

Methane as a source of carbon and energy for chironomid larvae

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Summary

Food webs are an important component of community and ecosystem structure. Early descriptions of food webs were usually simple and consisted of few taxa and limited numbers of links. Until recently, photosynthetic phytoplankton, algae and macrophytes were considered to be the only primary producers and thus the major carbon source in aquatic systems. Chemosynthesis was considered quantitatively less important. When the hydrothermal vent communities were first discovered, biologists were astonished by the density of biomass associated with the active vents, where photosynthesis was not possible. These findings led towards a new area of research. Today, it is accepted that in these special environments, microbial chemoautotrophic primary production can replace photosynthesis as the dominant source of ecosystem energy production. Recent work in freshwater ecosystems also indicated that chemosynthetically fixed carbon sources (i.e. methane) could be of importance. Based upon high variation in chironomid larval $\delta^{13}\text{C}$ values from eutrophic lakes with different mixing regimes, limnologists proposed differences in the methane cycle that provide benthic organisms with carbon to be responsible.

In this thesis, I particularly focus on interactions between chironomid larvae, as a model organism for benthic macroinvertebrates, and the methane carbon cycle in freshwater lakes. By comparing lakes with different morphologies and mixing regimes, it could be shown that the variation in chironomid larval $\delta^{13}\text{C}$ signatures previously found indeed relate to variations in conditions for methane production and oxidation in the sediment. The contribution of methane-derived carbon was higher in the dimictic Holzsee compared to the polymictic Großer Binnensee with its well-oxygenated water column, irrespective of an identical methanogenic and methanotrophic bacterial community (chapter I). By sampling both lakes during the year, I found seasonality in methane turnover activities and chironomid larval $\delta^{13}\text{C}$ values only in the stratified and not in the polymictic lake. Thus, both the methane oxidation activity and the availability of MOB biomass determined the importance of methane-derived carbon for the larval diet (chapter II). The analysis of larval $\delta^{13}\text{C}$ values and methane production from sediments at different water column depth provides additional evidence for a site-specific methanotrophic contribution to larval biomass within one lake. Based on larval distribution, the contribution of methane-

derived carbon via chironomids to pelagic and terrestrial predators was estimated to be substantial (chapter III).

Depleted $\delta^{13}\text{C}$ values of chironomid larvae can also be a consequence of larvae feeding upon other chemoautotrophic bacteria, apart from using methane as a carbon source. Sulphur stable isotopes were used to demonstrate that within a single lake, one of two congeneric chironomid species relies more upon methanotrophic biomass whereas the other depends on chemoautotrophic biomass (chapter IV). The application of hydrogen isotopes provided further evidence for the importance of methane-derived carbon in the nutrition of chironomid larvae and gave insight into the dominant methanogenic pathways operating in different sediment strata (chapter V).

By manipulating the isotopic signature of methane, I collected for the first time experimental evidence that chironomid larvae assimilate methane-derived carbon by feeding on MOB that are present in both, the sediment and the water column (chapter VI).

Zusammenfassung

Nahrungsnetze sind strukturierende Bestandteile von Lebensgemeinschaften und Ökosystemen. Anfängliche Beschreibungen von Nahrungsnetzen waren meist einfach und basierten auf wenigen Taxa mit einer begrenzten Anzahl von Verknüpfungen. Bis vor kurzem nahm man an, dass Phytoplankton, Algen und Makrophyten die einzigen Primärproduzenten seien und damit die hauptsächliche Kohlenstoffquelle in aquatischen Systemen darstellten. Als quantitativ wenig von Bedeutung wurde die Chemosynthese angesehen. Als die Lebensgemeinschaften an den Hydrothermalquellen der Tiefsee entdeckt wurden, waren Biologen erstaunt über die Menge an Biomasse, die dort in einem Bereich gefunden wurde, in dem Photosynthese nicht möglich ist. Diese Entdeckungen war die Geburtsstunde einer neuen Forschungsrichtung. Inzwischen ist es allgemein akzeptiert, dass in diesen speziellen Lebensräumen mikrobielle chemoautotrophe Primärproduktion die Photosynthese als dominierende Energiequelle in Ökosystemen ersetzen kann. Neuere Forschungen in Süßwasser-Ökosystemen konnten erste Hinweise erbringen, dass auch hier chemosynthetisch fixierter Kohlenstoff (wie z.B. Methan) als Kohlenstoffquelle von Bedeutung sein könnte. Aufgrund der großen Variation bei den $\delta^{13}\text{C}$ Werten in Chironomidenlarven aus eutrophen Seen mit unterschiedlichen Zirkulationstypen haben Limnologen vermutet, dass Unterschiede im Methanzzyklus, über den Organismen möglicherweise mit Kohlenstoff versorgt werden, verantwortlich sein könnten.

In dieser Arbeit habe ich mich besonders mit den Interaktionen zwischen Chironomidenlarven als einem Modellorganismus für benthische Makroinvertebraten und dem Methanzzyklus in Süßwasserseen beschäftigt. Durch den Vergleich von Seen mit unterschiedlicher Morphologie und unterschiedlichem Zirkulationstyp konnte gezeigt werden, dass die Variation, die zuvor bei den $\delta^{13}\text{C}$ Werten von Chironomidenlarven gefunden worden war, tatsächlich zu den entsprechenden Bedingungen für Methanproduktion und -oxidation im Sediment in Beziehung steht. Der Anteil an Kohlenstoff, der aus Methan stammte, war höher im dimiktischen Holzsee im Vergleich zum polymiktischen Großen Binnensee mit seiner mit Sauerstoff angereicherten Wassersäule, ungeachtet einer identischen methanogenen und methanotrophen bakteriellen Gemeinschaft (Kapitel I). Durch das

Beproben beider Seen während eines ganzen Jahres konnte ich im Gegensatz zu dem polymiktischen See, nur in dem stratifizierten See jahreszeitliche Variationen beim Methanumsatz und bei den $\delta^{13}\text{C}$ Werten der Chironomidenlarven messen. Daraus kann man schließen, dass die Bedeutung von aus Methan stammenden Kohlenstoff als Nahrungsquelle für Chironomidenlarven sowohl von dem Methanumsatz als auch von der Verfügbarkeit der Biomasse an Methan oxidierenden Bakterien abhängig ist (Kapitel II). Diese Arbeit konnte sogar innerhalb eines Sees einen tiefenabhängigen Unterschied des methanotrophen Beitrags zur larvalen Biomasse nachweisen. Das ergab sich aus der Analyse von $\delta^{13}\text{C}$ Werten bei Chironomidenlarven und der Methanproduktion in den Sedimenten aus unterschiedlichen Tiefen des Sees. Die Bedeutung von Methan als Kohlenstoffquelle nimmt einen noch höheren Stellenwert ein, wenn man bedenkt, dass aufgrund der hohen Abundanz der Larven vermutlich ein großer Teil des aus Methan stammenden Kohlenstoffs über die Chironomiden von pelagischen und terrestrischen Räubern aufgenommen wird (Kapitel III).

Die niedrigen $\delta^{13}\text{C}$ Werte von Chironomidenlarven könnten aber nicht nur durch die Nutzung von Methan als Kohlenstoffquelle, sondern auch durch den Verzehr von anderen chemoautotrophen Bakterien verursacht sein. Mit Hilfe von Schwefelisotopen konnte das auch gezeigt werden, nämlich dass in demselben See die eine von zwei nahe verwandten Chironomiden-Spezies mehr von methanotropher Biomasse lebt, während die andere von anderer chemoautotropher Biomasse abhängig ist (Kapitel IV). Durch den Einsatz von Wasserstoffisotopen konnte ich die Bedeutung des aus Methan stammenden Kohlenstoffs in der Nahrung von Chironomidenlarven weiter belegen und außerdem untersuchen, welche wesentlichen methanogenen Abbauewege in unterschiedlichen Sedimentschichten stattfinden (Kapitel V).

Durch Manipulation der Isotopensignatur von Methan konnte ich erstmalig experimentell zeigen, dass Chironomidenlarven Kohlenstoff aus Methan verwerten, indem sie Methan oxidierende Bakterien fressen, die sowohl im Sediment als auch in der Wassersäule zu finden sind (Kapitel VI).

Introduction

Although lakes constitute only ca. 0.01% of the Earth's total surface area of water, they have received an elevated level of scientific attention that is disproportionate to their coverage. Most probably this is because ecological interactions, interactions among organisms and between organisms and their environment can be studied as in a 'microcosm' (sensu Forbes 1887) and thus more easily than in any other ecosystem (Lampert & Sommer 1997). Studies on lakes provide some of the most complete investigations on structure, dynamics and energetics of food webs encompassing organisms from bacteria to vertebrates. One key group in lake food webs is the zoobenthos that encompasses all those species (mainly macroinvertebrates e.g. >0.5 mm) inhabiting the bottom of the solid-liquid interface.

Zoobenthos

Zoobenthic organisms are important in lake food webs, mainly for four reasons: first, they can occur in high numbers and thus form a main food source for many aquatic carnivores, like predatory invertebrates and fish. Second, they link the pelagic to the benthic food web, because they are fuelled by inputs of phytodetrital material from the pelagic zone (e.g. Graf 1989, Johnson & Wiederholm 1992), through a process known as pelagic-benthic coupling. They thus transform fine particulate organic matter into food for larger carnivores. Third, benthic macroinvertebrates may recycle nutrients to the open water by their excretions. Fourth, by bioturbation they promote the development of a rich microbial community with increased microbial activity and biomass (e.g. Aller & Yingst 1978, Dobbs & Guckert 1988a,b).

Benthic macroinvertebrates ingest a great diversity of food by various feeding mechanisms. Distinctions can be made between herbivory, defined as the ingestion of living vascular plant tissue or algae; carnivory, the ingestion of living animal tissue; and detritivory, the intake of non-living particulate organic matter and microorganisms associated with it (Cummins 1973, Hutchinson 1993). Many invertebrates are opportunistic omnivores – feeding at more than one trophic level. Such organic matter can originate from (a) sedimenting phytoplankton, (b) benthic aquatic macrophytes, (c) terrestrial arboreal and wetland vegetation and animal matter and

(d) attached microbial communities. Bacteria, associated with organic matter, are often considered a potential food resource for detritivores (e.g. Findlay et al. 1984, Carman & Thistle 1985, Kemp 1987), but bacteria that always occur in the sediment, are generally not considered as a quantitatively important carbon source for deposit-feeding macroinvertebrates (see Lopez & Levinton 1987, Goedkoop & Johnson 1994).

Sediment bacteria as a food source for zoobenthos

Currently, little is known about interactions between bacteria and their macroinvertebrate consumers (e.g. Brinkhurst & Chua 1969). Bacterial grazers were proposed to directly stimulate bacterial growth through grazing, because if density-regulated mechanisms exist, a constant removal of bacterial cells should enhance overall bacterial population production (Fenchel & Jørgensen 1977, Fry & Arnold 1982). Several studies have shown that macrozoobenthos grazing affects bacterial abundance and production, particularly at high grazer densities (Hargrave 1970, Johnson et al. 1989). In the few freshwater studies available, consumption of bacteria comprised only 5 and 11% of the energy demands of deposit-feeding macroinvertebrates (Johnson et al. 1989). In extremely heterotrophic environments, like blackwater streams, deposit-feeders can assimilate up to 50% of their carbon requirements from bacteria (Edwards & Meyer 1990).

The abundance and biomass of sediment bacteria seem to display little variation both between years and among years, (e.g. Boström & Törnblom 1990, Brunberg & Boström 1992). Therefore, it has been hypothesised that sediment bacteria may constitute a more reliable food resource than seasonally occurring pulses of settling phytodetritus, and that their relative contribution to invertebrate diets should increase during periods of low phytodetrital sedimentation, i.e. during summer stratification and winter. Evidence for such seasonal variation in the relative importance of bacteria came from Johnson et al. (1989), who found that a seasonal decline in the availability of algal carbon was accompanied by an increase in the relative contribution of bacterial carbon (4-21%) to the energy requirements of *Chironomus plumosus* larvae.

Two of the dominant microbial groups in lake sediments are methanogens (methane producing microorganisms) and methanotrophs (methane oxidising bacteria, MOB): groups that are both involved in the carbon cycle in freshwater lakes (e.g. Hanson & Hanson 1996, Shively et al. 2001, Chan et al. 2005).

Sediment bacteria as key players in the carbon cycle in freshwater lakes

In lakes (but also in ponds, wetlands and streams), large quantities of organic matter are degraded under anaerobic conditions through methanogenesis (Rudd & Taylor 1980, Ingvorsen & Brock 1982, Mattson & Likens 1993). Especially, lakes were found to be 'hot spots' of methane production and thus contribute largely (1-16%) to the global natural methane emissions (Bastviken et al. 2004). Several groups of bacteria degrade the organic matter via a complex food web. The final step is performed by methanogens that under anoxic conditions convert all organic carbon and carbon dioxide quantitatively to methane. In lakes, methane is almost exclusively produced from either acetate or H_2/CO_2 as precursors (Lovley & Klug 1983, Peters & Conrad 1996, Conrad 1999). Because deeper sediment layers typically contain organic matter that is more aged and depleted in labile compounds, degradation processes including CH_4 production rates are expected to decrease with sediment depth. Present evidence from lakes suggests that the majority of methane production occurs in anoxic sediments (Bartlett et al. 1988, Rudd & Hamilton 1978). Methane can be exported from the sediment either by ebullition or diffusion (e.g. Mattson & Likens 1990, Bastviken et al. 2004). As a result of diffusive export from the anoxic sediment, methane finally reaches the oxic sediment or the water column. There, a large proportion is likely to be oxidised by MOB (e.g. Bastviken et al. 2002) for which CH_4 serves as an energy and carbon source (Hanson & Hanson 1996). Hence, carbon that would be otherwise biologically unavailable for other organisms is cycled back into the food web. There are several reasons to believe that the methane pathway plays an important energetic role in freshwater ecosystems. Anoxic water and sediment are often rich in organic matter compared to overlying oxic water and anoxic metabolism may account for a substantial part (20-60%) of the carbon metabolism and the heterotrophic microbial production within freshwater environments (Hessen & Nygaard 1992, Mattson & Likens 1993, Boon & Mitchell

1995, Utsumi et al. 1998). Methanogenesis contributes to 30-80% of the anaerobic carbon mineralisation in lake waters and sediments (Rudd & Hamilton 1978, Fallon et al. 1980, Kuivila et al. 1988, Bédard & Knowles 1991), and actually as much as 90% of the methane produced is oxidised by MOB as it passes into the oxic zones within the sediment or water (Rudd & Taylor 1980, Frenzel et al. 1990, Oremland et al. 1993, Utsumi et al. 1998). This suggests that a large proportion of the methane may be converted to microbial biomass. Thus, MOB represent a potentially significant pathway for the recycling of methane-derived carbon back into aquatic food webs. That a considerable methanotrophic biomass develops is shown in reports from two lakes, which indicate that the production of organic carbon in oxic water layers by MOB may be similar to the carbon fixation by all heterotrophic bacteria (Hessen & Nygaard 1992) or to primary production (Utsumi et al. 1998).

Stable isotopes as a tool to analyse food webs

Stable isotope analyses can provide a powerful tool for investigating food webs and are being used increasingly in different ecosystems (Fry & Sherr 1984, Peterson & Fry 1987, Wada et al. 1987). Food web studies attempt to characterise the trophic relationship of animals or consumers and their energy sources (e.g. Pimm et al. 1991, Williams & Martinez 2000, Post 2002). An increasing number of studies used stable isotopes to characterise trophic relationships in freshwater (e.g. Yoshioka et al. 1994, Vander Zanden et al. 1999, Grey et al. 2001, Post 2002), marine (e.g. Pinnegar & Pollunin 2000, Levin & Michener 2002) and terrestrial animal communities (e.g. Ponsard & Arditì 2000, Scheu & Falca 2000). The two most widely used stable isotopes are carbon and nitrogen. The carbon isotope ratio of an animal reflects that of the organic source or primary producer, while the nitrogen isotope ratio can be used to infer the trophic level of an animal (DeNiro & Epstein 1978, 1981, Minagawa & Wada 1984). Unlike $\delta^{13}\text{C}$, which changes little as it is passed to consumers (+1‰ with each trophic level) (Peterson & Fry 1987), nitrogen signatures ($\delta^{15}\text{N}$) increase on average by +3.4‰ with each successive trophic level (DeNiro & Epstein 1981, Minagawa & Wada 1984). Combining these two tracers, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of animals can provide an indication of the carbon and nitrogen sources, fixation pathways and trophic level.

Also in lakes, stable isotope ratios of animals are increasingly used to analyse different diets and hence provide evidence for varying carbon pools/sources (e.g. pelagic vs. littoral vs. profundal). Lakes offer many opportunities for resolving whether the food web is supported by autochthonous production by photosynthetic phytoplankton (e.g. Hessen et al. 1990, Laybourn-Parry et al. 1994) or if the food web is based on allochthonous inputs of organic matter from their catchment areas (see reviews by Jones 1992, Hessen 1998).

