 4$, $p = 0.007$, Table 3).

$\delta^{13}C$ of methane

Methane carbon signatures in Lake Brandt did not differ between depths during winter mixing (Independent samples t-test, $t = 0.454$, $df = 4$, $p = 0.673$, Table 1). For experimental data, because final methane concentration was <0.5 ppm in both water treatments, methane $\delta^{13}C$ values were eliminated from analysis in those treatments. In the sediment treatment, the presence of ampicillin did not affect the final methane carbon signature (-58.93‰ without inoculation, -58.55‰ with inoculation) (Independent samples t-test, $t = -0.174$, $df = 1.161$, $p = 0.887$).

Methanotroph and methanogen DNA

Methanotroph and methanogen DNA concentrations in the Lake Brandt water column at 1.5 m are reported in Table 4. In Lake Brandt sediment, methanotroph concentration did not differ between oxic and anoxic zones (Independent samples t-test, $t = 0.870$, $df = 4$, $p = 0.433$, Table 5). However, methanogen concentration was ~2-fold greater in anoxic sediment (Independent samples t-test, $t = -11.923$, $df = 4$, $p < 0.001$, Table 5).

Concentrations of both methanotroph and methanogen DNA in water were higher in sediment treatments, while ampicillin limited methanotroph concentration. Filtered and unfiltered water samples had very low amounts of methanotroph and methanogen DNA consistent with the low concentrations of methane regardless of whether ampicillin was present, and water in the sediment treatment has similarly low levels of methanotroph DNA in the presence of ampicillin (Independent samples t-test, $t = -1.825$, $df = 7$, $p = 0.111$, Table 6). Regardless of antibiotic presence, concentration of methanogen DNA was significantly different between all treatments (One-way ANOVA, $p < 0.001$; Tukey's test, filtered water-unfiltered water, $p = 0.004$, unfiltered water-sediment, $p < 0.001$, filtered water-sediment, $p < 0.001$, Table 6). However, methanotroph DNA concentration was very low (< 0.7 ng/100 mL) in both filtered and unfiltered water, and much higher (78.61 ng/100 mL) in the sediment treatment water with no ampicillin (Independent samples t-test, $t = -12.21$, $df = 7$, $p < 0.001$, Table 6).

In the sediment treatment, the presence of an antibiotic did not affect the concentration of methanotroph or methanogen DNA present in sediment (Two-way ANOVA, no significant predictor, Table 7). Furthermore, methanotroph and methanogen concentrations were not significantly different between oxic and anoxic samples (Two-way ANOVA, no significant predictors, Table 7).

$\delta^{13}C$ of seston

Seston was significantly ^{13}C depleted (~6‰) in the sediment treatment when an antibiotic was not present (Figure 1) and very similar in all other treatments. A significant interaction between treatment and antibiotic was found to affect the seston

carbon signature (Two-way ANOVA, $R^2 = 0.927$). In the presence of ampicillin, there was no significant difference in carbon signature between treatments (One-way ANOVA, $p = 0.360$, Figure 1). However, without an antibiotic, the sediment treatment had a much more depleted carbon signature than water treatments (One-way ANOVA, $p < 0.001$; Tukey's test, filtered water-unfiltered water, $p = 0.320$, unfiltered water-sediment, $p = 0.001$, filtered water-sediment, $p < 0.001$, Figure 1).

$\delta^{13}C$ of Daphnia

Without ampicillin, the *Daphnia* carbon signature was considerably more depleted (~12‰) in the sediment treatment compared to both filtered and unfiltered water treatments (Bonferroni-corrected independent samples t-test, filtered water-unfiltered water, $p = 0.140$, unfiltered water-sediment, $p = 0.011$, filtered water-sediment, $p = 0.001$, Figure 2). There was no significant difference between treatments when an antibiotic was present (Bonferroni-corrected independent samples t-test, filtered water-unfiltered water, $p = 0.028$, unfiltered water-sediment, $p = 0.249$, filtered water-sediment, $p = 0.546$, Figure 2).

Isotopic carbon shift

In Lake Brandt during summer stratification, *Daphnia* carbon signatures were more depleted than those of seston (~1-4‰). However, carbon signatures of *Daphnia* became more enriched when compared to seston during winter mixing (~1‰). The mean isotopic difference of *Daphnia* and seston between summer and winter sampling dates

were significantly different (Independent samples t-test, t = 4.504, df = 2.157, p = 0.040, Table 8).

The carbon signature of *Daphnia* was 1-2‰ more enriched than the seston in all treatments except the sediment treatment without antibiotic, which was 1.56‰ more depleted than the seston (Table 9). $\delta^{13}C$ shifts between *Daphnia* and seston were not significantly different between treatments when an antibiotic was used (Independent samples t-test, t = -0.706, df = 7, p = 0.462). However, without ampicillin, the isotopic shift between *Daphnia* and seston was statistically different between water and the sediment treatments (Independent samples t-test, t = 3.247, df = 7, p = 0.014).

In the sediment treatment when no antibiotic was used, methane-derived carbon contributed to the diet of *Daphnia*. At our calculated fractionation rate of -0.38‰, methane-derived carbon accounted for 31.95 ± 3.5% of *Daphnia* diet. At a methanotrophic fractionation rate of 1‰, methane accounted for 36.55 ± 3.6% of *Daphnia* diet. This percent increased to 86.60 ± 4.7% and 133.30 ± 5.8% as fractionation was increased to 16‰ and 30‰, respectively.