Stable isotopes reveal evidence for chemosynthesis in marine environments

In certain environments – deep-sea hydrothermal vents, cold seeps and some organic rich coastal sediments – microbial chemosynthetic primary production can replace photosynthesis as the dominant source of ecosystem energy production (e.g. Cavanaugh et al. 1981, Brooks et al. 1987, Van Dover & Fry 1994). Stable isotope techniques have been an essential component of research regarding the energetic basis of chemosynthetic-based ecosystems (see reviews of Jannasch & Mottl 1985, Fisher 1990). In specific anoxic environments, such as deep-sea hydrothermal vents and cold seeps, it has been shown that macroinvertebrate communities attain their energy from the microbial oxidation of methane (Childress et al. 1986, Page et al. 1990, Paul et al. 1985). The low $\delta^{13}\text{C}$ of that macrofauna indicated that chemosynthetically fixed carbon sources (i.e. methane) must have significantly contributed to their diet (Rau & Hedges 1979). This is because the $\delta^{13}\text{C}$ of biogenic methane is extremely low ($\delta^{13}\text{C} \leq -60\text{‰}$) (Van Dover 2000, Coplen et al. 2002) compared to phytoplankton-derived carbon ($\delta^{13}\text{C} -15$ to -25‰) (Fry & Sherr 1984). Since $\delta^{13}\text{C}$ remains largely unaltered as it is transferred through food webs, the flow of methane-derived carbon can be easily detected in biological communities (e.g. Childress et al. 1986, Fisher 1990, Levin & Michener 2002). Initially, deep-sea vent communities were the only biological communities that were thought to be fuelled by methane-derived carbon. Although, it was known that methanotrophic communities inhabit many terrestrial and freshwater environments, such as soils, tundras, wetlands, streams and lakes (Fenchel & Finley 1995, Hanson & Hanson 1996), nobody realized the importance of chemoautotrophic primary production as a base (aside photosynthesis) for these food webs.

Stable isotopes reveal evidence for chemosynthesis in freshwater environments

Just over a decade ago, Bunn and Boon (1993) used stable isotope evidence to suggest that biogenic methane may be assimilated into higher trophic levels in freshwater food webs via methane oxidising bacteria (Fig. 1).

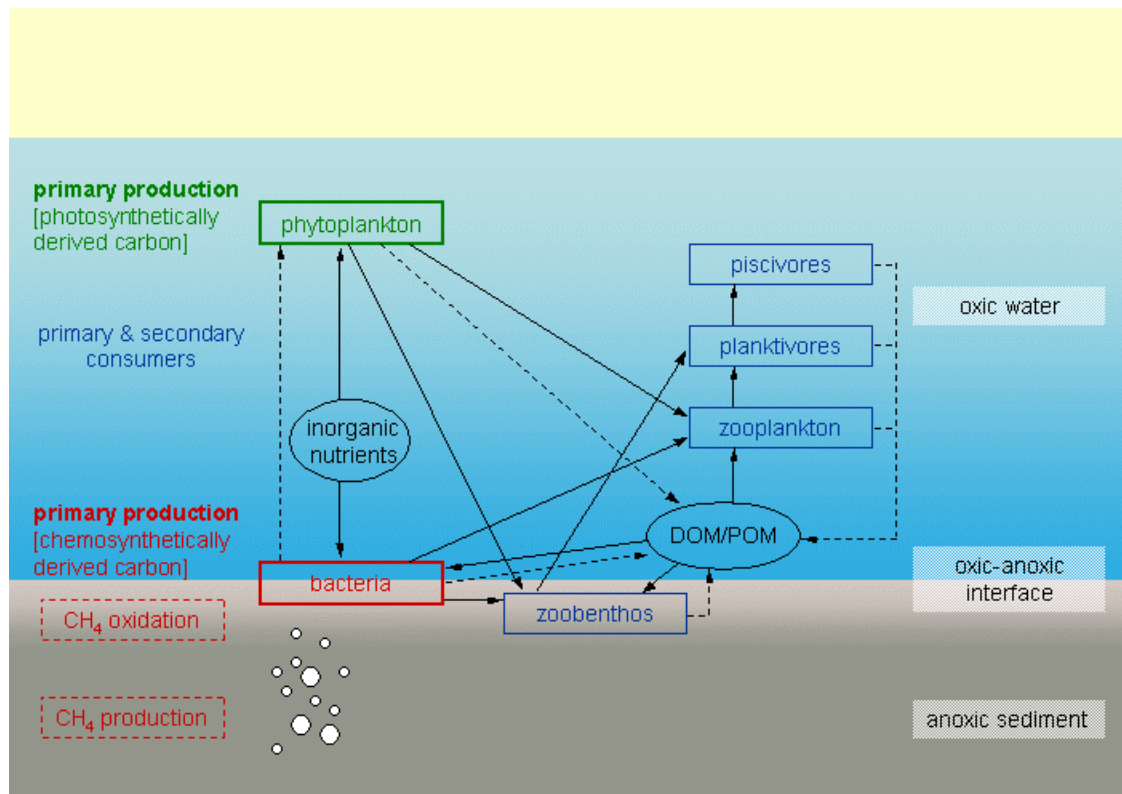


Figure 1: A generalised scheme of a lake food web including pathways for incorporation of chemosynthetic microbial production (arrow means 'C-transfer by uptake'; dashed arrow means 'C-release').

Stable carbon isotope data have shown that zooplankton in some lakes selectively feed upon food sources isotopically lighter than bulk particulate organic matter (POM), suggesting that methanotrophic bacteria can provide a significant food source for zooplankton (Bunn & Boon 1993, Jones et al. 1999, Bastviken et al. 2003, Fig. 2). More recently, several studies have further suggested the importance of methane as a carbon source especially for macroinvertebrates in freshwaters, for instance in billabong (flood-plain lake, Bunn & Boon 1993), stream (Kohzu et al. 2004) and lake food webs (Kiyashko et al. 2001, Grey 2002, Grey et al. 2004a,b, Kelly et al. 2004, Fig. 2).

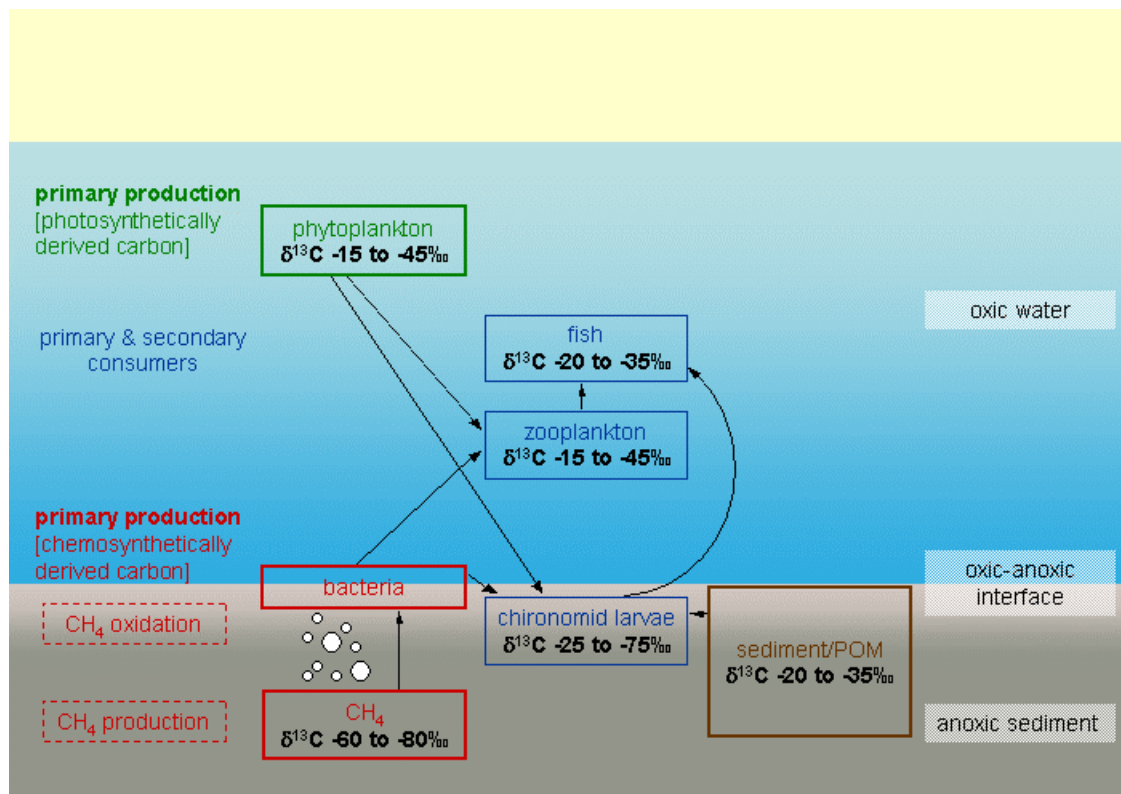


Figure 2: A generalised scheme of trophic interactions in a lake ecosystem and typical $\delta^{13}\text{C}$ isotopic signatures found during my thesis. Methane can play an important energetic role in both benthic and pelagic food webs.

Chironomid larvae, a model organism for lake zoobenthos

Chironomid larvae are keystone members of lake ecosystems (Coffman 1995). In soft sediments they often dominate the profundal community together with oligochaete worms. Their predominance in profundal benthic communities of nutrient rich lakes suggests that they may be important not only as a community member, but also as agents in the recycling of material lost from the water column. Immature stages live in the sediment, whereas pupal stages migrate through the water column to emerge as imagos (e.g. Frank 1982, Armitage et al. 1995, Fig. 3).

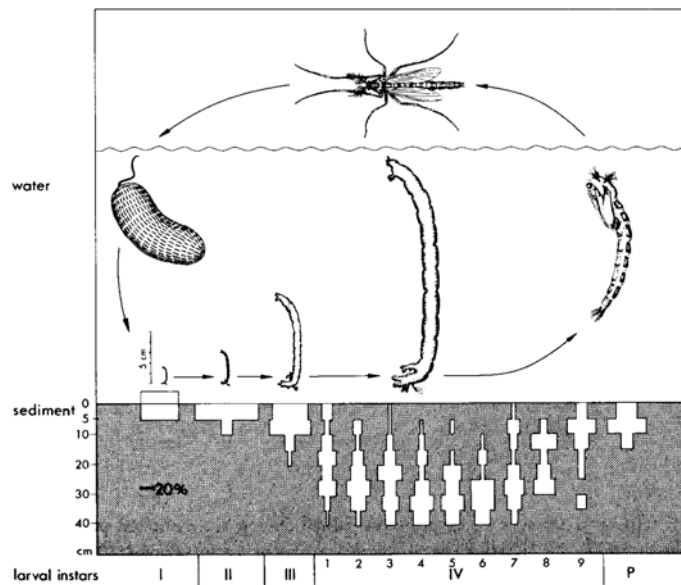


Figure 3: Life cycle of *Chironomus plumosus* and larval vertical distribution in the sediment of lake Federsee (taken from Frank 1982).

Even though larvae of different chironomid species are morphologically very similar, they construct and reside in different types of tubes. These larval tubes vary in structure that are linked to a variety of feeding modes, such as in two characteristic benthic species, *Chironomus plumosus* and *C. anthracinus* (Monakov 2003).

As a typical filter-feeder, *Chironomus plumosus* builds U-shaped dwelling tubes in the loose sediment. With excretions of the salivary glands it either produces a cone-shaped net (Fig. 4A) with a mesh gauge of 12 to 17 μm (Walshe 1947, 1951), or strings sticky threads across the tube lumen (McLachlan 1977). The larva generates a current of water by undulating its body, which transports oxygen and food in the tube, leading to a gradually filling of the net with algae and organic matter. Additionally, *C. plumosus* is known to switch to a deposit-feeding mode and collect suspended particulate matter at the sediment-water interface (Walshe 1947, McLachlan 1977, Hodkinson & Williams 1980). Larvae of the species *C. anthracinus* are typical deposit-feeders that inhabit cone-shaped (vertical I-shaped) tubes in the mud. By scraping the sediment surface, larvae feed directly from the oxidised top layer which is high in energy (Fig. 4B).

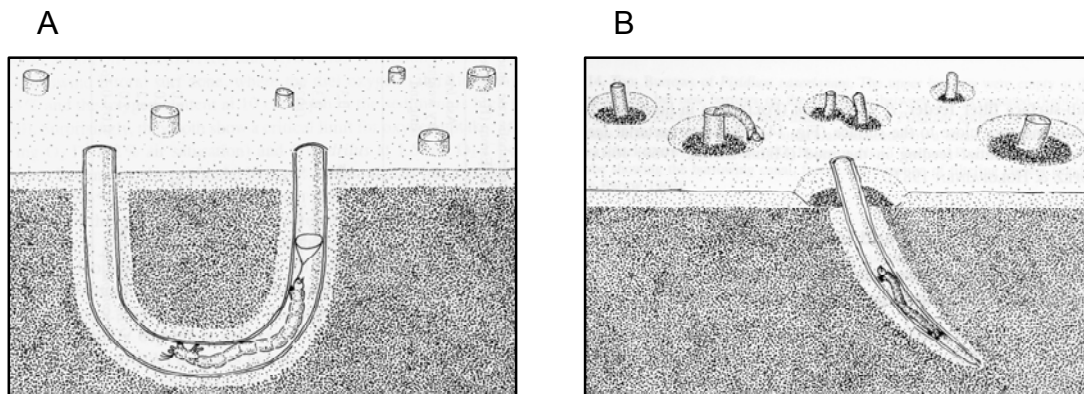


Figure 4: Cross-section through a *Chironomus plumosus* (A) and *C. anthracinus* (B) tube (oxic sediment – light; anoxic sediment – dark) (taken from Jónasson 2003).

Chironomid larvae are ideal organisms to study microbe-invertebrate interactions in freshwater lakes. First, they occur globally in high abundances in a variety of diverse lakes. Thus, they not only play an important functional role in lake food webs but are also easy to compare within and between lakes. Second, they show a high diversity in feeding modes and link the pelagic and benthic food webs in different ways. Third, and most importantly for this thesis, is that chironomid larvae were hypothesised to directly feed upon methanotrophic bacteria (Bunn & Boon 1993, Kiyashko et al. 2001, Grey et al. 2004a,b, Kelly et al. 2004). Thus the objective of this thesis is to resolve and elucidate connectivity among chironomid larvae and carbon-cycling in temperate freshwater lakes.

Thesis outline

The thesis is divided into six chapters. Each chapter represents an independent study that is structured into abstract, introduction, methods, results and discussion. Thereby, I examined if freshwater aquatic macroinvertebrates (e.g. chironomid larvae) are partly 'fuelled' by methane gas. This outline gives a short overview of the motivation and scientific approach for each study.

Chapter I

Several recent studies have reported chironomid larvae with extremely depleted $\delta^{13}\text{C}$ values in different lake ecosystems. As one possible explanation, chironomids were proposed to feed upon methanotrophic bacteria and thus incorporate methane-derived carbon. Higher abundances of these bacteria should therefore lead to a higher availability of ^{13}C -depleted microbial biomass and thus more depleted chironomids. To test this hypothesis, $\delta^{13}\text{C}$ signatures of chironomid larvae and methane turnover rates from two lakes with different morphologies and mixing regimes were compared. To exclude any effect of differing bacterial species composition influencing the study, the community structure of methanogens and methanotrophs from each lake was analysed and cell numbers of methanotrophs determined.

Chapter II

Pronounced seasonal changes in chironomid larvae $\delta^{13}\text{C}$ values are evident in several lakes. It has been suggested that this seasonality can be explained by differential incorporation of isotopically light methanotrophic biomass. Chapter I demonstrated that activity and size of the methanogenic and methanotrophic populations in the lake sediment influenced chironomid larval $\delta^{13}\text{C}$ values. Accordingly, I expected seasonal changes in chironomid $\delta^{13}\text{C}$ values to be linked to seasonal changes in the methane cycling activity. To test this hypothesis methane production and oxidation rates were determined in parallel to stable isotope analyses of chironomid larvae and methane in two lakes with two different mixing regimes over an annual cycle.

Chapter III

Previous studies sampling chironomid larvae at different depths in arctic and temperate lakes found a consistent pattern in larval $\delta^{13}\text{C}$. Offshore chironomids were generally more ^{13}C -depleted compared to nearshore chironomids. The authors of these studies speculated that differences in chironomid $\delta^{13}\text{C}$ could be attributed to different conditions within the sediment affecting the proportion of methanotrophic bacteria in their diet. To test this hypothesis I examined differences in methane production in littoral, sublittoral and profundal sediments of one lake and the subsequent incorporation of methane-derived carbon into chironomid larvae. In addition, larval distribution and other site-specific parameters were used to estimate potential methane-mediation via the abundant chironomid biomass to higher terrestrial predators.

Chapter IV

Besides the consumption of methanotrophs, another potential carbon pathway resulting in macroinvertebrates with depleted $\delta^{13}\text{C}$ values could be via the consumption of chemoautotrophs that assimilate respired carbon. In this study it was hypothesised that the analyses of sulphur stable isotopes can provide further insight into linkages between sediment microbial flora and macroinvertebrate consumers. Two co-occurring chironomid species, differing in their feeding strategies, from two lakes were thus analysed for sulphur stable isotopes to identify the differential assimilation of methanotrophic and chemoautotrophic bacteria.

Chapter V

Methanogenic degradation of organic matter is an important microbial process in lake sediments. One means to identify the dominant methanogenic pathway is to perform stable isotope analyses of methane. In this chapter I explore whether hydrogen isotopes can be used as an additional biogeochemical tracer in macroinvertebrate tissues. I aimed to determine whether differences in the chironomid $\delta^2\text{H}$ reflected the predominant CH_4 production pathway, which fuelled the methanotrophic population.

Chapter VI

Recent studies and the chapters above have indicated that methane plays a major role in the nutrition of chironomid larvae. Although interactions between chironomid larvae and methanotrophs have been inferred (the stable isotope data provides convincing but indirect evidence), none have been demonstrated experimentally. Thus, by using ^{13}C -labelled methane, lake sediment and chironomid larvae in a controlled laboratory experiment we intended to track the carbon flow from methane through bacteria to chironomid larvae. In this study, stable isotopes in combination with bacterial biomarkers (phospholipid fatty acids, PLFAs), diagnostic for methanotrophs, were used.

chapter I



Methane cycling in lake sediments and its influence on chironomid larval $\delta^{13}\text{C}$

ABSTRACT

Stable carbon isotope analysis of chironomid larvae gave rise to the hypothesis that methane oxidising bacteria can provide an important food source for higher trophic levels in lakes. To investigate the importance of the methane cycle for the larval stable carbon signatures, isotope analysis and microbiological and biogeochemical investigations were combined. The study was based on a comparison of a dimictic lake (Holzsee) and a polymictic, shallow lake (Großer Binnensee), both located in northern Germany. Both lakes are inhabited by *Chironomus plumosus* larvae, which exhibited a stronger ^{13}C -depletion in Holzsee than in Großer Binnensee, indicating a greater contribution of methane-carbon in the former. Indeed, the processes involved in the microbial methane cycle were found to be more active in Holzsee, showing higher potential methane production and methane oxidation rates. Consistently, cell numbers of methane oxidising bacteria were with $0.5\text{--}1.7 \times 10^6$ cells $\text{g}_{\text{dw}}^{-1}$ about one order of magnitude higher in Holzsee than in Großer Binnensee, too. Molecular analysis of the microbial community structure revealed no differences in the methanotrophic community between the two lakes, with a clear dominance of type I methanotrophs. The methanogenic population seemed to be adapted to the prevailing substrate in the respective lake (H_2/CO_2 in Holzsee and acetate in Großer Binnensee), even though differences were minor.

In conclusion, the stronger larval ^{13}C -depletion in Holzsee was not reflected in differences in the microbial community structure, but in the activity and size of the methanogenic and methanotrophic populations in the lake sediment.

INTRODUCTION

Microbial methane production and oxidation are important processes in the mineralisation of organic material. They are controlling the sedimentary methane cycle relevant for the emission of the greenhouse gas methane to the atmosphere. A recent survey on methane emissions from lakes showed, that lakes contribute to 6-16% of global natural methane emissions (Bastviken et al. 2004). Besides its importance for the carbon export from lakes, methane oxidation leads to the build up of microbial biomass, which can be used as internal carbon source at the basis of the trophic system in the lake.

In metabolic steps along the food web, the preferred reaction of ^{12}C carbon leads to an enrichment of the lighter isotope ^{12}C compared to ^{13}C in organic compounds, enabling food web studies by stable carbon isotope analysis. The carbon isotope fractionation of methanogenic archaea is pronounced and leads to a distinct, very depleted signature of the methane produced, with $\delta^{13}\text{C}$ signatures as low as -50‰ to -110‰ (Whiticar 1999). The distinct carbon signature of biogenic methane can be used as a tracer for the recycling of methane-carbon into the food web of lakes. Indeed, recent food web studies based on stable carbon isotope analyses of chironomid larvae revealed a possible link between the microbial methane loop in sediments and higher trophic levels in aquatic systems (Bunn & Boon 1993, Kiyashko et al. 2001, Grey et al. 2004a, Kelly et al. 2004). The ^{13}C -depletion found for the larval biomass compared to mean $\delta^{13}\text{C}$ values of sediment and particulate organic matter led to the hypothesis, that chironomid larvae assimilated a dietary food source based on methane oxidising bacteria (MOB), which had converted isotopically light biogenic methane into biomass. A comparison of larval $\delta^{13}\text{C}$ and corresponding lake characteristics supported this hypothesis (Kelly et al. 2004).

In this study, we investigated the methane cycle and the isotopic signature of chironomid larvae in parallel, to get a more detailed picture of the interaction of both. For this purpose we chose two eutrophic lakes with contrasting morphology: Großer Binnensee (GB), a shallow, well mixed (polymictic) lake and Holzsee (Hz), a summer stratified (dimictic) lake with regularly occurring anoxia in the hypolimnion, both located in northern Germany. We hypothesised, that methane is produced in the

sediments of both lakes, but in the dimictic lake Hz with higher rates, which fuel a more active methane oxidation, thus leading to higher cell numbers and more biomass of methane oxidising bacteria (MOB) in the sediment. This higher availability of MOB biomass as food source for chironomid larvae then leads to a more pronounced ^{13}C -depletion of the larvae in the dimictic lake. On the other hand, differences in larval $\delta^{13}\text{C}$ between lakes could also be dependent on differences in the methane $\delta^{13}\text{C}$ of these lakes, which is strongly influenced by the metabolic pathway used for methane production (Whiticar 1999). Moreover, the fractionation between methane and the biomass of methane oxidising bacteria (MOB) depends on the prevailing family of MOB, resulting in no fractionation for type II MOB biomass compared to the signature of methane if methane is limiting, and a fractionation of -13‰ to -30‰ for type I MOB (Summons et al. 1994, Jahnke et al. 1999). Thus, besides the quantitative aspect of the interaction between methane turnover and larval $\delta^{13}\text{C}$, the community structure of methanogens and methanotrophs is important to estimate the isotopic signature of the methane-based food source. Therefore, we investigated possible qualitative differences in the methanogenic and methanotrophic community by denaturing gradient gel electrophoresis (DGGE) as fingerprinting method.

MATERIALS AND METHODS

Sampling sites

The two lakes investigated, Großer Binnensee (GB) and Holzsee (Hz), are located in the Holsteiner Lake District, northern Germany (54°19'40''N, 10°37'30''E for GB and 54°09'36''N, 10°11'04''E for Hz). Their most important characteristics are summarized in Table 1. Holzsee is a dimictic lake with regular oxygen depletion in the hypolimnion throughout the summer stratification period. Großer Binnensee is shallow (mean water depth 2 m) and polymictic. Sediment and chironomid larvae were sampled in August 2003 at a water depths of 6 m in Hz and 3 m in GB. Oxygen and temperature profiles were measured with a WTW oxygen probe EOT 190 (Oximeter Oxi 191, WTW, Germany).

Table I-1: Characteristics^a of dimictic Holzsee and polymictic Großer Binnensee.

Lake	Surface Area (km ²)	Max Depth (m)	Mean Depth (m)	DOC (mg C l ⁻¹)	N:P	pH	O ₂ (mg l ⁻¹)	T (°C)
Großer Binnensee	4.8	3.0	1.9	8.2	31	8.0	10.6 / 9.2	23.9 / 23.8
Holzsee	0.2	6.9	3.7	5.0	24	7.9	9.5 / 0.1	25.6 / 12.8

^aData represent mean values for the years 1991–2000 (Stähr et al. 2002) except for oxygen concentrations and temperatures, which were measured at the water surface (left) and above the sediment surface (right) during the current study.

Preparation of chironomid larvae and sediment samples for stable carbon isotope analysis

Fourth instar *Chironomus plumosus* larvae were sieved *in situ* from sediments collected using an Ekman grab. The larvae were left overnight in 0.2 µm filtered tap water at ambient temperature for gut clearance. Faecal material was removed to prevent coprophagy and fixed with *para*-formaldehyde for fluorescence *in situ* hybridisation (FISH). Individual larvae were oven-dried at 60°C, homogenised and stored for subsequent stable isotope analysis by continuous flow isotope ratio mass spectrometry as described by Grey et al. (2004a). Sediments were acidified (0.5 M HCl) for 24 h to remove inorganic carbon according to Midwood and Boutton (1998), and prepared and analysed as for chironomids. The isotopic ratios were expressed in the delta notation: $\delta^{13}\text{C} = 10^3(R_{\text{sa}}/R_{\text{st}} - 1)$ with $R = {}^{13}\text{C}/{}^{12}\text{C}$ of sample (sa) and standard (st), respectively (Grey et al. 2004a, Krüger et al. 2001).

Potential methane production rates

To account for small-scale heterogeneity in the sediment, triplicate sediment cores were taken adjacent to the area sampled for chironomids. Cores were closed with rubber stoppers and transported to the laboratory immediately after sampling, where they were divided into layers of 0-6, 6-12 and 12-20 cm sediment depth. The triplicate depth layers were combined for further analyses. Sediments were converted into slurries by the addition of autoclaved and deoxygenated tap water in a ratio of 1:1 (w/w).

Three replicates of 50 ml slurry from each sediment layer were flushed with N₂ and incubated in serum bottles to determine the initial potential methane production

rates (MPR), and to collect the methane for stable carbon isotope analysis by gas chromatograph-combustion-isotope ratio mass spectrometry as described by Krüger et al. (2001). These incubations are in the following referred to as “initial incubations”. After this initial incubation, a “substrate and inhibitor experiment” was set up with the slurries. The three replicates of each sediment layer were mixed and divided into 15 replicates of 9 ml in Hungate tubes with a total volume of 16 ml. All tubes were sealed with butyl stoppers and repeatedly flushed with N₂ to remove residual O₂. Substrates or inhibitor were added to triplicates of each sediment layer as described below and three parallels were used without addition as controls. As substrates, acetate (10 mM) and H₂/CO₂ (50% / 10% (v/v)) were used, to study effects on MPRs and to assess their importance as substrates in the sediment. The fraction of methane produced from H₂/CO₂ (f_{H₂}) was determined using methyl fluoride (CH₃F 99%, ABCR Karlsruhe, Germany) as a specific inhibitor of acetate-dependent methanogenesis (Frenzel & Bosse 1996, Janssen & Frenzel 1997, Conrad & Klose 1999). The f_{H₂} was calculated by comparing the MPR after addition of methyl fluoride (final concentration 0.5%) to those in control tubes. Bromoethanesulfonate (BES 10 mM) was added to another 3 replicates to inhibit methane production completely (Balch & Wolfe 1979) and to get an accumulation of the precursor acetate.

At the end of the “substrate / inhibitor experiment” after 3 weeks incubation, 2 ml slurry samples were taken from control, methyl fluoride, and BES tubes, and centrifuged at 13000 rpm for 4 min. The supernatant was frozen at –20°C for acetate analysis by high performance liquid chromatography (HPLC) (Krumböck & Conrad 1991). After further enrichment of methanogens in the control tubes for additional two weeks, slurry samples (0.7 ml) were taken for DNA extraction and stored at –20°C.

All incubations were conducted in the dark at 8°C. Headspace gas samples were taken twice daily in the substrate / inhibitor experiment or each second day in initial incubations after heavy shaking of the tubes by hand, and analysed for methane and methyl fluoride on a gas chromatograph (HP 5890 series II, Agilent Technologies, Palo Alto, USA) equipped with an HP Plot Q capillary column (Agilent Technologies, Palo Alto, USA) and a flame ionization detector. Methane production rates were calculated by linear regression of the methane increase with incubation time, and expressed in nmol CH₄ g_{dw}⁻¹ h⁻¹ of sediment. The MPR calculated were

statistically analysed for differences between lakes and depths by 2-Way ANOVA tests (SigmaStat 3.1).

Methane oxidation rates and cell counts of methane oxidising bacteria

Potential methane oxidation rates (MOR) were measured in the same sediment layers as described for methane production. Triplicate ten ml slurries were transferred into sterile serum bottles (total volume 50 ml) and sealed with butyl stoppers. Methane was added to a final concentration of 3% in the headspace. Bottles were incubated at 8°C in the dark on a rotary shaker at 120 rpm. Methane depletion was followed by sampling the headspace, with the first sample taken 15 min after amendment with methane, followed by sampling at 2 h intervals. Potential methane oxidation (MOR) were calculated by linear regression from the depletion curves and are given as mean \pm standard deviation of the triplicate incubations per sediment layer in $\mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ h}^{-1}$ of sediment. The MOR calculated were statistically analysed for differences between lakes and depths by 2-Way ANOVA tests (SigmaStat 3.1). At the end of the experiment, enrichment cultures for methane oxidising bacteria (MOB) were set up with 1 ml of the slurry of each parallel in modified NMS medium (see below). Additionally, 0.7 ml aliquots of the slurries were frozen for DNA extraction and 0.3 ml aliquots fixed with *para*-formaldehyde for FISH. These samples are referred to as MOR samples.

Cell numbers of MOB were determined by most probable number (MPN) technique in the three depth layers of the sediments. Each sample was diluted in 2-fold steps in 8 replicates (microtiter plates) and in two parallel plates as described by Eller and Frenzel (2001), using nitrate mineral salt medium with 1/10 of standard nitrate concentration (modified after Whittenbury et al. 1970). Inoculated plates were incubated in gas tight jars at 10°C for at least 8 weeks in the dark, with one parallel microtiter plate incubated in an atmosphere with 20% CH₄ in air, and the other, in ambient air as control for heterotrophic growth. Cell numbers were analysed by t-tests to verify that differences were statistically significant.

Enrichment cultures for methane oxidising bacteria

After a short term enrichment of methane oxidising bacteria (MOB) in the sediment slurries with 3% methane in the headspace for about one week (measurement of potential methane oxidation rates), 1 ml of the slurries was transferred into 10 ml of nitrate mineral salt medium with 1/10 of standard nitrate concentration as used for most probable number determinations. In these incubations, 20% methane was added to the headspace and the cultures were incubated at 8°C in the dark on a rotary shaker at 120 rpm. The enrichment was subcultured three times with 1 ml inoculum in 10 ml medium, each subcultivation took place after approximately three weeks, until no sediment particles were remaining in the culture. After that, two further subcultivations with 0.7 ml inoculum in 10 ml medium were carried out before the bacterial biomass was harvested by centrifugation for DNA extraction (6 ml culture) and fluorescence in situ hybridisation (FISH) (4 ml culture). DNA extraction and FISH was carried out as described for sediment samples.

Fixation and fluorescence in situ hybridization (FISH)

For fluorescence in situ hybridisation (FISH), samples of sediment slurries (0.3 ml), cultures or larval faeces were fixed with 4% *para*-formaldehyde solution and stored in ethanol : PBS (1:1 (v/v)) at -20°C (Eller & Frenzel 2001). Prior to hybridisation, 2 µl of fixed sample were mixed with 8 µl melted 0.2% agarose, transferred to 8-well coated slides and dried at 46°C for about 15 min. Autofluorescence in the sediment samples was quenched with toluidine blue (Eller & Frenzel 2001). Samples were simultaneously hybridised with the universal eubacterial probe Eub338 (Amann et al. 1990), and probes M α 450 for *Methylocystaceae*, and M γ 84 and 705 for *Methylococcaceae* (Eller et al. 2001), and additionally DNA stained with DAPI (4',6-Diamidino-2-phenylindole, Sigma, Germany). Probes were synthesised and labelled with fluorochromes (Fluorescein-isothiocyanat, CY3, CY5) by Thermo Hybaid / Interaktiva (Germany). Coverslips were mounted with a 5 + 1 mixture of Citifluor (Citifluor Ltd, UK) and Vecta shield (Vector Laboratories Inc, Canada) and fixed with colourless nail varnish. Slides were analysed using a Zeiss axioplan 2 microscope (Zeiss, Germany), equipped with a 100 x oil immersion lens, 1.25 x Optovar and F-

View digital black and white camera. AnalySIS 3.0 software (Soft Imaging System GmbH, Germany) was used for image analysis.

DNA extraction and PCR amplification

The DNA extraction was based on cell lysis with 10% lauryl-sulfate solution (10% SDS in 0.5 M Tris/HCl, pH 8.0, 0.1 M NaCl) and horizontal shaking for 45 s (Vortex Genie 2 with adapter 13000-V1, MoBio Laboratories Inc., USA) after addition of zirconium-silica beads, followed by DNA purification using NH₄-acetate and isopropanol precipitations as described in detail by Henckel et al. (1999).

To amplify the 16S rRNA gene of eubacteria, the primer sets GM3 / GM4 (Murray et al. 1996) and 533f / 907r (Weisburg et al. 1991) were used. The 16S rDNA of methanotrophs was amplified using the primers MethT1d F / MethT1b R for type I MOB and 27 F / MethT2 R for type II MOB, followed by a nested PCR approach for DGGE analysis (primer set 358 F GC / 517 R), all previously described by Wise et al. (1999).

Methanogenic archaea were investigated via the genes for the key enzyme methyl-coenzyme M reductase (Mcr), using the primers and PCR conditions described in Hales et al. (1996) and Springer et al. (1995) with the GC-clamp (Wise et al. 1999) attached to the forward primer. The amplification of mcrA-genes was followed by DGGE analysis as described in detail by Watanabe et al. (2004) and Inagaki et al. (2004), applying a denaturing gradient of 30–60%.

DGGE and phylogenetic analysis of band sequences

DGGE analysis was carried out in a Dcode System (BioRad, Germany) at 60°C, with denaturing gradients used according to the description of the respective authors named for the primer sets above (80% urea/formamide corresponding to 5.6 M urea and 32% deionised formamide). For a better band resolution an acrylamid gradient was applied in parallel to the denaturing gradient, ranging from 6 to 8% acrylamid. Gels were run for 20 h at 60°C and 100 V, stained with SYBR-Gold (Molecular Probes, USA) for 45 min in the dark, and scanned with a Syngene PTS 20 M imager (Synoptics LTD, UK). Bands were excised, re-amplified and sequenced (performed

by MWG, Germany), and sequences analysed as described previously by Eller and Frenzel (2001). Then they were checked for next relatives by BLAST search in the recent Genbank Data Library (<http://www.ncbi.nlm.nih.gov/BLAST/>), and aligned and phylogenetically analysed with the ARB software package (Ludwig et al. 2004). The database used was based on the ssu-Jan03 database and updated with methanotrophic and related sequences, containing approximately 26000 sequences in total.