CHAPTER IV

DISCUSSION

Depleted carbon signatures relative to seston in crustacean zooplankton have been repeatedly observed in freshwater systems (del Giorgio and France 1996; Jones et al. 1999; Grey et al. 2000). However, the trophic basis of the relatively low $\delta^{13}C$ for freshwater zooplankton remains unclear. As of yet, no study has experimentally evaluated the impact of sediment methane production and subsequent methane-oxidation on the $\delta^{13}C$ of *Daphnia magna*, while also suppressing methane uptake by methanotrophic bacteria.

Implications of methane concentration and DNA analysis

Methane concentrations over the three winter sampling dates from October to November of 2009 in Lake Brandt only differed between depths on November 9th (Table 1). However, this difference is likely unimportant to *Daphnia* because methane concentrations were low during winter mixing (<8.5 ppm) and *Daphnia* $\delta^{13}C$ was not depleted relative to seston at that time. This is probably due to mixing and colder temperatures during winter in the monomictic reservoir. Methane accumulates in the hypolimnion during summer stratification but sharply decreases during winter mixing (Eckert and Conrad 2007). Rudd and Hamilton (1978) found that over 90% of hypolimnetic methane remains until winter turnover. Moreover, during summer stratification, methanogenesis was also much higher (568 mg/m^2/day) in the lacustrine

section of Lake Brandt than during winter/spring mixing (25 mg/m^2/day) (Wade 2007). Methanogenesis varies in lakes based on many factors, including temperature and organic carbon inputs. However, all factors are not known. Decreased rates of methanogenesis during winter mixing in Lake Brandt may be due to colder bottom waters in the reservoir, slowing metabolic rates of methanogens (Zeikus and Winfrey 1976). Furthermore, increased inorganic sedimentation rates during winter may inhibit methanogenesis (Wade 2007). Therefore, thermal stratification in the reservoir influenced vertical methane distribution and various factors, such as temperature and sedimentation rates, may account for low methane concentrations on our sampling dates.

The presence of anoxic sediment facilitated the production of methane in the experimental system (Table 2). Average methane concentration almost doubled during the four-day incubation in sediment treatments, whereas methane concentration was initially near zero and decreased in the both water treatments over the experimental period (Table 2). Without sediment, no methane production occurred and that present initially would have diffused from the tanks during the experiment or been oxidized resulting in a decrease in methane concentration. During winter sampling dates in Lake Brandt, we found that methane concentration was much lower than measured in the sediment treatment (Table 1). A previous Lake Brandt study measured methane production over a 24-hour period from cores collected during winter/spring mixing and also found much lower methane concentrations than detected in this experiment (< 3 ppm) (Wade 2007). Longer sediment incubation time before initial measurements (10-12 days) may account for higher methane accumulation in this experiment. Furthermore,

greater incubation temperatures of sediment treatments (~25°C) when compared to Lake Brandt temperatures at 4 m (~18°C) may have attributed to greater methane production (Kelly and Chynoweth 1981).

In Lake Brandt sediment, methanogens were more abundant in anoxic than oxic environments (Table 5). This finding is to be expected given that methanogens are strict anaerobes and during winter mixing, superficial sediments may be partially oxidized. Surprisingly, methanogens also were present in the aerobic water column (Table 4). During the winter sampling dates, it was fairly windy and turbidity in the reservoir was high. This high turbidity may have caused superficial sediments to become disturbed, increasing the flux of methanogenic cells into the water column.

Real-time PCR analysis detected considerable amounts of methanogen DNA in both oxic and anoxic sediments with no antibiotic effect in the experimental system (Table 7). Also, methanogen DNA in the water column of the sediment treatment was greater than in treatments without sediment (Table 6). This may be due to dispersal of cells from the sediment or the high sediment suspension within this treatment caused by daily jostling of the water column. The unfiltered water treatment had significantly more methanogen DNA present than filtered water (Table 6). Despite this difference, methanogen DNA was very low in both treatments that lacked sediment. Paerl (1975) found that available particulate substrates increase attached microbial growth in both marine and freshwater systems. Therefore, methanogens attached to suspended sediment and particles in the unfiltered water likely accounts for this difference. However, since

methanogens are strict anaerobes and the aquarium water was aerobic, the methanogen DNA would not have been derived from live cells.

Methanotroph DNA concentration in Lake Brandt water column was low compared to the sediment treatment without ampicillin (Table 4). A possible reason for this is the relatively low methane concentrations in the reservoir during winter mixing. Previous studies have found that methanotroph abundance decreases considerably when methane concentrations are limited because of a lack of a sustainable energy source (Harrits and Hanson 1980; Schnell and King 1995). Furthermore, because of complete oxygenation of the water column and low rates of methanogenesis during winter, rates of methanotrophy were, most likely, higher at the sediment water interface where methane availability was greater. Wade (2007) found that, in the lacustrine section of Lake Brandt, methane oxidation was much lower during mixing (4 $mg/m^2/day$) than during summer stratification (381 $mg/m^2/day$), consistent with the difference in methanogenesis. However, Rudd and Hamilton (1978) found an immediate increase in methane oxidation during fall turnover, noting that over 90% of yearly methane oxidation occurred during fall turnover. Consequently, increased methane oxidation and ebullition during and directly after fall turnover will lead to the eventual decline in methane concentration, inhibiting the methanotroph population within the lake (Harrits and Hanson 1980).

Methanotrophic bacterial abundance was extremely low in water treatments when compared to the control sediment treatment due to low methane concentrations (Table 6). Minimal amounts of methane limit the available energy source for methanotrophs, resulting in a smaller sustainable population in the water column. However,

methanotrophs were numerous when methane concentrations were high and an antibiotic was not present.