The phylogenetic tree for type I MOB (*Methylococcaceae*) was calculated with full length sequences by Neighbour Joining (ARB). Partial sequences derived from DGGE bands were added to this tree using the Maximum Parsimony algorithm and only the positions present in the partial sequences, resulting in 155 valid columns. The tree topology was kept constant during addition of partial sequences (Ludwig et al. 1998).

Nucleotide accession numbers

The partial sequences of methanotroph 16S rDNA (DGGE bands) used for tree calculation have the accession numbers AJ863013–AJ863037.

RESULTS

Isotope analyses

Individual larvae collected in Holzsee (Hz) exhibited $\delta^{13}\text{C}$ values between -35.2 and -55.0‰ (mean \pm SD: $-46.9 \pm 4.1\%$, $n = 31$). Larvae were ^{13}C -depleted by about 20‰ compared to the sediment isotope values of -27.2‰ determined for all three layers in Hz (Table 2A). The extent of larval isotopic variability and the degree of ^{13}C -depletion in Großer Binnensee (GB) were less pronounced than in Hz. Mean larval $\delta^{13}\text{C}$ from GB was $-30.6 \pm 1.4\%$ ($n = 19$), while the sediment $\delta^{13}\text{C}$ was around -18‰ (Table 2A).

Table I-2A: $\delta^{13}\text{C}$ values of methane produced in sediment slurries from different depth in initial incubations compared to $\delta^{13}\text{C}$ signatures of the organic carbon fraction in sediment samples.

	$\delta^{13}\text{C}$ Signatures (‰)			
	Holzsee		Großer Binnensee	
	CH ₄	Sediment	CH ₄	Sediment
0-6 cm	-74.2 ± 0.7	-27.2	-72.6 ± 1.1	-17.6
6-12 cm	-79.2 ± 0.3	-27.2	-69.2 ± 0.4	-18.2
12-20 cm	-82.3 ± 1.0	-27.2	-56.9 ± 1.5	-17.7
Larvae	-46.9 ± 4.1		-30.6 ± 1.4	

Methane production rates and methane $\delta^{13}\text{C}$

In the initial sediment incubations from GB, methane production started only after an adaptation phase of 500-1000 h, compared to 200-300 h in Hz. Potential methane production rates (MPR) in Hz were highest at the sediment surface, with 39.7 ± 4.2 nmol CH₄ h⁻¹ g_{dw}⁻¹ in the top 0–6 cm of the sediment, compared to 19 ± 0.3 and 9 ± 2.1 nmol CH₄ h⁻¹ g_{dw}⁻¹ in 6-12 and 12-20 cm depths (Fig. 1). In GB the potential MPR were relatively lower and showed a different pattern with depth, with highest rates of 7.8 ± 0.4 nmol CH₄ h⁻¹ g_{dw}⁻¹ recorded in the intermediate sediment layer (Fig. 1). A 2-Way ANOVA analysis of the MPR revealed significant differences between the two lakes, the depth layers of the two lakes and between the depth layers within each lake. Also, a statistically significant interaction between lake and depth was found. The methane produced in these initial incubations differed in its $\delta^{13}\text{C}$ -value with sediment depth and revealed opposing depth patterns for the two lakes (Table 2A). In Hz, methane became more ¹³C-depleted with sediment depth (from -74‰ in the top to -82‰ in the deepest layer), whereas in GB methane was less ¹³C-depleted with depth (-73‰ to -57‰).

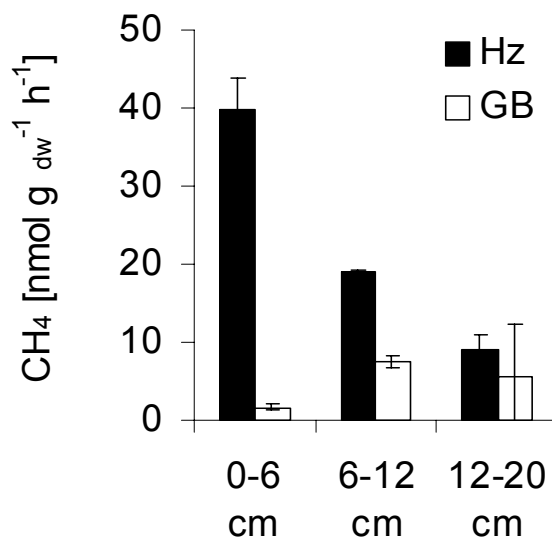


Figure I-1: Potential methane production rates (MPR) in sediment slurries of Holzsee (Hz) and Großer Binnensee (GB). Rates were determined from initial slurry incubations of the three sediment depth layers 0–6 cm, 6–12 cm and 12–20 cm (mean \pm SE, $n = 3$).

In the substrate and inhibitor experiment, methane production was strongly stimulated by the addition of acetate and H_2/CO_2 in all three depth layers of both lake sediments, with H_2/CO_2 leading to significantly higher MPRs than acetate addition (Fig. 2). The differences in MPRs between the sediment depth layers within one lake and within one substrate treatment were not always statistically significant.

The overall much higher capacity for methane production detected in Hz might be due to the presence of higher cell numbers of methanogens than in GB sediment, which might be maintained by the overall preferable conditions for methanogenesis in Hz, with an anoxic water column above the sediment and high amounts of organic carbon introduced to the lake from the surrounding trees and pasture. In the top two layers of Hz sediment, 44% of the methane was produced from H_2/CO_2 in contrast to 20% in the 12-20 cm layer. In the GB sediment, the proportion of methane produced by CO_2 -reduction was calculated to be 59, 33 and 73% for the 0-6, 6-12 and 12-20 cm layers, respectively (Table 2B).

The inhibition of methane production by BES addition did not result in an accumulation of acetate in the sediment porewater.

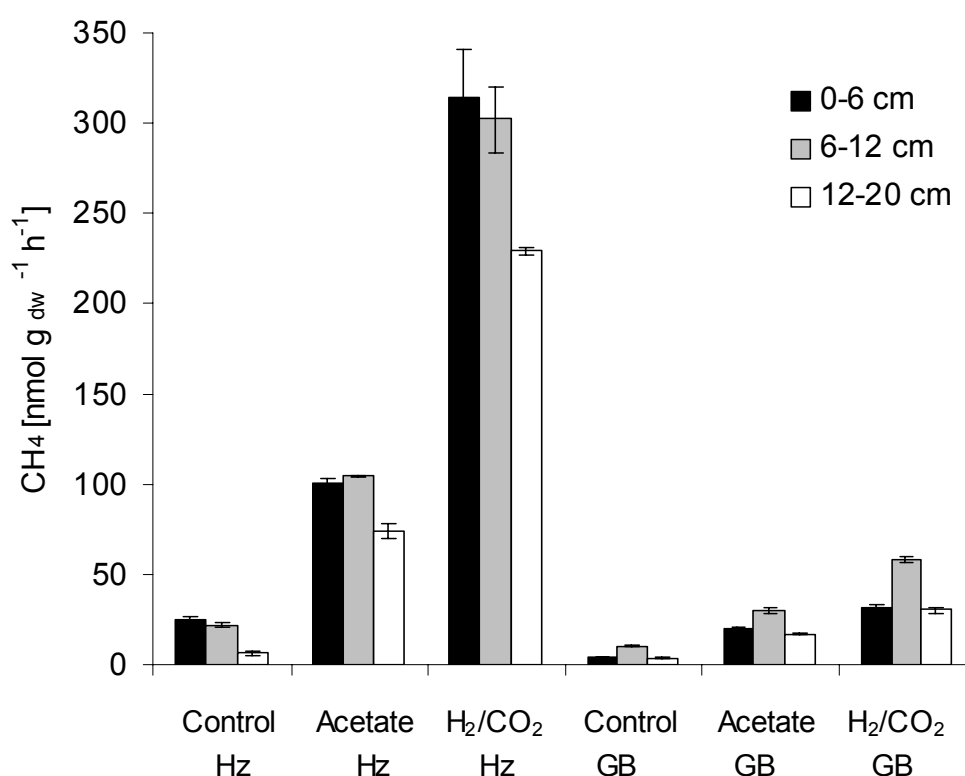


Figure I-2: Induction of methane production in sediment slurries from Holzsee (Hz) and Großer Binnensee (GB) after substrate addition, compared to unamended controls. Rates were determined from prolonged slurry incubations (substrate / inhibitor experiment) of the three sediment depth layers 0–6 cm, 6–12 cm and 12–20 cm (mean \pm SE, n = 3).

Table I-2B: Comparison of $\delta^{13}\text{C}$ values of methane and CO₂ produced in sediment slurries from different depth during prolonged incubations (substrate / inhibitor experiment), and the percentage of methane produced from H₂/CO₂ (f_{H_2}), determined with CH₃F as specific inhibitor of acetoclastic methanogenesis.

	Holzsee			Großer Binnensee		
	f_{H_2}	$\delta^{13}\text{C}$ of CH ₄	$\delta^{13}\text{C}$ of CO ₂	f_{H_2}	$\delta^{13}\text{C}$ of CH ₄	$\delta^{13}\text{C}$ of CO ₂
0-6 cm	43.6%	-70.4 \pm 1.2‰	-15.8 \pm 9.4‰	59.2%	-71.8 \pm 4.2‰	-17.1 \pm 6.1‰
6-12 cm	43.9%	-74.4 \pm 0.9‰	-19.1 \pm 9.0‰	33.3%	-68.1 \pm 2.9‰	-17.8 \pm 6.7‰
12-20 cm	20.2%	-82.8 \pm 1.1‰	-18.9 \pm 7.3‰	72.9%	-71.3 \pm 1.3‰	-13.9 \pm 3.7‰

Diversity of methanogens

In order to analyse the methanogenic community, DGGE fingerprints of sediment samples and slurries were obtained. In the sediment samples of both lakes, a limited methanogenic diversity with 2-4 dominant bands was detected. Furthermore, negligible difference between different depth layers of the sediment was observed. In GB, sequences of excised bands were most closely related to *Methanospirillum hungatei*, *Methanoculleus thermophilus* or *bourgensis*. A third band appeared in the

deeper layers (6-12 and 12-20 cm) related to *Methanosarcina acetivorans*. In the deepest layer, sequences most closely related to those detected in forest soil were found (Galand et al. 2002). In Hz, sequenced bands again showed greatest homology with these forest soil sequences. Another two bands occurring in all samples clustered with *Methanospirillum hungatei* and an uncultured *Methanomicrobiaceae* (strain Ebac, Springer et al. 1995). The prolonged incubation of the sediment in the control tubes of the substrate and inhibitor experiment resulted in an increasing dominance of hydrogenotrophic *Methanocorpusculum*/*Methanoculleus* in Hz, and acetoclastic *Methanosarcina* species in GB, indicating an adaptation of the methanogenic communities to different substrates.

Methane oxidation rates and cell numbers of methanotrophic bacteria

In general, methane oxidation (MO) started immediately, except in the 12-20 cm layer from GB, where it started after approximately 18 h. The differences in potential methane oxidation rates (MORs) between the two lakes were statistically significant, whereas the differences between the depth layers within the lakes were not significant (Fig. 3A).

The constant MO activity with sediment depth was not reflected in the MOB cell counts, which showed highest cell numbers in the 6-12 cm sediment layer in both lakes (Fig. 3B). In Hz, MOB cell numbers were significantly higher ($(4.9 \pm 0.8) \times 10^5$ to $(16.9 \pm 3.4) \times 10^5 \text{ g}_{\text{dw}}^{-1}$) in all three sediment depths than in GB, where $(1.4 \pm 0.3) \times 10^5$ to $(3.5 \pm 0.6) \times 10^5 \text{ g}_{\text{dw}}^{-1}$ were found (Fig. 3B). The differences in cell numbers between the depth layers within the lakes were also significant. To account for the different densities of the sediment layers (total dry weight per volume), cell numbers were also calculated per volume. This calculation resulted in about one order of magnitude lower cell numbers than per gram dry weight (with 0.56×10^5 to $1.8 \times 10^5 \text{ ml}^{-1}$ and 2.0×10^4 to $4.2 \times 10^4 \text{ ml}^{-1}$ for Hz and GB, respectively), but similar patterns with sediment depth.

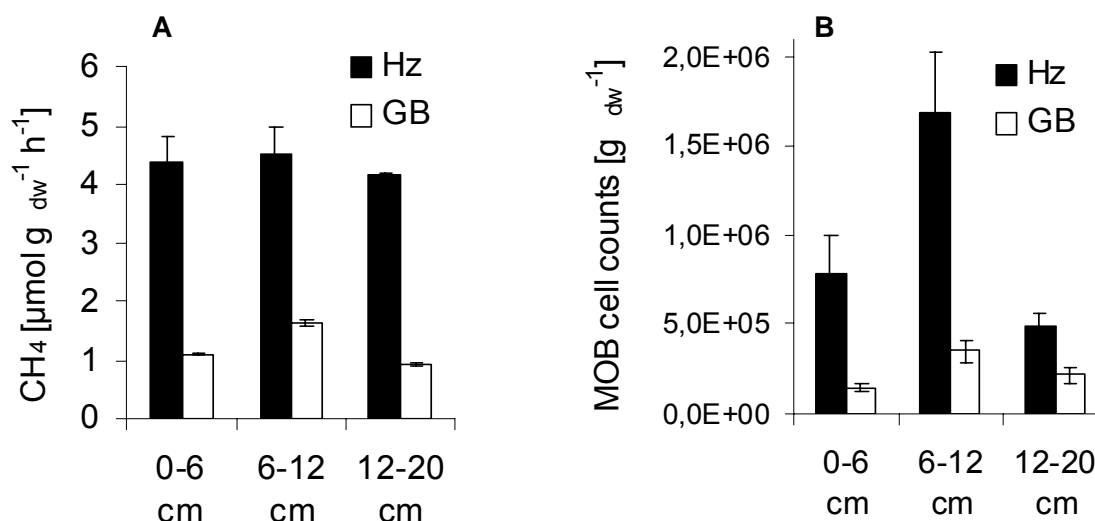


Figure I-3: (A) Potential methane oxidation rates (MOR) in sediment slurries from Holzsee (Hz) and Großer Binnensee (GB). Rates were determined from oxidic slurry incubations after addition of 3% methane. Three sediment depth layers were investigated (0–6 cm, 6–12 cm and 12–20 cm, mean \pm SE, $n = 3$). (B) Cell numbers of methane oxidising bacteria (MOB) in the sediment of Holzsee (Hz) and Großer Binnensee (GB). Numbers were calculated by most probable number (MPN) determination for the three sediment depth layers 0–6 cm, 6–12 cm and 12–20 cm (mean \pm SE, $n = 8$).

Diversity of methanotrophic bacteria

To investigate the population structure of methane oxidising bacteria (MOB), the diversity in the sediment samples, MOR incubations, enrichment cultures and gut content of chironomid larvae was investigated by fluorescence in situ hybridisation (FISH) and DGGE analysis. FISH with specific probes for the two MOB families revealed a dominance of type I MOB in the sediment samples of both lakes, while type II cells were only scarcely found (Fig. 4A). This was supported by FISH analysis of sediments fixed after MOR measurements, and of enrichment cultures, which only contained type I MOB (Fig. 4B, C). Intact type I MOB cells were found in the gut content of chironomid larvae from both lakes (Fig. 4D), and it was possible to get enrichment cultures of MOB from the gut content.

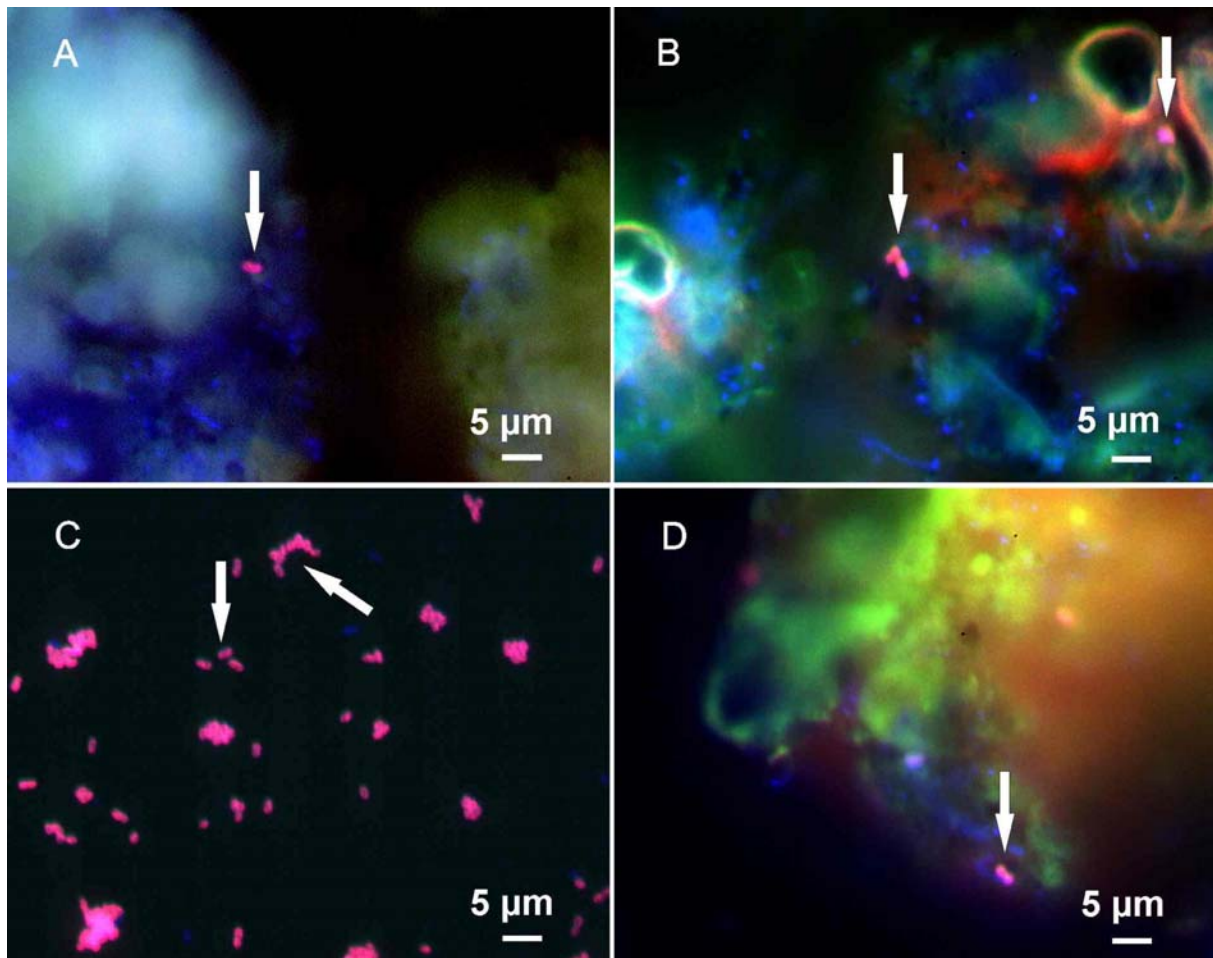


Figure I-4: Specific detection of methane oxidising bacteria (MOB) by fluorescence in situ hybridisation (FISH). MOB were detected by parallel hybridisation with the type I specific probe mix $M\gamma 84/M\gamma 705$ labelled with Cy3 (red), the type II specific probe $M\alpha 450$ labelled with FLUOS (green) and the eubacterial probe $Eub338$ (Cy5, signal only shown in 4 C in blue, but in all cases present). Additionally, DNA was stained with DAPI (blue, except 4 C). The images show the overlay of the three colours, resulting in a pink signal for type I MOB cells, indicated with arrows. (A) Sediment sample, depth layer 0-6 cm, Großer Binnensee. (B) Short time enrichment of MOB during measurements of potential methane oxidation rates (MOR), sample from 0-6 cm sediment depth, Holzsee. (C) Enrichment culture from sediment of 6-12 cm depth, Holzsee. (D) Gut content of chironomid larvae, collected after gut clearance, Großer Binnensee.

In agreement with the results from microscopic analysis, the PCR assays resulted in amplicons of the predicted size only for type I MOB. DGGE analysis of type I MOB specific PCR products revealed a simple community with only few bands per sample (Fig. 5, insert). Sediment and MOR samples from both lakes and all sediment layers showed the same DGGE banding patterns. However, the patterns of enrichment cultures were different to those of the sediment samples, indicating a cultivation-based change in the community structure. Enrichment cultures from both

lakes exhibited the same DGGE banding patterns. All sequences from DGGE bands (as marked in the insert in Fig. 5) clustered within the genus *Methylobacter*, despite different electrophoretic mobility.

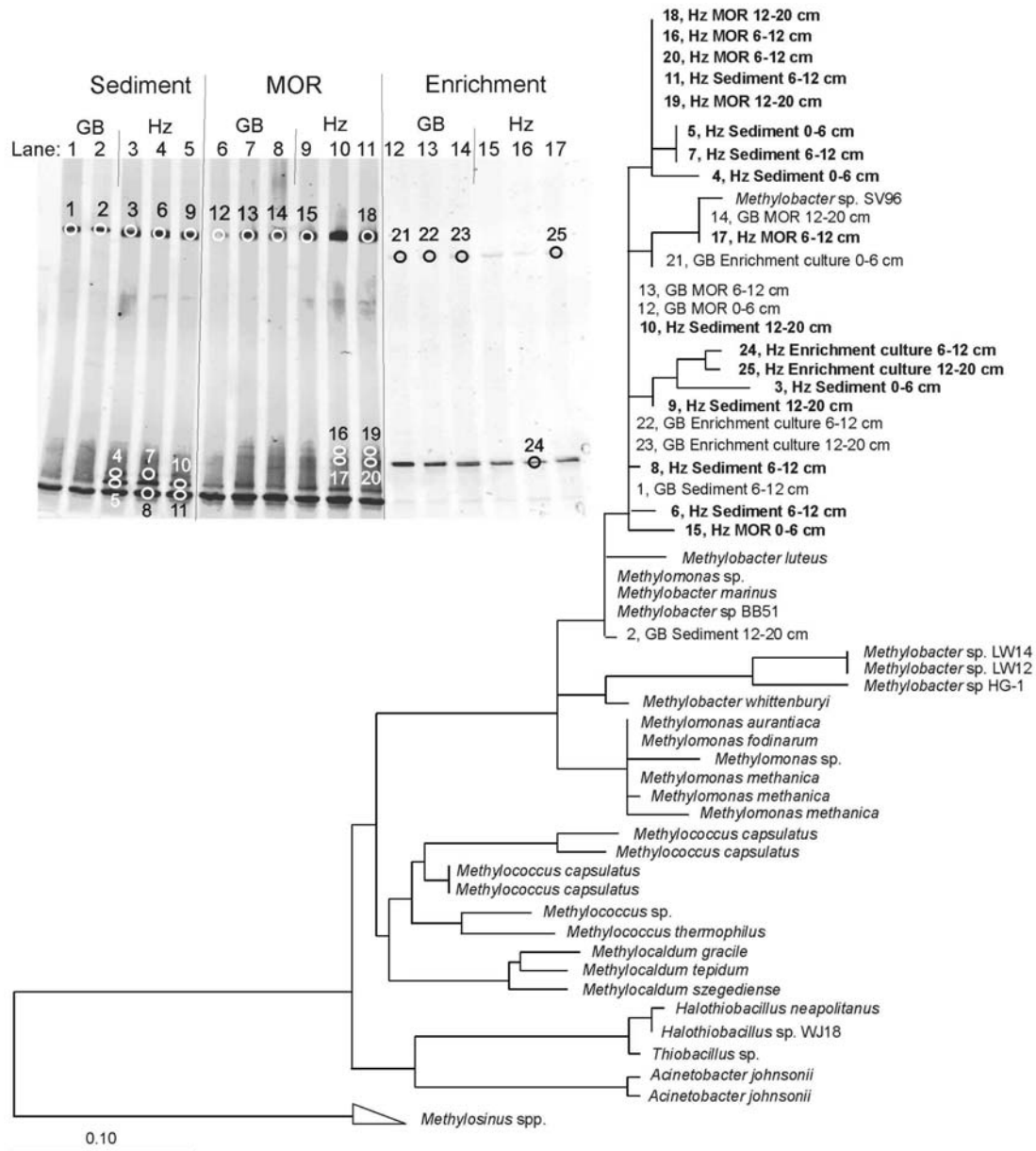


Figure I-5: Population structure of type I methane oxidising bacteria (MOB) as detected by DGGE analysis. The inserted picture shows the banding patterns in DGGE analysis after amplification of the 16S rRNA gene of type I MOB. Sequenced bands are marked and numbered. The dendrogram was calculated in ARB with full length sequences by Neighbour Joining. Partial sequences derived from DGGE bands were added to this tree using the Maximum Parsimony algorithm and only the positions present in the partial sequences, resulting in 155 valid columns. The tree topology was kept constant during addition of partial sequences. Hz: Holzsee, GB: Großer Binnensee, MOR: Sediment incubated with 3% methane for about one week to determine potential methane oxidation rates, Enrichment: Enrichment culture for MOB in modified NMS medium at 8°C, several transfers.

DISCUSSION

At the end of the summer, high activities of methanogens were expected for both lakes, because stable anoxia in the hypolimnion of the dimictic lake Holzsee (Hz) had developed and an enrichment of organic matter in the sediment of the polymictic lake Großer Binnensee (GB) could be expected. We assumed that higher rates of methane turnover in the stratified lake lead to higher cell numbers of methane oxidising bacteria (MOB) and thus give rise to a stronger ^{13}C -depletion in the larval biomass than found in the polymictic lake (Kelly et al. 2004). Indeed, the potential methane production rates (MPR) measured in sediment samples were about 5-fold higher, and the larval biomass was by 16‰ more ^{13}C -depleted in stratified Hz than in polymictic GB (Fig. 1). Normalised to the sediment volume, the MPR measured in Hz sediment were with $2.25 \pm 0.22 \text{ nmol CH}_4 \text{ cm}^{-3} \text{ h}^{-1}$ in the same range as rates measured in sediment of the eutrophic Lake Dagow (Casper 1996, Glissmann et al. 2004), but about 10-fold higher than rates measured in sediment of the eutrophic lake Plußsee (Nüsslein & Conrad 2000).

Methanogenic community structure and methane $\delta^{13}\text{C}$

In addition to the overall availability of methane-carbon (as reflected in MPR), its $\delta^{13}\text{C}$ signature is an important information to characterise it as a basal food source. This signature is dependent mainly on the pathway used for methane production, resulting in $\delta^{13}\text{C}$ values of -80 to -110‰ and -50 to -60‰ for hydrogenotrophic and acetogenic methanogenesis, respectively (Whiticar 1999, Conrad 2005). The methane $\delta^{13}\text{C}$ of Hz sediment incubations was more negative than of GB sediment (Table 2A). With the exception of the 12-20 cm layer in GB, all methane $\delta^{13}\text{C}$ were more negative than those generally reported for acetoclastic methanogenesis, indicating the importance of hydrogenotrophic methanogenesis in both lakes. The methanogenic community structure reflected these methane signatures, with a dominance of hydrogenotrophic methanogens belonging to *Methanomicrobiaceae* and *Methanocorpusculaceae* in Hz and the top layers of GB sediment and a higher proportion of acetoclastic *Methanosarcinaceae* in the 12-20 cm layer of GB. This higher abundance of hydrogenotrophic methanogens was also in accordance with the stronger stimulation

of methane production by H_2/CO_2 than by acetate in both lakes (Fig. 2). In eutrophic Lake Dagow, in contrast, acetoclastic methanogens were dominant (Casper 1996, Glissmann et al. 2004). Furthermore, H_2/CO_2 dependent methanogenesis is energetically less attractive than acetogenic at low temperatures (Schulz & Conrad 1996, Glissmann et al. 2004). Thus, the methane produced in Hz and GB sediment incubations exhibited relatively negative $\delta^{13}C$ signatures compared to mean values reported for other freshwater systems (Bunn & Boon 1993 and citations therein, Whiticar 1999).

Over a prolonged incubation period during the substrate and inhibitor experiment, the methane $\delta^{13}C$ showed only minor changes, except for the 12-20 cm layer of GB. Here the $\delta^{13}C$ signature became substantially lighter (Table 2A and B), indicating a shift of the methanogenic pathways towards hydrogen as substrate (Whiticar 1999). The methanogenic community composition, however, showed an increase in *Methanosarcina* related sequences. Nevertheless, these methanogens are able to utilise both, H_2/CO_2 and acetate (Krzycki et al. 1987). Besides a shift in community composition, the observed change of methane $\delta^{13}C$ might be explained by a ^{13}C -depletion in the substrate acetate as a consequence of homoacetogenesis (Gelwicks et al. 1989). Unfortunately, the inhibition of methane production by BES addition did not result in an accumulation of acetate in the sediment porewater, preventing a ^{13}C -analysis of this precursor, but indicating a strong competition for this substrate in the sediments of both lakes. The analysis of carbon dioxide present in the incubations showed no substantial differences in $\delta^{13}C$ signatures between the lakes and the different depth layers (Table 2B). Therefore, the observed changes in methane $\delta^{13}C$ most likely were caused by the development of different methanogenic communities in the differing lake environments.

The importance of methane oxidation in the lakes

Our hypothesis of higher methane turnover rates in the stratified lake was also confirmed for the methane oxidation activity. Potential methane oxidation rates (MOR) were about 4 times higher in stratified Hz than in polymictic GB (Fig. 3A). The higher activity measured in Hz sediment compared to GB correlated with higher methanotrophic cell numbers (Fig. 3B), thus leading to a higher availability of

methane derived microbial biomass in stratified Hz. The rates detected in the two lakes were in the same range as MOR in the sediment of a pristine lake (Suominen et al. 1998), but lower than in Lake Washington ($19\text{--}25 \mu\text{mol g}_{\text{dw}}^{-1} \text{h}^{-1}$, Auman et al. 2000). Concerning MOB cell numbers, only a few investigations with MPN counts are available for comparison. Compared to soil from a flooded rice field in Northern Italy (Eller et al. 2005b), the lake sediments investigated here were inhabited by 1-2 orders of magnitude less methanotrophs per gram dry weight. In landfill cover soil, $10^8\text{--}10^{11} \text{ g}_{\text{dw}}^{-1}$ MOB cells were detected (Gebert et al. 2003), despite similar methanotrophic activity as in the lake sediments. To avoid an underestimation of MOB cell numbers by cultivation based methods, the methane oxidation activity can be used to estimate cell numbers. With a specific activity factor of $0.5\text{--}3 \times 10^{-9} \mu\text{mol CH}_4 \text{ cell}^{-1} \text{ h}^{-1}$ (Costello et al. 2002), cell numbers of $0.3\text{--}3.3 \times 10^9 \text{ MOB g}_{\text{dw}}^{-1}$ and $1.4\text{--}9.0 \times 10^9 \text{ g}_{\text{dw}}^{-1}$ were calculated for GB and Hz, respectively. These high cell numbers demonstrate, that MOB could provide a substantial carbon source for higher organisms in aquatic systems.

Interestingly, high initial potential MORs were not only detected in the top layers of the sediment, where optimal conditions for methanotrophs were expected, but also in the deeper layers investigated. These substantial initial rates indicate, that the MOB were in an active state in situ. As MOB grow preferentially at oxic/anoxic interfaces in flooded environments (Frenzel et al. 1990, King 1994, Calhoun & King 1998, Segers 1998), these high rates indicate the presence of oxygen in the usually anoxic main body of the sediment, which could be achieved by bioturbation and/or bioirrigation. Chironomid larvae are well known for their bioturbating and irrigating activity (Granéli 1979, Jørgensen & Revsbech 1985, Frenzel 1990), thus creating better growth conditions for their probable food source methanotrophic bacteria. Enhanced methane oxidation due to the presence of and bioturbation by chironomid larvae has been shown for rice field soil (Kajan & Frenzel 1999). To calculate the increase of the sediment surface by larval bioturbation, the natural abundance of *C. plumosus* per square meter was determined, resulting in 1600 and 190 individuals m^{-2} in GB and Hz, respectively. We assumed a mean length of the tubes of 0,2 m and an inner diameter of $2 \times 10^{-3} \text{ m}$ and used the coat surface of a cylinder for calculation. With these parameters, larval bioturbation increased the sediment surface 3-fold in GB, but only 1.2-fold in Hz. The strong effect of bioturbation on the sediment

structure in GB could be an important factor, influencing the degradation pathways of organic matter and reducing the carbon flow into the microbial methane cycle, resulting in the lower MPR and MOR measured. Thus, besides the recycling of organic carbon in the water column of shallow lakes, the oxygenation of the sediment by bioturbation could influence the importance of methane as a basal food source in lakes.

In both lakes, exclusively type I MOB with highest sequence similarity to the genus *Methylobacter* were detected by PCR based DGGE in the original sediment, in sediment after MOR measurement and in enrichment cultures (Fig. 4 and 5). Even though this DNA based method does not allow to conclude on the active community, the dominance of type I MOB even in enrichments indicates, that type II MOB were only rarely if at all present in the original sediment samples. Additionally, the results obtained by DGGE were verified by no signals for type II MOB using FISH, which should have resulted in bright signals with vegetative cells. We therefore conclude, that type I MOB were even the dominant active group of MOB in situ, which convert methane into microbial biomass. This dominance of type I MOB could affect the MOB biomass $\delta^{13}\text{C}$, due to their assimilation of methane via the ribulose monophosphate pathway. Jahnke et al. (1999) showed, that type I MOB biomass was ^{13}C -depleted by -12.6‰ to -23.9‰ compared to methane, whereas type II MOB were ^{13}C -enriched when methane was growth limiting. Summons et al. (1994) found an isotope fractionation of -16‰ to -30‰ for *Methylococcus capsulatus* biomass compared to methane, depending on the growth stage and cell density of the culture. Therefore, although not measured, the MOB biomass $\delta^{13}\text{C}$ in the two lake sediments investigated here was most likely depleted by 13-30‰ compared to methane.

The factors influencing the dominance of either family of MOB are not well known. In some lakes and systems with low temperature, type I MOB were found to be dominant (Boschker et al. 1998, Börjesson et al. 2004), but in other investigations they were not (Auman et al. 2000, Kevbrina et al. 2001, Trotsenko et al. 2002, Bussmann et al. 2004). Another important factor for the competition of MOB is the availability of nitrogen. Type I MOB were reported to prevail in systems with low methane and high nitrogen concentrations (Graham et al. 1993, Bodelier et al. 2000), conditions which probably occurred during most of the year in the eutrophic and phosphor limited lakes investigated here.