Methanotrophic bacteria in the water column of the sediment treatments were limited by ampicillin, while sediment methanotrophs were unaffected (Tables 6 and 7). However, Schnell and King (1995) found that ampicillin (0.5 mg/mL) limited methane uptake by almost 50% by methanotrophs in forest soils. Use of a lower concentration of ampicillin (100 μg/mL) in the present experiment may have resulted in relatively low antibiotic concentration below superficial sediment layers. However, it was clearly effective in limiting methane oxidation in the water column, and this concentration of the antibiotic was used because it was found to have no negative effect on the physiological state of *Daphnia* (Campbell et al. 2004; personal communication with Carolina Biological Supply Company), whereas effects of increased concentrations have not been evaluated.

A higher concentration of methanotroph DNA was detected within both oxic and anoxic sediment than was present in the water treatments (Tables 6 and 7). This is consistent with Rothfuss et al. (1997), who examined several bacterial groups in the sediments of Lake Constance and found methanotrophs present 30 cm below the sediment surface. Limited methane oxidation occurred down to 7.5 cm below the sediment surface, but dormant cells below this depth could be stimulated to begin methane oxidation when presented with oxygen (Rothfuss et al. 1997). Therefore, we assume that within oxic sediment methanotrophs were actively oxidizing methane that

was being produced in anoxic sediment, while methanotrophs in anoxic sediment were dormant.

Methane oxidation was found to occur in the sediment treatment when an antibiotic was not used. Methane concentration of the sediment treatment when ampicillin spiking occurred was almost 3 times greater than without an antibiotic (Table 3). This result implies that ampicillin limited methanotrophic bacteria, therefore, halting methane oxidation, leading to an accumulation of methane within the water column. When methanotrophs were not limited, however, methane concentration was much lower, indicating active methane oxidation. Similarly, Wade (2007), using dimethyl fluoride to limit methane oxidation in Lake Brandt sediment cores, found that during summer stratification, methane concentration was 3-7 times higher in treated cores than control cores.

Carbon stable isotope values

Lake Brandt methane carbon signatures did not differ between 1.5 and 4 m depths, consistent with a mixed water column (Table 1). The methane carbon signatures measured within the lake were fairly enriched (-46 to -37‰) when compared to reported biogenic methane values (-80 to -52‰) (Bunn and Boone 1993). Our data are consistent with relatively high fractionation associated methane oxidation and diffusion, resulting in depleted ^{12}C of methane in the water column. Bastviken et al. (2003) found that the carbon signature of methane was more enriched in surface waters of several small Swedish lakes when compared to bottom waters during both summer stratification and winter mixing, inferring abundant methane oxidation all year. In this study at 4 m depths,

methane carbon signatures were not depleted leading to the assumption that methane oxidation was occurring at greater depths, most likely, at the sediment water interface.

In laboratory experiments, addition of an antibiotic did not affect the carbon signature of methane in the sediment treatment. Therefore, fractionation by methanotrophs within the control treatment was low. The calculated value for methanotrophic fractionation was negative (-0.38‰), indicating enrichment in ^{12}C instead of ^{13}C during methane oxidation in our study. However, note that replicate measurements of $\delta^{13}C$ methane overlapped broadly between tanks with and without antibiotic, so this cannot be interpreted as a meaningful negative fractionation value; negative methanotrophic fractionation has not been reported in the literature (review: Whiticar 1999) and would not be expected.

The isotopic shift between *Daphnia* and seston is affected by seasonality in Lake Brandt. During winter turnover, *Daphnia* carbon values are slightly enriched compared to seston, consistent with expected carbon fractionation in the diet. However, during summer stratification, *Daphnia* were ^{13}C depleted when compared to seston (Table 8). During stratification of eutrophic lakes, methane oxidation is greatest at the anoxic-oxic boundary in the thermocline but can also occur throughout the epilimnion (Rudd and Hamilton 1978; Harrits and Hanson 1980). Vertical migration of *Daphnia* to this boundary may allow ample foraging on methanotrophic bacteria during summer stratification (Taipale et al. 2007). When winter turnover occurs, as discussed previously, a high volume of trapped methane should be oxidized or evaded from the lake surface. Eventually, low methane concentrations within lakes will result, reducing substrate

availability for methanotrophic bacteria in the water column. This, in turn, limits this potential food source for *Daphnia* during mixing. Methanotroph availability for *Daphnia* may also be limited because methanotrophic abundance is highest at the sediment-water interface during winter. Furthermore, increased inorganic carbon loading in the winter may inhibit methanogenesis, decreasing the available carbon source for methanotrophs. An alternate hypothesis for this *Daphnia* carbon depletion in the summer is the incorporation of depleted microalgae (Hamilton et al. 1992). However, in our experimental data, we show that methane-derived carbon played an important role in carbon depletion of *Daphnia* when compared to bulk seston, and under the experimental conditions, algae were not isotopically light.

The impacts of seasonal stratification and turnover events on *Daphnia* carbon signatures have been examined elsewhere. Three separate studies conducted on Lake Mekkojärvi, a small humic lake in Finland, found that during autumn turnover *Daphnia* consumption of methanotrophic bacteria was very high compared to summer stratification (Kankaala et al. 2006; Taipale et al. 2007; Taipale et al. 2009), in contrast to the pattern for *Daphnia* $\delta^{13}C$ that we have observed in Lake Brandt. This difference may be due to much higher rates of methane oxidation (peak 495 $mg/m^2/day$) in Lake Mekkojärvi during mixing when compared to Lake Brandt (4 $mg/m^2/day$).