Internal recycling of carbon in the lake and chironomid larval $\delta^{13}\text{C}$ signatures

Besides a higher proportion of methane-carbon in the diet, the stronger ^{13}C -depletion of methane and larval biomass in Hz might also be caused by a longer recycling of carbon in this lake, leading to an overall depletion in $\delta^{13}\text{C}$ signatures. The methane $\delta^{13}\text{C}$ from initial sediment incubations of the two lakes showed a difference of 10‰, which was also found for the organic carbon fraction in sediment samples (Table 2), indicating a general ^{13}C -depletion of the organic carbon fraction in Hz compared to GB. However, the larval $\delta^{13}\text{C}$ differed by 16‰ between the lakes, with -47‰ and -31‰ in Hz and GB, respectively. Thus, the larval biomass showed a depletion of 20‰ compared to the sediment in Hz and of only 13‰ in GB, supporting the postulated higher proportion of methane-carbon in the larval diet in Hz.

To explain their depleted $\delta^{13}\text{C}$ signature, the larvae need to assimilate methane derived carbon. This could be achieved by feeding on MOB and digestion of the bacterial cells (Kelly et al. 2004). The presence of intact type I MOB cells in the larval gut clearance (Fig. 4D) indicates, that MOB might survive the gut passage and might even be a part of the gut microflora of the larvae. Nevertheless, the cleared gut content might have passed faster than naturally through the larvae, since it was collected from larvae laying over night in filtered tap water at room temperature.

To estimate the percentage of methane-carbon contributing to larval biomass, we used a simple 2-source, isotope mixing model (Phillips & Gregg 2001), assuming sediment carbon and MOB as the only food sources. The MOB $\delta^{13}\text{C}$ value used for our calculations was derived from the weighted mean methane value for the 20 cm of sediment with an isotope fractionation of -16‰ or -30‰ applied (Summons et al. 1994). With a mean methane $\delta^{13}\text{C}$ of -78.9‰ and -65.3‰ for Hz and GB, respectively, the $\delta^{13}\text{C}$ of MOB biomass was calculated to span from -94.9‰ to -108.9‰ in Hz and from -81.3‰ to -95.3‰ with a -16‰ or -30‰ fractionation applied, respectively. For the sediment $\delta^{13}\text{C}$, weighted mean values of -27.2‰ and -17.8‰ were used for Hz and GB, respectively. According to the model, and not accounting for larval trophic fractionation, methane-carbon contributed 24.1-29.1% of larval biomass in Hz, and 16.5-20.2% in GB. Kelly et al. (2004) applied a 16‰ fractionation factor in the model and reported similar values to Hz for *C. plumosus* from

comparable lake types (Esthwaite and Blelham Tarn in the UK), but lower values from L. Neagh (<10%), which is polymictic and thus comparable to GB.

Conclusions

The more negative $\delta^{13}\text{C}$ signatures of chironomid larvae found in the stratified lake Hz compared to polymictic GB corresponded to higher methane turnover rates and higher MOB cell numbers in the sediment. The higher uptake of methane-carbon was confirmed by calculations based on a 2-source isotope-mixing model, resulting in a contribution of methane-carbon to larval biomass of 24.1-29.1% and 16.5-20.2% in Hz and GB, respectively. No difference in the population structure of MOB was found between the two lakes investigated, thus no specialised MOB community was linked to the larval carbon signature. Therefore, the comparison of the microbial methane cycle in the lake sediments and the $\delta^{13}\text{C}$ signature of the *C. plumosus* larvae revealed a connection based on the quantity of the microbial methane turnover present in the sediment. The interaction between larvae and microorganisms was most likely based on feeding or farming, not symbiosis, but further investigations are needed to unravel the carbon transfer pathway.



chapter II



Are seasonal changes of $\delta^{13}\text{C}$ signatures in chironomid larval biomass related to changes in the microbial methane cycle?

ABSTRACT

Methane-derived carbon has been shown to be an important carbon source for macroinvertebrates in several studies of lake ecosystems using stable isotopes. Furthermore, season and lake morphology appear to influence the importance of methane as a carbon source. However, rarely have the dynamics of the methane cycle been measured concurrently to isotope signatures of chironomid larvae. We examined the methane dynamics in two lakes with contrasting mixing regimes (polymictic and dimictic), while monitoring corresponding changes in chironomid larval $\delta^{13}\text{C}$ throughout an annual cycle. Both, methane turnover rates and abundance of methane oxidising bacteria were higher in the dimictic lake, where correspondingly lower larval $\delta^{13}\text{C}$ values of -44.2‰ to -61.7‰ were recorded. In contrast, methane production and oxidation rates, as well as cell numbers of methane oxidising bacteria were always lower in the polymictic lake. Corresponding larval $\delta^{13}\text{C}$ values ranged from -32.3 to -29.6‰ . Furthermore, seasonal variation in larval $\delta^{13}\text{C}$ was more pronounced in the dimictic lake ($-50.1 \pm 5.9\text{‰}$) compared to the polymictic lake ($-31.1 \pm 1.2\text{‰}$), reflecting the amplitude of turnover rates. This suggests strongly that lake characteristics have an influence on methane turnover rates and, in conjunction with season, affect the fraction of incorporation of methane-derived carbon into freshwater food webs via macroinvertebrates during the year.

INTRODUCTION

Stable isotope analyses are increasingly used in aquatic ecosystem studies to elucidate food web structures, and sources and pathways of nutrients through ecosystems (e.g. Peterson & Fry 1987, Cabana & Rasmussen 1996, Grey et al. 2001). Since the $\delta^{13}\text{C}$ signature of biogenic methane is extremely negative compared to phytoplankton-derived carbon (Fry & Sherr 1984), the flow of this carbon source can be traced through biological communities (e.g. Childress et al. 1986, Fisher 1990, Levin & Michener 2002). A study by Bunn and Boon (1993) indicated that biogenic methane may be assimilated into higher trophic levels in freshwater food webs via methane oxidising bacteria (MOB). More recently, several studies have suggested the importance of methane as a carbon source for macroinvertebrates in freshwater ecosystems, and especially for the widespread and numerous occurring chironomid larvae (Kiyashko et al. 2001, Grey 2002, Grey et al. 2004a, Kelly et al. 2004, Kohzu et al. 2004).

High variability in the $\delta^{13}\text{C}$ signatures of chironomid larval biomass between different, but also within one chironomid species has been observed and intraspecies variation has been studied in detail for *Chironomus plumosus* and *Chironomus anthracinus* (Grey et al. 2004a, Kelly et al. 2004). These authors found that this variation seemed to be related to lake morphology, the water depth at the sampling point within one lake and the sampling time point (season). Kelly et al. (2004) sampled six eutrophic lakes of different morphology and modelled the methane-derived contribution to *C. plumosus* larval carbon to range from 6 up to 58%.

Although the aforementioned studies provide good isotopic evidence for the incorporation of methane-derived carbon into freshwater food webs, direct correlations of the microbial methane cycle and larval $\delta^{13}\text{C}$ signatures are rare. Only one study directly comparing these parameters in two lakes of different morphology is available (Eller et al. 2005a). The authors showed for one sampling time point at the end of the summer stratification period that higher methane production, oxidation rates and more negative larval biomass $\delta^{13}\text{C}$ signatures occurred in the dimictic lake. It was assumed by Grey et al. (2004b) that the difference in lake mixing regime should lead to differences in the seasonal pattern of methane turnover between the lakes, which consequently should be reflected in seasonal changes of larval $\delta^{13}\text{C}$

signatures. To test this assumption, larvae and the microbial methane turnover activities were investigated over an entire year in parallel in the two lakes also studied by Eller et al. (2005a). The following specific questions were addressed: (1) Do the lakes exhibit differences in the seasonality of methane turnover and $\delta^{13}\text{C}$ signatures of produced methane? (2) Do the larval $\delta^{13}\text{C}$ signatures reflect the differences and seasonal changes in methane turnover rates and / or methane $\delta^{13}\text{C}$ signatures? (3) How much of the chironomid larval biomass carbon can be attributed to biogenic methane?

To answer these questions, sediment cores were sampled every second month in the two lakes and analysed for methane production and oxidation rates, as well as the number of MOB. Additionally, the $\delta^{13}\text{C}$ signatures of organic matter in the sediment and the methane produced from the sediment were determined, to define the signatures of potential food sources for chironomid larvae. In parallel, chironomid larvae of the species *C. plumosus* were sampled and analysed for their $\delta^{13}\text{C}$ signature.

MATERIALS AND METHODS

Two eutrophic lakes in northern Germany were selected as study sites because of their differing morphology and its consequence for the water column mixing regime. Großer Binnensee (N54°20' E10°37') is a shallow, exposed and polymictic lake, whereas Holzsee (N54°10' E10°11'), although shallow, has a smaller surface area and is protected by surrounding woodland so that it stratifies and exhibits regular oxygen depletion in the hypolimnion. Some environmental characteristics of the lakes are given in Table 1. Samples were collected from August 2003 to August 2004 at two months intervals. On each sampling date the same sites were revisited. Vertical profiles for temperature and oxygen concentration were determined in the water column in 1 m depth intervals using a WTW oxygen probe EOT 190 (Oximeter Oxi 191, WTW Germany).

Table II-1: Environmental characteristics recorded for Großer Binnensee and Holzsee. Temperature, oxygen concentrations and pH values are given for water above sediment. Means were calculated from data recorded two-monthly from August 2003 to August 2004.

Parameter	Unit	Großer Binnensee	Holzsee
Surface Area	km ²	4.8	0.2
Depth (max / mean)	m	3.0 / 1.9	6.9 / 3.7
Temperature	°C	13.1 ± 8.0	10.4 ± 5.1
O ₂	mg l ⁻¹	10.2 ± 1.6	3.9 ± 4.2
pH		8.5	7.7
DOC ^a	mg C l ⁻¹	8.2	5.0
N:P ^a		31	24
Trophic state ^a		highly eutrophic	eutrophic

^a Source of data: Stähr et al. (2002)

Sampling of chironomid larvae and sediment for stable carbon isotope analyses

Chironomid larvae were collected using an Ekman grab and sieved from the surrounding sediment (2 mm mesh) *in situ* (Grey et al. 2004a). Sampling water depth was 3 m in Großer Binnensee and 6.5 m in Holzsee. Only *Chironomus plumosus* 4th instar larvae were selected and transported to the laboratory in lake water. Larvae were then placed into filtered water for 24 h to allow gut clearance (Feuchtmayr & Grey 2003). Excess faecal material was removed periodically to prevent coprophagy. Individual larvae (20 to 30) were then placed into separate wells of cell culture plates and dried at 60°C for 24 h and stored in a dessicator. Only in October 2003 sampling for 4th instar larvae in Holzsee was not successful.

Sediment was sampled adjacent to the sampling point for the larvae two days before the sampling of the larvae. Four to six sediment cores were taken to account for lateral sediment heterogeneity. Cores were sealed with rubber stoppers and transported directly to the laboratory, where they were divided into three depth layers: the surface (0-6 cm), the intermediate (6-12 cm), and the deepest (12-20 cm) sediment layer. Composite samples for each depth layer were created by combining the material from at least four cores and converted into slurries by adding autoclaved tap water (1:1, w/w). During the whole preparation process, nitrogen was flushed

over the samples to prevent oxidation. For carbon stable isotope analysis, sediment samples were oven dried at 60°C, homogenized and stored in a dessicator.

Stable carbon isotope signatures were analysed using a Carlo-Erba NA1500 elemental analyser coupled to a Micromass Isoprime continuous flow isotope ratio mass spectrometer. Prior to combustion, samples were weighed into tin-cups (0.5-0.7 mg for chironomids and 1.5-2.0 mg for sediment). The isotopic ratios were expressed in the delta notation: $\delta^{13}\text{C} = 10^3 (R_{\text{sa}}/R_{\text{st}} - 1)$ with $R = {}^{13}\text{C}/{}^{12}\text{C}$ of sample (sa) and standard (st), respectively (Grey et al. 2004a). The reference material used was a secondary standard of known relation to the international standard of Vienna Pee Dee belemnite. Repeated analyses of an internal reference ($n = 50$) resulted in typical precision of $\pm 0.1\text{‰}$.

Methane production rates

Methane production rates (MPR) were determined for each sediment depth layer, derived from combined sediment samples as described above. Three replicates of 50 ml slurry from each sediment layer were transferred into sterile serum bottles (total volume 120 ml), sealed with butyl stoppers and flushed with N_2 gas for 30 min to remove residual oxygen. Incubation was performed at 8°C in the dark. Methane production was followed by headspace sampling with subsequent gas chromatographic analysis (GC-FID) and MPR calculated by linear regression of the methane increase with incubation time (Eller et al. 2005a). At the end of these incubations, the produced methane was collected for stable carbon isotope analysis by gas chromatograph-combustion isotope ratio mass spectrometry (GC-C-IRMS) as described by Krüger et al. (2001).

Methane oxidation rates

To determine potential methane oxidation rates (MOR), slurries of each sediment layer were prepared as described above and transferred in triplicate (10 ml) into sterile serum bottles (total volume 60 ml). After re-oxidation of the headspace with synthetic air, bottles were sealed with butyl stoppers and methane added to a concentration of 3% (v/v) in the headspace. The bottles were incubated at 8°C in the

dark on a rotary shaker at 120 rpm. Methane depletion was followed by headspace sampling and subsequent GC analysis, with the first sample taken 15 min after methane addition and subsequent sampling in 2 h intervals until final depletion. Methane oxidation rates were calculated by linear regression of the methane depletion over time (Eller et al. 2005a).

Cell counts of methane oxidising bacteria (MOB)

Cell numbers of methane oxidising bacteria (MOB) were estimated by the most probable number (MPN) method for all three sediment layers as described by Eller et al. (2005a). Each slurry sample was diluted in 2-fold steps in 8 replicates (microtiter plates) and in two duplicate plates. One of these plates was incubated in an atmosphere containing 20% CH₄ in air, the other in ambient air as control for heterotrophic growth. Cell numbers and standard deviations for the eight replicate dilution series from one depth layer sample were calculated from positive dilutions by using the code number system and table of Rowe et al. (1977).

Data analysis and modelling

Two-way analysis of variance (ANOVA) was used to test methane production and oxidation rates (MPR and MOR) for significant differences between the two lakes and the different sediment depths for each month. Additionally, mean values of MOB cell numbers per sediment layer determined over the year were compared between the two lakes using a one-way ANOVA. Differences in larval $\delta^{13}\text{C}$ signatures between the two lakes and between the sampling dates for one lake were tested for significance with one-way ANOVA. Tukey tests provided post-hoc comparison of means. All statistical tests were performed using STATISTIKA, Version 6.0 (StatSoft).

To estimate the percent contribution of biogenic methane to the carbon content of chironomid larvae, a simple two-source isotope mixing model (Phillips & Gregg 2001) was applied, assuming sediment and methane oxidising bacteria (MOB) as the only food sources for chironomid larvae. For these calculations, only the stable carbon isotope data of sediment organic carbon and methane derived from the upper two sediment layers (0-12 cm sediment depth) were used, because in sediment

cores larvae were only seldom detected below this depth (personal observation). For each sampling date, means of the $\delta^{13}\text{C}$ signatures of methane produced in the two sediment layers were used to calculate the $\delta^{13}\text{C}$ signature of MOB biomass by applying a fractionation factor of -16‰ for the methanotrophic metabolism (Summons et al. 1994, Kelly et al. 2004, Eller et al. 2005a).

RESULTS

The two eutrophic lakes Großer Binnensee (polymictic) and Holzsee (dimictic) exhibited the stratification patterns expected during the annual sampling cycle, i.e. a summer stratification in Holzsee, and a well mixed water body throughout the year in Großer Binnensee (Stähr et al. 2002). The sediment-water interface was oxic in Großer Binnensee throughout the year and anoxic for approximately 120 days during autumn 2003 and summer 2004 in Holzsee (Fig. 1A-B).

Seasonal changes and inter-lake differences in $\delta^{13}\text{C}$ signatures of chironomid larvae

Chironomus plumosus larvae sampled in Großer Binnensee showed a limited seasonal variability in their $\delta^{13}\text{C}$ signature of 2.7‰ (min -32.3‰ and max -29.6‰ , Fig. 1C), with slightly more negative values during the winter period. In Holzsee in contrast, large seasonal changes of 17.5‰ were detected for larval $\delta^{13}\text{C}$ signatures (min -61.7‰ and max -44.2‰ , Fig. 1D), with markedly more negative values over the winter. Throughout the year, larvae sampled in Holzsee showed consistently more negative $\delta^{13}\text{C}$ signatures than larvae sampled in Großer Binnensee (one-way ANOVA, $P < 0.001$). The annual mean larval $\delta^{13}\text{C}$ signatures were $-31.1 \pm 1.2\text{‰}$ and $-50.1 \pm 5.9\text{‰}$ for larvae from Großer Binnensee and Holzsee, respectively, a difference of 19‰ . Additionally, the inter-individual variability of larval $\delta^{13}\text{C}$ signatures differed between the lakes, with a low variability at one sampling time point (0.8 to 1.8‰) in Großer Binnensee compared to 4.1 to 9.8‰ in Holzsee.