The carbon signature of seston was highly depleted when sediment but no antibiotic was present within experimental treatments (Figure 1). Without an antibiotic, there was high methanotroph DNA concentration in the water column of this treatment.

Therefore, attachment of methanotrophic bacteria to POM explains the depletion of the seston carbon signature.

Like the $\delta^{13}C$ of seston, the carbon signature of *Daphnia* was associated with the abundance of methanotrophic bacteria within the water column. $\delta^{13}C$ of *Daphnia* was highly depleted when methanotroph abundance was high, which resulted from high methane production from the sediment and no limitation by ampicillin (Figure 2). Furthermore, average *Daphnia* carbon signature was depleted when compared to the carbon signature of the seston only in this treatment (Table 9). As *Daphnia* should be 0-1‰ more enriched than their food source, we assume that *Daphnia* are assimilating isotopically depleted methane-derived carbon in the sediment treatment when an antibiotic did not limit methanotroph presence. *Daphnia* in treatment groups without abundant methanotrophs fractionated carbon (0-1‰ more enriched) as expected by Peterson and Fry (1987).

In this study, the two-source mixing model estimated that methane contributed to 31-87% of *Daphnia* diet in the experimental sediment treatment when an antibiotic was not used. Percent contribution of methane to the diet of *Daphnia* was >100% when the methanotroph fractionation value of 30‰ was used. Therefore, we assume that this is an impractical methanotroph fractionation factor in this study. With the calculated fractionation value of -0.38‰, methane contributed to almost 32% of *Daphnia* diet. The other fractionation values of methanotrophs yielded percentages (37% and 87%) that fell between previously reported ranges of methane contribution. Previous studies examining the potential impact of methane-derived carbon on *Daphnia* have also found significant

evidence of incorporation of methane-derived carbon through consumption of methanotrophic bacteria by *Daphnia*. In bottle experiments, Kankaala et al. (2007) found that as *Daphnia* densities were increased, methanotroph abundance decreased. Methanotrophic bacterial contribution to *Daphnia* diet was also found to be between 5-15% in three small Swedish lakes and as high as 87% in a small humic lake during autumn turnover (Bastviken et al. 2003; Taipale et al. 2007). Therefore, incorporation of methane-derived carbon into the diet of *Daphnia* in this study is corroborated.

CHAPTER V

CONCLUSION

Results of this study indicate that seasonal changes within water bodies may change methane production and oxidation in turn affecting *Daphnia* diet composition. Additionally, it was found that increased methane concentrations within the water column can sustain large populations of methanotrophic bacteria. When methanotroph abundance is high, incorporation of methane-derived carbon into the diet of *Daphnia* occurs, resulting in depleted carbon isotopic signatures of *Daphnia* when compared to bulk seston. However, when methanotroph abundance is limited, carbon signatures of *Daphnia* reflect expected fractionation values (0-1‰) between seston and *Daphnia*. Results of this study, therefore, suggest that methane-derived carbon can account for the depleted carbon signatures of *Daphnia* observed throughout freshwater systems.

Research of methane-derived carbon influences on zooplankton feeding regimes is limited in scope, especially in reservoirs. However, we do know that methane-derived carbon within pelagic food webs is important in freshwater carbon cycling (Bastviken et al. 2003; Bastviken et al. 2008; Kankaala et al. 2006; Kankaala et al. 2007). As global warming has become an important focus of scientific studies, further research is needed to examine the implications of the consumption of methane-oxidizers and the resulting impact on methane fluxes into the atmosphere from freshwater systems.

REFERENCES

Bastviken, D., J. Ejlertsson, I. Sundh, L. Tranvik. 2003. Methane as a source of carbon and energy for lake pelagic food webs. Ecology 84: 969-981.

Bastviken, D., J. J. Cole, M. L. Pace, M. C. Van de Bogert. 2008. Fates of methane from different lake habitats: connecting whole-lake budgets and CH_4 emissions. Journal of Geophysical Research 113.

Brendelberger, H. 1991. Filter mesh size of cladocerans predicts retention efficiency for bacteria. Limnology and Oceanography 36: 884-894.

Brooks, J. L. and S. I. Dodson. 1965. Predation, body size, and composition of plankton. Science 150: 28-35.

Bunn, S. E. and P. I. Boon. 1993. What sources of organic carbon drive food webs in billabongs? A study based on stable isotope analysis. Oecologia 96: 85-94.

Burns, T. P. 1989. Lindeman's contradiction and the trophic structure of ecosystems. Ecology 70: 1355-1362.

Burns, C. W. 1968. The relationship between body size and filter feeding cladocera and the maximum size of particle ingested. Limnology and Oceanography 13: 675-678.

Campbell, A. K., K. Wann, S. Matthews. 2004. Lactose causes heart arrhythmia in the water flea *Daphnia pulex*. Comparative Biochemistry and Physiology, Part B 139: 225-234.

Chopelet, J., P. U. Blier, F. Dufresne. 2008. Plasticity of growth rate and metabolism in *Daphnia magna* populations from different thermal habitats. Journal of Experimental Zoology 309A: 553-562.

del Giorgio P. A. and R. L. France. 1996. Ecosystem-specific patters in the relationship between zooplankton and POM or microplankton $\delta^{13}C$. Limnology and Oceanography 41: 359-365.

DeMott, W. R. 1982. Feeding selectivities and relative ingestion rates of *Daphnia* and *Bosmina*. Limnology and Oceanography 27: 518-527.

Eckert, W. and R. Conrad. 2007. Sulfide and methane evolution in the hypolimnion of a subtropical lake: a three-year study. Biogeochemistry 82: 67-76.

Edmondson, W. T. 1957. Trophic relations of the zooplankton. Transactions of the American Microscopical Society 76: 225-245.

Fry, B. 2006. Stable Isotope Ecology. New York: Springer.

Gentzel, T. 2010. The effects of methane producers and consumers on the diet of Chironomus larvae in an arctic lake. MS Thesis. University of North Carolina, Greensboro.

Gophen, M. and W. Geller. 1984. Filter mesh size and food particle uptake by *Daphnia*. Oecologia 64: 408-412.

Gophen, M. and B. Gold. 1981. The use of inorganic substances to stimulate gut evacuation in *Daphnia magna*. Hydrobiologia 80: 43-45.

Grey, J., R. I. Jones, D. Sleep. 2000. Stable isotope analysis of the origins of zooplankton carbon in lakes of differing trophic state. Oecologia 123: 232-240.

Hamilton, S. K., W. M. Lewis, S. J. Sippel. 1992. Energy sources for aquatic animals in the Orinoco River floodplain: evidence from stable isotopes. Oecologia 89: 324-330.

Harrits, S. M. and R. S. Hanson. 1980. Stratification of aerobic methane-oxidizing organisms in Lake Mendota, Madison, Wisconsin. Limnology and Oceanography 25: 412-421.

Hessen, D. O., T. Anderson, A. Lyche. 1990. Carbon metabolism in a humic lake: pool sizes and cycling through zooplankton. Limnology and Oceanography 35: 84-99.

Holmes, A. J., A. Costello, M. E. Lidstrom, J. C. Murrell. 1995. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiology Letters 132: 203–208.

Jones, R. I., J. Grey, D. Sleep, L. Arvola. 1999. Stable isotope analysis of zooplankton carbon nutrition in humic lakes. Oikos 86: 97-104.

Kankaala, P., S. Taipale, J. Grey, E. Sonninen, L. Arvola, R. Jones. 2006. Experimental $\delta^{13}C$ evidence for a contribution of methane to pelagic food webs in lakes. Limnology and Oceanography 51: 2821-2827.

Kankaala, P., G. Eller, R. I. Jones. 2007. Could bacterivorous zooplankton affect lake pelagic methanotrophic activity? Fundamental and Applied Limnology, Archiv für Hydrobiologie 169: 203-209.

Kelly, C. A. and D. P. Chynoweth. 1981. The contribution of temperature and of the input of organic matter in controlling rates of sediment methanogenesis. Limnology and Oceanography 26: 891-897.

Kirk, K. L. 1991. Inorganic particles alter competition in grazing plankton: the role of selective feeding. Ecology 72: 915-923.

Knisely, K. and W. Geller. 1986. Selective feeding of four zooplankton species on natural lake phytoplankton. Oecologia 69: 86-94.

Lindeman, R. L. 1942. The trophic-dynamic aspect of ecology. Ecology 23: 399-417.

Moss, B. 1970. Seston composition in two freshwater ponds. Limnology and Oceanography 15: 504-513.

Paerl, H. W. 1975. Microbial attachment to particles in marine and freshwater ecosystems. Microbial Ecology 2: 73-83.

Peterson, B. J. and B. Fry. 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology and Systematics 18: 293-320.

Peterson, B. J., J. E. Hobbie, J. F. Haney. 1978. *Daphnia* grazing on natural bacteria. Limnology and Oceanography 23: 1039-1044.

Quiblier-Llobéras, C., G. Bourdier, C. Amblard. 1996. A qualitative study of zooplankton grazing in an oligo-mesotrophic lake using phytoplanktonic pigments as organic markers. Limnology and Oceanography 4: 1767-1779.

Ramsey, F. L. and D. W. Schafer. 2002. The Statistical Sleuth: A Course in Methods of Data Analysis. California: Duxbury Thomson Learning.

Rothfuss, F., M. Bender, R. Conrad. 1997. Survival and activity of bacteria in a deep, aged lake sediment (Lake Constance). Microbial Ecology 33: 69-77.

Rudd, J. W. M. and R. D. Hamilton. 1978. Methane cycling in a eutrophic shield lake and its effects on whole lake metabolism. Limnology and Oceanography 23: 337-348.

Salonen, S. and T. Hammar. 1986. On the importance of dissolved organic matter in the nutrition of zooplankton in some lake waters. Oecologia 68: 246-253.

Schaefer, E. F. 1997. A DNA assay to detect the toxic dinoflagellate *Pfiesteria piscicida*, and the application of a PCR based probe. MS Thesis. University of North Carolina, Greensboro.

Schnell, S. and G. King. 1995. Stability of methane oxidation capacity to variations in methane and nutrient concentrations. FEMS Microbiology Ecology 17: 285-294.

Taipale, S., P. Kankaala, R. I. Jones. 2007. Contributions of different organic carbon sources to *Daphnia* in the pelagic foodweb of a small polyhumic lake: results from mesocosm DI^{13}C-additions. Ecosystems 10: 757-772.

Taipale, S., P. Kankaala, H. Hämäläinen, R. I. Jones. 2009. Seasonal shifts in the diet of lake zooplankton revealed by phospholipid fatty acid analysis. Freshwater Biology: 54: 90-104.

Wade, E. M. 2007. The importance of biogenic methane and sedimentation to benthic Chironomid larvae in four reservoirs. MS Thesis. University of North Carolina, Greensboro.