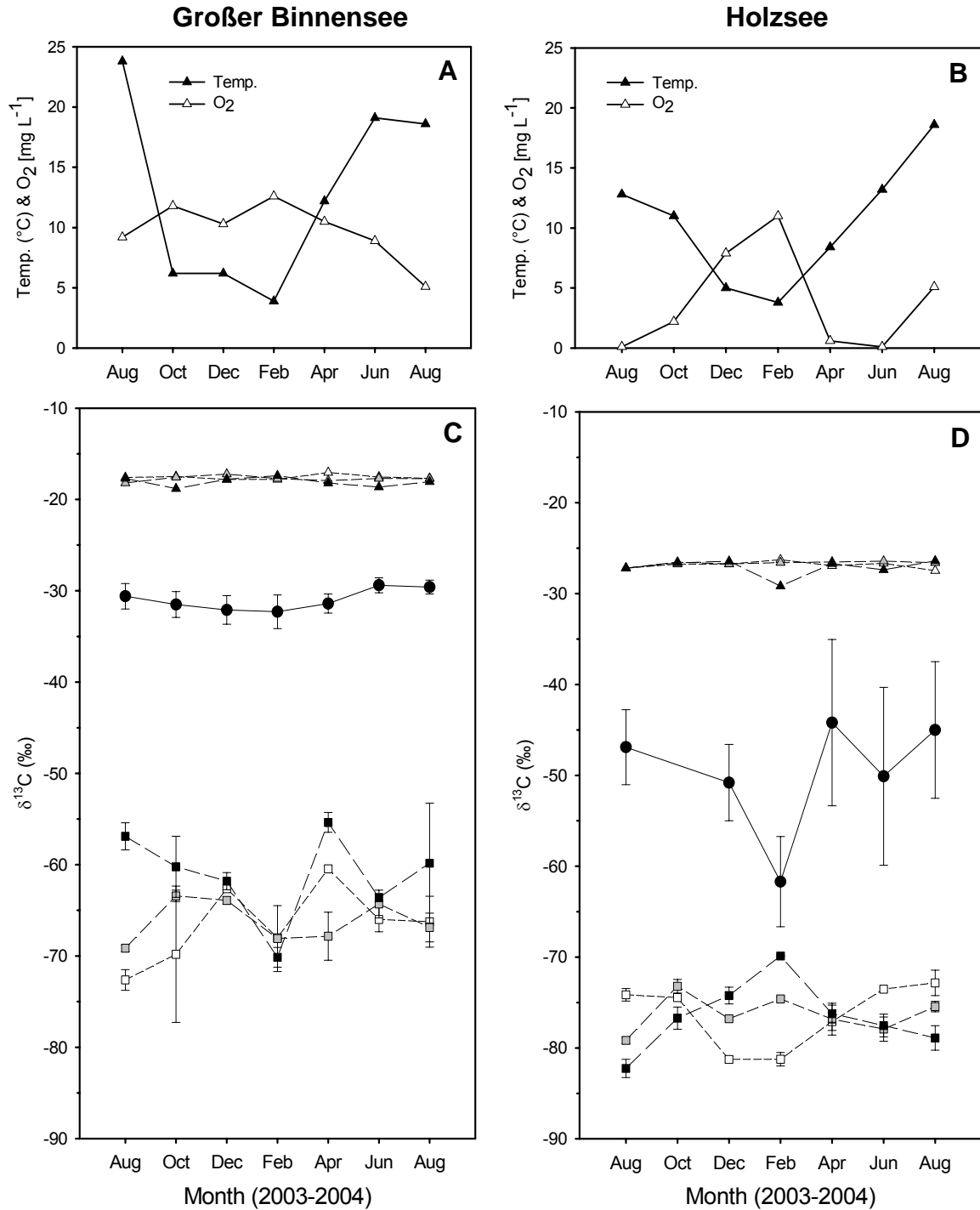


Figure II-1: Seasonal variation of physico-chemical parameters and isotopic signatures measured in Großer Binnensee and Holzsee over an annual cycle: (A-B) Temperature and oxygen concentrations in the water column directly above the sediment surface, (C-D) Isotopic signatures ($\delta^{13}\text{C}$) of organic carbon fraction in the sediment, *Chironomus plumosus* larval biomass (mean \pm SD) and methane produced in sediment slurries (mean \pm SD).

Carbon isotopic signatures of sediment organic carbon and methane

In both lakes, no distinct depth pattern in $\delta^{13}\text{C}$ signatures of sediment organic carbon was found (-17.6‰ to -18.0‰ in Großer Binnensee and -26.6‰ to -27.3‰ in Holzsee). The sediment carbon $\delta^{13}\text{C}$ signatures were consistently at least 10‰ less negative than the larval biomass in both lakes (Fig. 1C-D). Additionally, the sediment signatures showed no seasonal pattern, but remained stable throughout the annual period in both lakes and in all three depth layers analysed (differences between months were not significant, $P > 0.10$). Throughout the year, sediment $\delta^{13}\text{C}$ values were consistently more negative in Holzsee than in Großer Binnensee, with annual means of $-26.9 \pm 0.3\text{‰}$ and $-17.8 \pm 0.1\text{‰}$, respectively, a difference of 9‰ between the two lakes.

Methane produced in the lake sediments showed a seasonal variation in $\delta^{13}\text{C}$ signatures (Fig. 1C-D). In Großer Binnensee, most negative values of methane with -70‰ occurred in August 2003 in 0-12 cm sediment depth and in February 2004 in all three depth layers (Fig. 1C). Additionally, large differences between the $\delta^{13}\text{C}$ signatures of methane produced in the different sediment layers at one sampling time point were found (max. -56.9 to -72.6‰ in August 2003), but no consistent seasonal pattern in relation to sediment depth occurred (Fig. 1C). Mean methane $\delta^{13}\text{C}$ signatures in all three sediment layers varied between -61.2 and -68.8‰ throughout the year.

In Holzsee, methane $\delta^{13}\text{C}$ signatures became more negative with sediment depth during the summer period, whereas in winter (December 2003 and February 2004) this trend was reverse, with methane produced in the deepest sediment layer exhibiting the most positive $\delta^{13}\text{C}$ signatures (Fig 1D). Mean $\delta^{13}\text{C}$ values in all three depths varied between -74.8 and -78.5‰, thus the overall seasonal variability was lower than in Großer Binnensee.

Over the annual cycle, methane $\delta^{13}\text{C}$ signatures of the three sediment layers ranged between -55 to -73‰ for Großer Binnensee (annual mean $-64.6 \pm 2.4\text{‰}$) and -73 to -88‰ for Holzsee (annual mean $-76.4 \pm 1.3\text{‰}$), respectively (Fig. 1C), with a mean difference of 12‰ between the two lakes.

Seasonal changes in methane production rates

To study seasonal changes in methane production in the two lakes, methane production rates (MPR) were determined in the three sediment layers. In Großer Binnensee, seasonal changes of MPR in the upper two sediment layers showed no clear annual pattern, ranging from 1.7 to 8.6 nmol CH₄ g_{dw}⁻¹ h⁻¹ and 3.1 to 9.0 nmol CH₄ g_{dw}⁻¹ h⁻¹ in the surface and intermediate layer, respectively (Table 2). Only the deepest sediment layer in Großer Binnensee exhibited a clear maximum in MPR in February. In Holzsee, only the MPR in the surface sediment layer showed substantial seasonal changes, with highest rates in August 2003 and June 2004, and the lowest in February 2004 (Table 2). Methane production rates were always significantly different ($P < 0.01$) between the two lakes and the three depths at one sampling time point (exception February 2004).

The pattern of MPR across the three sediment layers generally remained characteristic for the lakes (Table 2). In Großer Binnensee, highest annual mean MPR were found in the intermediate sediment layer (6-12 cm, 8 nmol CH₄ g_{dw}⁻¹ h⁻¹), whereas lower rates were detected in the surface and deepest layer (5 and 4 nmol CH₄ g_{dw}⁻¹ h⁻¹, respectively). In Holzsee, a different depth pattern of MPR was found, with highest MPR in the surface layer (annual mean of 25 nmol CH₄ g_{dw}⁻¹ h⁻¹), decreasing with increasing sediment depth to annual means of 16 nmol CH₄ g_{dw}⁻¹ h⁻¹ and 7 nmol CH₄ g_{dw}⁻¹ h⁻¹, respectively. In general, mean MPR calculated for all three sediment depth layers at each sampling time point were almost 3 to 4 times lower in Großer Binnensee than in Holzsee.

Methane oxidation rates and biomass of methane oxidising bacteria

The methane oxidation rates (MOR) in Großer Binnensee showed only small seasonal changes. A small maximum occurred at the end of the summer period in all three sediment layers, with an additional maximum in February 2004 in the deeper two layers (Table 2). Beside these seasonal differences, MOR were comparable for all three sediment layers, ranging between 0.2 and 1.6 μmol CH₄ g_{dw}⁻¹ h⁻¹ (Table 2). Annual mean MOR for the respective layer showed only slight differences between the surface and the intermediate layer (0.8 and 0.9 μmol CH₄ g_{dw}⁻¹ h⁻¹, respectively),

whereas in the deepest layer, rates decreased by almost 50% to $0.5 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ h}^{-1}$.

Corresponding to MOR, cell numbers of methane oxidising bacteria (MOB) in Großer Binnensee showed no substantial differences between the sediment layers. Annual mean cell numbers for surface, intermediate and deepest layer of 1.6, 2.1 and 1.4×10^5 cells $\text{g}_{\text{dw}}^{-1}$ were detected, respectively. Only small seasonal changes with slightly increased cell numbers at the end of the summer period (August 2003, 2004), and a second maximum in the surface and the intermediate layer in February 2004 and in the deepest layer in April 2004 occurred (Table 2).

In Holzsee, MOR in the surface and intermediate layer increased from 2.8 ± 0.3 and 2.9 ± 0.2 in December 2003 to 5.6 ± 0.3 and 4.5 ± 0.1 April 2004, respectively, but no clear annual trend was detected for the deepest layer (Table 2). In this lake, a similar depth pattern was found for MOR as for MPR, with highest mean rates detected throughout the year in the surface layer ($4.2 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ h}^{-1}$), gradually decreasing to 3.7 and $2.7 \mu\text{mol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ h}^{-1}$ in the intermediate and the deepest layer, respectively. Significant differences ($P < 0.05$) between the two lakes for all three depth layers were found during the whole year. Methane oxidation rates (MOR) in Großer Binnensee were 3 to 4 times lower than in Holzsee (Table 2).

In contrast to MOR, cell numbers of methane oxidising bacteria (MOB) in Holzsee showed no clear seasonal trend. Additionally, only small differences between the three sediment layers were detected (Table 2). Nevertheless, annual means of cell counts for MOB in Holzsee gradually decreased from the top to the bottom layer, reflecting the depth pattern detected for MOR. In the surface layer in Holzsee, almost four times more MOB cells ($6.3 \times 10^5 \text{ g}_{\text{dw}}^{-1}$) were found than in Großer Binnensee. Differences in MOB cell numbers between the two lakes in each depth were significant for all sampling time points ($P < 0.05$).

Table II-2: Comparison of methane production rates (MPR), methane oxidation rates (MOR) and cell numbers of methane oxidising bacteria (MOB) over an annual cycle in sediment cores of Großer Binnensee and Holzsee. MPR, MOR ($n = 3$) and MOB ($n = 8$) are given as means \pm standard deviation. **Bold and underlined** marks maximum, **bold** minimum values per depth.

Date	Depth (cm)	Großer Binnensee			Holzsee		
		MPR [nmol CH ₄ g _{dw} ⁻¹ h ⁻¹]	MOR [μmol CH ₄ g _{dw} ⁻¹ h ⁻¹]	MOB [10 ⁵ g _{dw} ⁻¹]	MPR [nmol CH ₄ g _{dw} ⁻¹ h ⁻¹]	MOR [μmol CH ₄ g _{dw} ⁻¹ h ⁻¹]	MOB [10 ⁵ g _{dw} ⁻¹]
08/2003	0-6	1.7 ± 0.4	1.1 ± 0.01	1.4 ± 0.3	39.7 ± 4.2	4.4 ± 0.44	7.8 ± 2.1
	6-12	7.8 ± 0.4	1.6 ± 0.05	3.5 ± 0.8	18.9 ± 0.3	4.5 ± 0.45	16.9 ± 3.4 (max)
	12-20	3.5 ± 4.9	0.9 ± 0.03	3.1 ± 0.5	9.0 ± 2.1	4.2 ± 0.04	4.8 ± 0.7
10/2003	0-6	5.2 ± 1.1	0.9 ± 0.05	1.2 ± 0.2	27.0 ± 1.9	3.0 ± 0.32	8.3 ± 2.5
	6-12	9.0 ± 1.2	0.8 ± 0.04	2.5 ± 0.4	16.5 ± 1.6	2.8 ± 0.59	5.1 ± 1.1
	12-20	1.1 ± 0.3	0.3 ± 0.01	0.7 ± 0.1	5.9 ± 0.6	1.9 ± 0.10	3.0 ± 0.5
12/r2003	0-6	5.0 ± 0.4	0.7 ± 0.02	0.7 ± 0.2	17.1 ± 6.2	2.8 ± 0.31	3.3 ± 0.5
	6-12	5.3 ± 0.3	0.6 ± 0.02	0.6 ± 0.3	20.7 ± 0.8	2.9 ± 0.18	5.0 ± 1.0
	12-20	1.6 ± 0.2	0.3 ± 0.03	0.6 ± 0.2	12.0 ± 2.3	2.6 ± 0.29	0.7 ± 0.3
02/2004	0-6	5.9 ± 0.1	0.5 ± 0.04	3.1 ± 0.9	11.6 ± 0.6	4.4 ± 0.18	7.6 ± 1.3
	6-12	15.8 ± 9.9 (max)	1.2 ± 0.04	2.2 ± 0.5	13.4 ± 0.6	3.6 ± 0.15	6.0 ± 1.1
	12-20	8.3 ± 1.1	0.9 ± 0.02	1.1 ± 0.4	1.9 ± 0.5	1.2 ± 0.13	3.1 ± 0.6
04/2004	0-6	8.6 ± 0.5	0.8 ± 0.16	0.6 ± 0.2	29.0 ± 2.6	5.6 ± 0.33	5.8 ± 1.6
	6-12	3.1 ± 1.1	0.4 ± 0.03	1.2 ± 0.2	16.6 ± 2.6	4.5 ± 0.12	3.0 ± 1.2
	12-20	5.2 ± 0.7	0.2 ± 0.05	2.1 ± 0.2	10.4 ± 0.9	3.7 ± 0.10	6.4 ± 1.1
06/2004	0-6	2.7 ± 0.3	0.6 ± 0.03	1.4 ± 0.4	33.7 ± 0.9	4.6 ± 0.08	3.7 ± 0.6
	6-12	8.3 ± 0.9	0.8 ± 0.01	2.0 ± 0.5	12.6 ± 1.8	3.3 ± 0.19	2.3 ± 1.1
	12-20	0.9 ± 0.8	0.3 ± 0.02	0.8 ± 0.2	4.1 ± 0.6	1.8 ± 0.09	2.8 ± 0.6
08/2004	0-6	6.3 ± 1.8	1.1 ± 0.10	3.6 ± 0.6	18.2 ± 1.1	4.9 ± 0.07	7.3 ± 1.3
	6-12	6.7 ± 0.8	1.0 ± 0.07	2.5 ± 0.3	14.4 ± 0.6	4.2 ± 0.64	1.1 ± 0.5
	12-20	5.7 ± 5.7	0.5 ± 0.01	1.3 ± 0.5	6.3 ± 0.7	4.0 ± 0.04	5.6 ± 1.1
Variation from 08/03 to 08/04	0-6	1.7 – 8.6	0.5 – 1.1	0.6 – 3.6	11.6 – 39.7	2.8 – 5.6	3.3 – 8.3
	6-12	3.1 – 9.0 (15.8)	0.4 – 1.6	0.6 – 3.5	12.6 – 20.7	2.8 – 4.5	1.1 – 6.0 (16.9)
	12-20	0.9 – 8.3	0.2 – 0.9	0.6 – 3.1	1.9 – 12.0	1.2 – 4.2	0.7 – 6.4

DISCUSSION

Seasonal patterns of methane turnover compared to larval $\delta^{13}\text{C}$ signatures

In the polymictic lake Großer Binnensee, only small seasonal changes in chironomid larval $\delta^{13}\text{C}$ values were detected, as was expected from lake characteristics and the study by Grey et al. (2004b). In parallel, only small changes in methane turnover rates were observed during the entire year in this lake, indicating a correlation between methane turnover activities and the larval diet. However, the annual mean larval $\delta^{13}\text{C}$ values were with -31‰ not markedly negative, indicating only a limited importance of methane derived carbon for the larval diet.

Indeed, MPR in Großer Binnensee were 3 to 4 times lower than in the dimictic lake Holzsee (Table 2) and in another dimictic eutrophic lake, Lake Dagow (Casper

1996, Glissmann et al. 2004). These low MPR and the limited seasonality of this process coincided with an oxygenated sediment-water interface throughout the year (Fig. 1A), inhibiting this strictly anaerobic process. Additionally, chironomid larvae were detected in high densities in this lake (Eller et al. 2005a), leading to oxygen penetration into deeper sediment layers due to bioturbation (Granéli 1979, Jørgensen & Revsbech 1985, Frenzel 1990), consequently inhibiting methane production even in these otherwise anoxic regions. Furthermore, the relatively high sulphate concentrations detected in Großer Binnensee (up to 0.5 mmol l⁻¹, data not shown) could lead to an inhibition of methanogens due to substrate competition with sulphate reducing bacteria (Chidthaisong & Conrad 2000). The limited availability of methane, in turn, probably led to the low MOR and MOB cell numbers detected (Table 2), even though the oxygenated sediment-water interface represented preferable conditions for this process. Thus, even though Großer Binnensee is a eutrophic lake, the microbial methane turnover rates were relatively low and seasonally constant.