Watts, E. and M. Petri. 1981. A scanning electron microscope study of the thoracic appendages of *Daphnia magna straus*. Journal of Natural History 15: 463-473.

Whalen, S. C. 2005. Biogeochemistry of methane exchange between natural wetlands and the atmosphere. Environmental Engineering Science 22: 73-94.

Whiticar, M. J. 1999. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chemical Geology 161: 291-314.

Wright, A.-D. G., C. Pimm. 2003. Improved strategy for presumptive identification of methanogens using 16S riboprinting. Journal of Microbiological Methods 55: 337-349.

Zeikus, J. G. and M. R. Winfrey. 1976. Temperature limitation of methanogenesis in aquatic sediments. Applied and Environmental Microbiology 31: 99-107.

APPENDIX A. TABLES

Table 1: Average methane data from Lake Brandt by sampling date and depth. Methane concentrations did not differ between depths on the first 2 sampling dates (Independent samples t-test, $t = -0.326$, $df = 4$, $p = 0.761$; $t = 2.455$, $df = 4$, $p = 0.070$, respectively). Methane concentration was significantly higher at 4 m on November 9th (Independent samples t-test, $t = -10.352$, $df = 4$, $p < 0.001$). $\delta^{13}C$ of methane did not differ between 1.5 m and 4 m (Independent samples t-test, $t = 0.454$, $df = 4$, $p = 0.673$). Mean values reported with ± one standard error (n = 3).

Sampling Date	Sampling Depth	Methane Concentration (ppm)	$\delta^{13}C$ of Methane (‰)
Oct-14-09	1.5 m	7.07 ± 0.37	-45.17
	4 m	7.27 ± 0.45	-45.05
Oct-28-09	1.5 m	3.44 ± 0.10	-38.02
	4 m	2.23 ± 0.26	-37.69
Nov-9-09	1.5 m	5.79 ± 0.07	-41.01
	4 m	8.46 ± 0.25	-46.02

Table 2: Average methane concentration before addition and after extraction of *Daphnia* by treatment with calculated difference. Mean difference in methane concentration was significantly different between filtered and unfiltered water and highly suggestive of a difference in water and sediment treatments (Pair-wise permutation test, filtered-unfiltered water, $p = 0.001$, filtered water-sediment, $p = 0.021$, unfiltered water-sediment $p = 0.021$). Mean values reported ± one standard error ($n = 6$).

Treatment	Time	Methane Concentration (ppm)
Filtered Water	Before	0.48 ± 0.05
	After	0.42 ± 0.03
		- 0.06 ± 0.06
Unfiltered Water	Before	1.22 ± 0.09
	After	0.38 ± 0.02
		- 0.84 ± 0.09
Unfiltered Water + Sediment	Before	749.01 ± 263.47
	After	1184.33 ± 235.63
		+ 435.32 ± 250.13

Table 3: Final average methane concentration by treatment and antibiotic presence. The methane concentration in the sediment treatment was significantly higher than the other two treatments, with and without ampicillin (ANOVA, $p < 0.001$, 0.001; Tukey's test, filtered water-unfiltered water, $p = 0.905$, 0.919, unfiltered water-sediment, $p < 0.001$, 0.001, filtered water-sediment, $p < 0.001$, 0.001, respectively). Ampicillin did not limit methane production in filtered or unfiltered water treatments (Independent samples t-test, $t = 0.262$, $df = 4$, $p = 0.807$, $t = 0.098$, $df = 4$, $p = 0.927$, respectively). In the sediment treatment with ampicillin, final methane concentration was significantly higher than without antibiotic spiking (Independent samples t-test, $t = -4.999$, $df = 4$, $p = 0.007$). Mean values shown with ± one standard error (n = 3).

Treatment	Antibiotic	Methane Concentration (ppm)
Filtered Water	-	0.43 ± 0.06
	+	0.41 ± 0.07
Unfiltered Water	-	0.38 ± 0.01
	+	0.38 ± 0.04
Unfiltered Water + Sediment	-	695.14 ± 184.95
	+	1673.52 ± 63.97

Table 4: Average methanotroph and methanogen DNA (ng/100 mL) in Lake Brandt at 1.5 m by sampling date.

Sampling Date	Average Methanotroph DNA (ng/100 mL)	Average Methanogen DNA (ng/100 mL)
Oct-14-09	2.26 ± 0.30	4.09 ± 0.67
Oct-28-09	2.64 ± 0.29	5.75 ± 0.86
Nov-9-09	2.32 ±0.38	4.82 ± 1.88

Table 5: Average methanotroph and methanogen DNA (ng/0.25 g) in Lake Brandt in oxic and anoxic sediment. Methanotroph abundance did not differ between oxic and anoxic sediment (Independent samples t-test, $t = 0.870$, $df = 4$, $p = 0.433$). Anoxic sediment had significantly more methanogens than oxic sediment (Independent samples t-test, $t = -11.923$, $df = 4$, $p < 0.001$). Values reported with ± one standard error.

Sampling Date	Oxygen Presence	Average Methanotroph DNA (ng/0.25g)	Average Methanogen DNA (ng/0.25g)
Oct-14-09	Oxic	13.23 ± 2.54	81.23 ± 20.04
	Anoxic	4.09 ± 0.08	151.88 ± 9.68
Oct-28-09	Oxic	4.49 ± 0.94	87.81 ± 21.67
	Anoxic	9.81 ± 1.61	149.61 ± 37.92
Nov-9-09	Oxic	7.14 ± 0.31	74.87 ± 22.81
	Anoxic	1.75 ± 0.24	138.83 ± 30.37

Table 6: Average methanotroph and methanogen DNA (ng/100 mL) in water samples by treatment and antibiotic presence. Methanotroph DNA was significantly greater in the sediment treatment when ampicillin was absent (Independent samples t-test, $t = -12.210$, $df = 7$, $p < 0.001$). Methanogen DNA abundance was significantly different between all treatments, with no antibiotic effect (ANOVA, $p < 0.001$; Tukey's test, filtered water-unfiltered water, $p = 0.004$, unfiltered water-sediment, $p < 0.001$, filtered water-sediment, $p < 0.001$). Mean values reported with ± one standard error ($n = 3$).

Treatment	Antibiotic Presence	Average Methanotroph DNA (ng/100 mL)	Average Methanogen DNA (ng/100 mL)
Filtered Water	-	0.14 ± 0.04	0.12 ± 0.02
	+	0.14 ± 0.05	0.10 ± 0.04
Unfiltered Water	-	0.39 ± 0.18	0.47 ± 0.14
	+	0.50 ± 0.67	0.69 ± 0.18
Unfiltered Water + Sediment	-	78.61 ± 53.96	13.79 ± 5.35
	+	0.98 ± 0.70	2.53 ± 0.34

Table 7: Average sediment methanotroph and sediment methanogen DNA values (ng/ 0.25 g) in the sediment treatment by oxygen and antibiotic presence. Oxygen nor antibiotic presence had significant effects on methanotrophic or methanogenic abundance in sediment. Mean values reported with ± one standard error (n = 3).

Oxygen Presence	Antibiotic Presence	Average Methanotroph DNA (ng/0.25 g)	Average Methanogen DNA (ng/0.25 g)
Oxic Sediment	-	8.58 ± 3.38	37.19 ± 20.53
	+	12.88 ± 2.31	74.90 ± 6.67
Anoxic Sediment	-	4.40 ± 2.26	41.00 ± 21.24
	+	5.97 ± 4.99	57.81 ± 30.03

Table 8: Average carbon isotopic shift between *Daphnia* and seston in Lake Brandt by sampling date. The isotopic difference of *Daphnia* and seston was significantly different between seasons (Independent samples t-test, t = 4.504, df = 2.157, p = 0.040). Mean values reported with ± one standard error.

Sampling Date	Average Carbon Isotopic Shift between *Daphnia* and Seston (‰)
Oct-14-09	+ 1.12
Oct-28-09	+ 1.67
Nov-9-09	+ 1.40
Aug-9-07	- 3.88
April-3-08	- 1.35
April-10-08	- 1.62

Table 9: Average carbon isotopic shift between *Daphnia* and seston based on treatment and antibiotic presence. Without the presence of the antibiotic, there was a significant difference in the isotopic shift of *Daphnia* and seston between water and sediment treatments (Independent samples t-test, t = 3.247, df = 7, p = 0.014). With ampicillin, there was no significant differences between treatments (Independent samples t-test, t = -0.706, df = 7, p = 0.462). Mean values reported with ± one standard error (n = 3).

Treatment	Antibiotic Presence	Average Carbon Isotopic Shift between *Daphnia* and Seston (‰)
Filtered Water	-	+ 1.94 ± 1.10
	+	+ 1.98 ± 0.46
Unfiltered Water	-	+ 1.49 ± 0.44
	+	+ 1.31 ± 0.13
Unfiltered Water + Sediment	-	- 1.56 ± 0.96
	+	+ 1.94 ± 0.27

Table 10: Average $\delta^{15}N$ of seston and *Daphnia* in treatments and Lake Brandt. Mean values reported with ± one standard error (n = 3).

Treatment	Antibiotic Presence	$\delta^{15}N$ of Seston (‰)	$\delta^{15}N$ of *Daphnia* (‰)
Filtered Water	-	4.20 ± 0.90	10.95 ± 1.00
	+	4.79 ± 0.43	13.67 ± 1.94
Unfiltered Water	-	3.72 ± 0.38	11.51 ± 1.05
	+	4.63 ± 0.16	13.16 ± 0.30
Unfiltered Water + Sediment	-	2.46 ± 0.70	3.25 ± 0.90
	+	3.81 ± 0.72	5.15 ± 0.22
Lake Brandt (Oct-14-09)		5.26	5.89
Lake Brandt (Oct-28-09)		4.51	4.04
Lake Brandt (Nov-9-09)		4.18	3.92

APPENDIX B. FIGURES

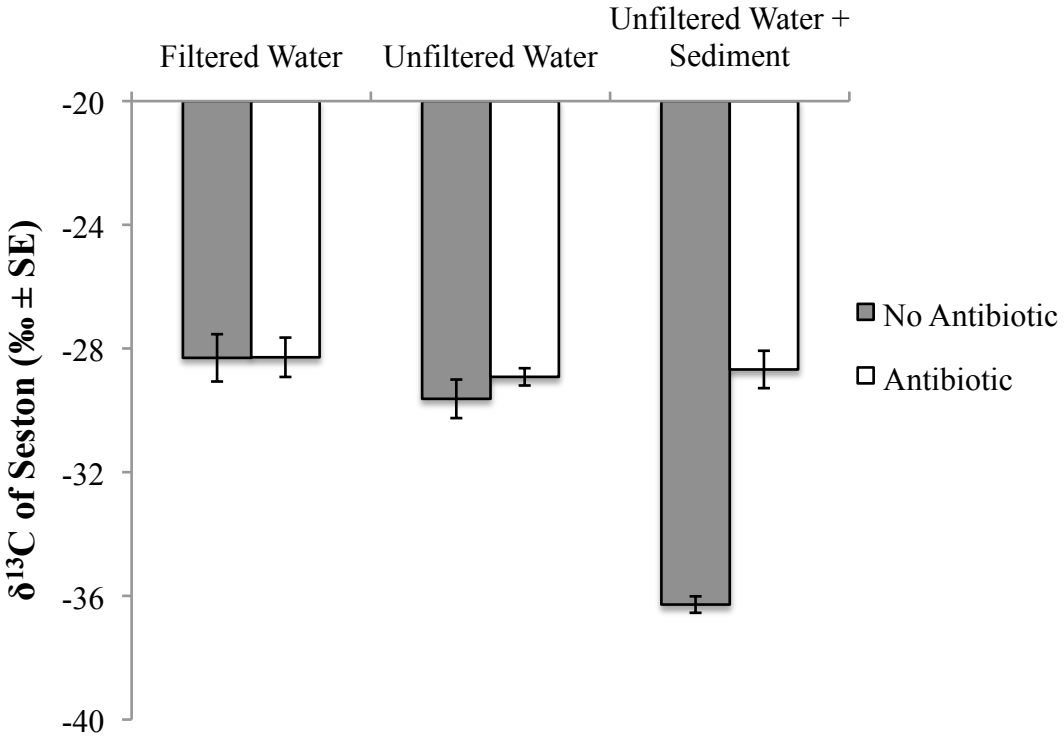

Figure 1: Average $\delta^{13}C$ of seston by treatment and antibiotic presence. Without ampicillin, the carbon signatures of the seston in the sediment treatments were significantly depleted when compared to those in water treatments (ANOVA, $p < 0.001$; Tukey's test, filtered water-unfiltered water, $p = 0.320$, unfiltered water-sediment, $p = 0.001$, filtered water-sediment, $p < 0.001$). Seston $\delta^{13}C$ signatures did not vary between treatments when antibiotic spiking occurred (ANOVA, $p = 0.360$). Mean values reported with ± one standard error ($n = 3$).

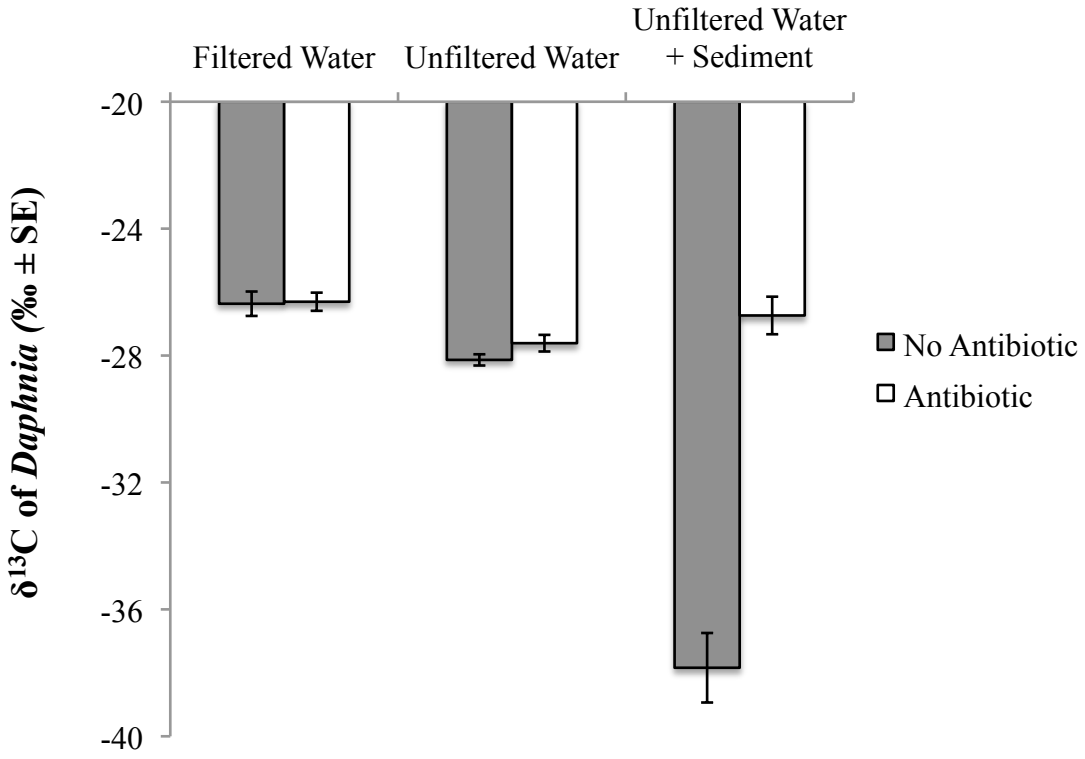

Figure 2: Average $\delta^{13}C$ of *Daphnia* by treatment and antibiotic presence. In the absence of an antibiotic, the *Daphnia* in the sediment treatment group were significantly depleted when compared with the other treatments (Bonferroni-corrected independent samples t-test, filtered water-unfiltered water, $p = 0.140$, unfiltered water-sediment, $p = 0.011$, filtered water-sediment, $p = 0.001$). The carbon signatures of *Daphnia* did not differ between treatments when ampicillin was present (Bonferroni-corrected independent samples t-test, filtered water-unfiltered water, $p = 0.028$, unfiltered water-sediment, $p = 0.249$, filtered water-sediment, $p = 0.546$). Mean values reported with ± one standard error ($n = 3$).