Theoretically, the larval $\delta^{13}\text{C}$ signatures detected in Großer Binnensee could also be reached without methane derived carbon as part of the larval diet, but by the inclusion of biomass of chemoautotrophic bacteria (Freeman et al. 1990, Hollander & Smith 2001, Kohzu et al. 2004) as discussed in detail in Grey and Deines (2005). These bacteria might be enriched in parallel to MOB in the special microbial habitat associated to the oxic / anoxic interfaces in the tubes build by the larvae (Fig. 4, Kajan & Frenzel 1999, Grey & Deines 2005 and references therein).

Nevertheless, compared to the sediment $\delta^{13}\text{C}$ values of -18‰, larval signatures were by 13‰ more depleted. The seasonal variation in methane $\delta^{13}\text{C}$ signatures (Fig. 1C) was not reflected in the larval signatures. Thus, the theoretical importance of methane derived carbon for larval nutrition as calculated with a two-source isotope mixing model (Phillips & Gregg 2001) reached annual mean values of $20.6 \pm 2.2\%$ (Fig. 2).

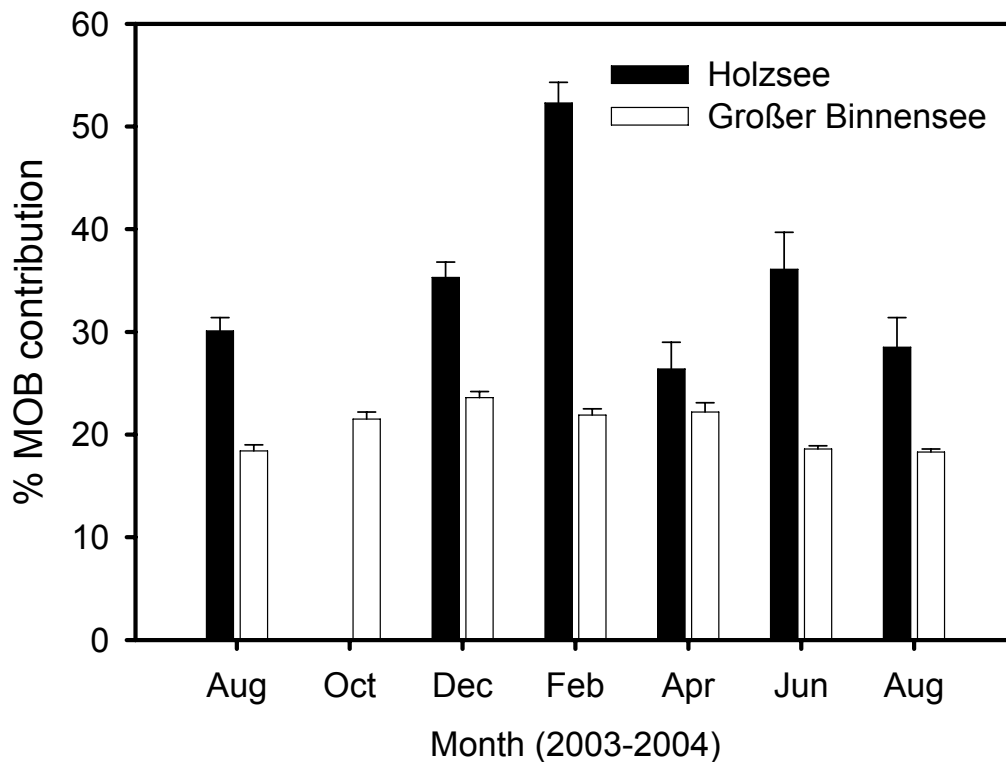


Figure II-2: Estimated percent contribution of methane oxidising bacteria (MOB) to *Chironomus plumosus* larval biomass carbon in Holzsee and Großer Binnensee. Values were calculated using a two-source isotope mixing model (Phillips & Gregg 2001), assuming sediment and methane oxidising bacteria as only carbon sources for the larvae (mean \pm SE).

In Holzsee, strong seasonal variation of larval $\delta^{13}\text{C}$ signatures occurred, with the most negative values of -62‰ detected in February 2004 (Fig. 1D), indicating a high contribution of methane derived carbon to the larval diet at this time of the year. Nevertheless, the seasonal changes for MPR detected in the surface sediment (0-6 cm) showed a reverse trend to the larval $\delta^{13}\text{C}$ signatures, with lowest MPR in February and highest in June 2004 (Table 2). At the latter time point, the larvae had their most positive $\delta^{13}\text{C}$ signatures (Fig. 1D). Thus, changes in MPR were not correlated to the larval $\delta^{13}\text{C}$ signatures.

The decrease of MPR in Holzsee in autumn could be explained by the re-oxygenation of the sediment-water interface, due to the autumn circulation of the water body (Fig. 1B), inhibiting methane production. In April 2004, anoxic conditions in the water above the sediment were re-established in Holzsee (Fig. 1B). Presumably, at this time point high substrate concentrations in the sediment following a spring phytoplankton bloom (Stähr et al. 2002) allowed high MPR. Thus, the

decrease in larval $\delta^{13}\text{C}$ signatures in Holzsee during the winter coincided with an increasing lack of phytoplankton in the water column and their increase in spring coincided with the phytoplankton spring bloom, respectively. This suggested a high proportion of algal biomass in the larval diet in early spring, and shows a seasonally varying importance of methane-derived carbon. Nevertheless, over the year larval $\delta^{13}\text{C}$ signatures were on average by 23.2‰ more negative than the sediment carbon, indicating that methane derived carbon in the diet of chironomids in Holzsee was not only important after the autumn overturn of the water column (Grey et al. 2004b), but also during periods when algal biomass was available.

In contrast to MPR, MOR in the upper two sediment layers of Holzsee (0-12 cm) increased steadily from December 2003 to April 2004 (Table 2), showing a seasonal trend fitting to an increased importance of methane derived carbon for the larval diet in the winter. Additionally, most negative $\delta^{13}\text{C}$ signatures of produced methane were detected in December 2003 and February 2004 in the surface sediment (Fig. 1D), in parallel to high MOB cell numbers (Table 2). This co-occurrence might have led to a high availability of MOB biomass with a very negative $\delta^{13}\text{C}$ signature. The comparison of the seasonal patterns of MPR, MOR and larval $\delta^{13}\text{C}$ signatures in Holzsee strongly indicates that the larvae predominantly feed on MOB biomass in the surface sediment. Therefore, based on this first comprehensive data set covering an entire year, we conclude that methane oxidation activity and the biomass of MOB are the factors primarily determining the importance of methane-derived carbon for the larval diet. This finally confirms the assumptions based on carbon isotope signatures alone by other authors (Bunn & Boon 1993, Grey et al. 2004a,b, Kelly et al. 2004). Furthermore, this also fits to the observations that higher numbers of *Eubacteria* (including MOB) than *Archaea* (including methanogens) are associated to chironomid larvae (Eller et al. submitted).

The higher methane turnover rates detected throughout the year in Holzsee compared to Großer Binnensee confirmed for these two lakes the hypothesis of Kelly et al. (2004) that the microbial methane cycle is of higher importance for the overall carbon cycle in dimictic than in polymictic lakes. This was also reflected in the differences of the annual mean $\delta^{13}\text{C}$ signatures between the two lakes, with the sediment differing by 9‰, methane by 12‰ and larval biomass by 19‰, indicating a

higher contribution of methane derived carbon to the larval diet in the dimictic lake Holzsee.

Correlation of methane turnover and larval $\delta^{13}\text{C}$ signatures at single sampling time points

In addition to the overall seasonal patterns, methane turnover rates and larval $\delta^{13}\text{C}$ signatures were compared for each single sampling time point and for each lake. For this purpose, mean values of MPR and MOR for the first two sediment layers (0-12 cm) were used. The analysis of all MPR and MOR and the corresponding larval $\delta^{13}\text{C}$ signatures at all single time points and from both lakes revealed, that chironomid larval $\delta^{13}\text{C}$ signatures were significantly and negatively correlated with MPR ($P < 0.01$) and MOR ($P < 0.001$), with the more negative larval $\delta^{13}\text{C}$ signatures corresponding to higher MPR and MOR (Fig. 3). The data sets from the two lakes were clearly separated, with higher methane turnover rates and more negative larval $\delta^{13}\text{C}$ signatures occurring in the dimictic lake Holzsee (Fig. 3). On average, chironomid larvae in (the dimictic lake) Holzsee were 1.5 to 2 times more reliant on methanotrophic biomass than larvae in (the polymictic lake) Großer Binnensee (Fig. 2).

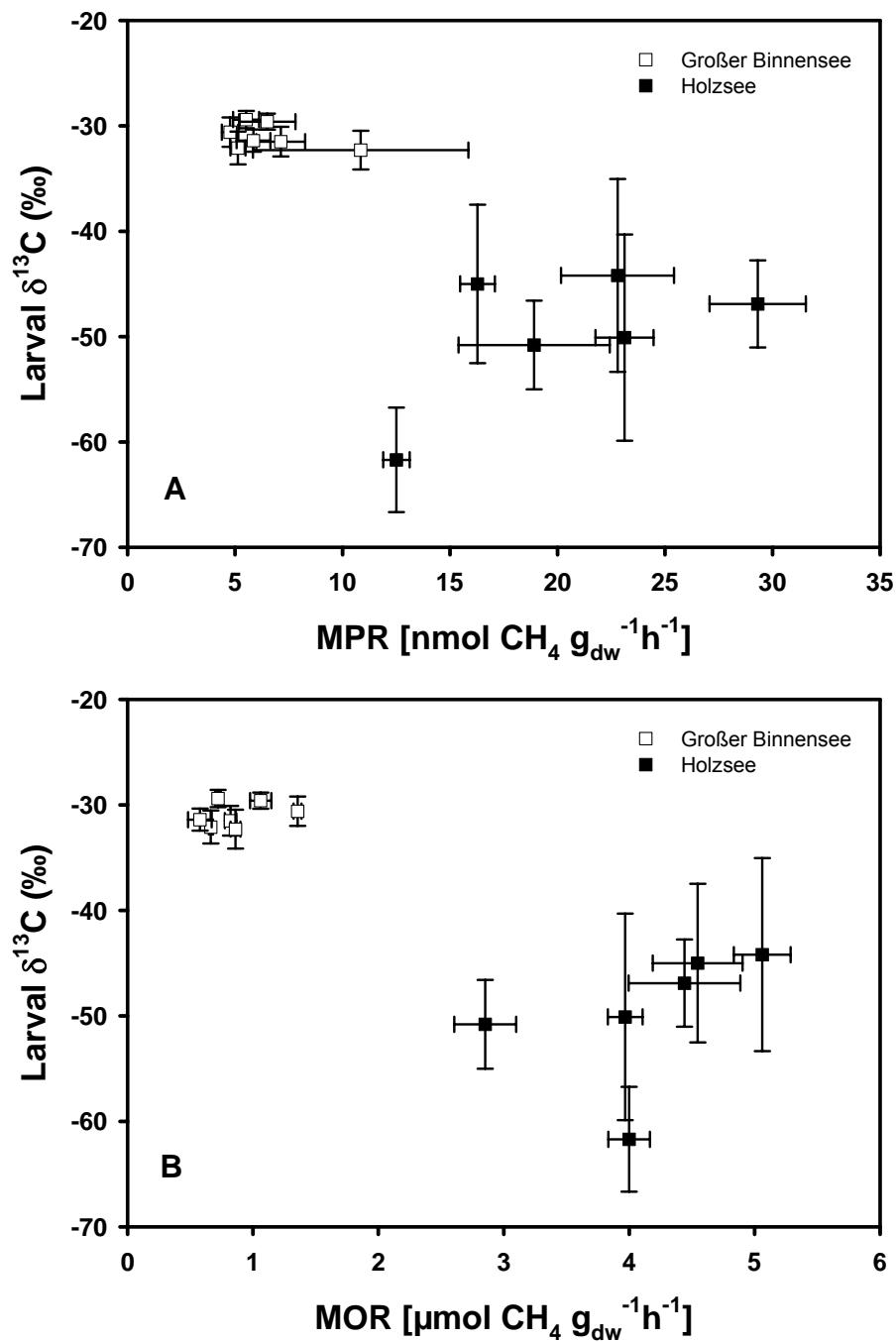


Figure II-3: Correlation between (A) methane production rates (MPR) and (B) methane oxidation rates (MOR) of the upper two sediment layers (0-12 cm) with $\delta^{13}\text{C}$ values of chironomid larval biomass. Regression line for (A) $y = -0.89x - 28.08$, $r^2 = 0.49$ and (B) $y = -4.94x - 27.91$, $r^2 = 0.68$.

However, even though seasonal changes in MOR and larval $\delta^{13}\text{C}$ signatures seemed to be correlated (Fig. 1 and Table 2), the data pairs for single sampling time points in Holzsee showed no negative correlation between MPR or MOR and larval $\delta^{13}\text{C}$ signatures (Fig. 3). This seems to contradict the hypothesis that higher methane

turnover rates lead to more negative larval $\delta^{13}\text{C}$ signatures. Nevertheless, the in situ situation in the lake sediment differs extremely from the sampling situation analysed here. In situ, the larvae live in their ventilated tubes and promote the growth of a specialized microbial community in this microenvironment as mentioned above, including most probably an enrichment of MOB (Fig. 4, Kajan & Frenzel 1999). However, the sediment samples taken for analysis of MPR and MOR did not exactly represent these tubes and thus most likely did not reflect the methane turnover rates present in close vicinity of the larvae. Additionally, it is not known how long it takes for the larval $\delta^{13}\text{C}$ signatures to reflect changes in the $\delta^{13}\text{C}$ signature of their food. Thus, methane turnover rates of one sampling time point might not correlate to larval $\delta^{13}\text{C}$ signatures of the same sampling time point, but to larval signatures of e.g. one week later. Moreover, the high inter-individual variability in larval $\delta^{13}\text{C}$ signatures detected in Holzsee indicated, that the larvae fed on carbon sources markedly different in their $\delta^{13}\text{C}$ signatures, like the strongly ^{13}C depleted MOB biomass compared to less depleted algal biomass or sediment (Grey et al. 2004b). The strong heterogeneity in larval carbon sources was not reflected in the bulk sediment samples, and thus no correlation of methane turnover rates and larval $\delta^{13}\text{C}$ signatures at single time points was found in Holzsee.

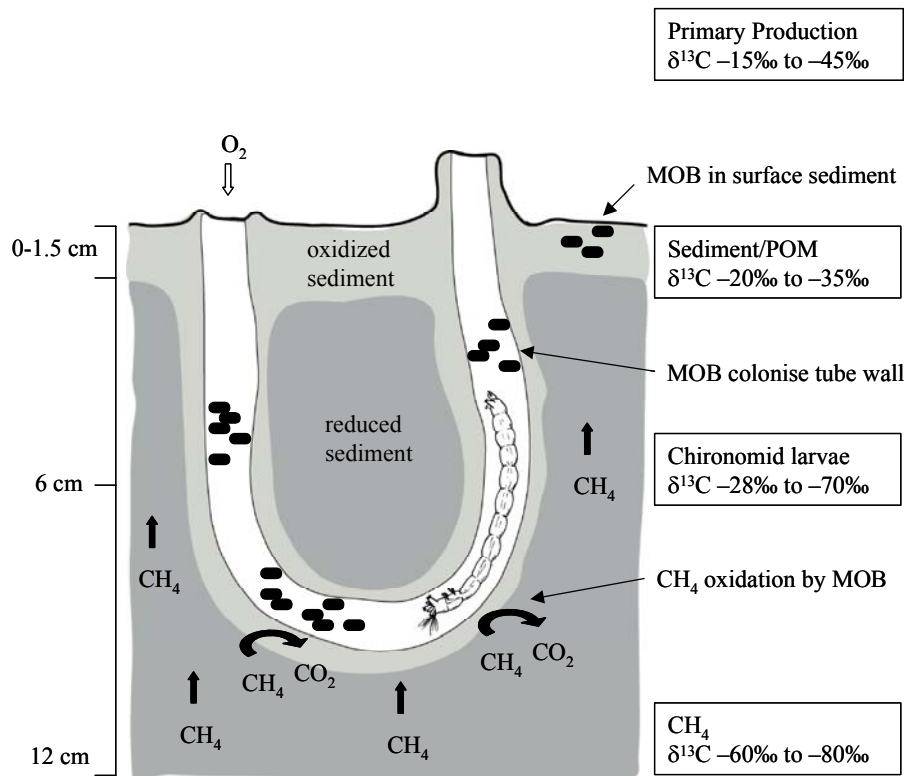


Figure II-4: Cross-section through a *Chironomus plumosus* tube. The chironomid is thought to maintain an interface of oxic-anoxic conditions in the tube by pumping water through and thus creating a microhabitat for methane oxidising bacteria (MOB). In boxes typical $\delta^{13}\text{C}$ values found in our study are given and possible carbon pathways are indicated by arrows.

Conclusions

The comparison of chironomid larval $\delta^{13}\text{C}$ signatures and methane turnover activities in a polymictic and a stratifying lake revealed that seasonal changes in both parameters occurred only in the stratifying lake. A detailed investigation of the methane cycle indicated, that besides the overall importance of the methane cycle in the sediment, the importance of methane-derived carbon for the larval diet was primarily determined by methane oxidation activity and the availability of biomass of MOB. Nevertheless, at single sampling time points direct correlations of larval signatures and methane turnover rates were not found. This could be due to the sampling technique that did not fully explore the larval tubes and their specialized microbial community; and/or due to a time delay until larval $\delta^{13}\text{C}$ signatures reflect isotopic changes in their food (i.e. not in equilibrium) or in their relative importance. Therefore, further studies should focus on the microenvironments of the tubes and the time needed for chironomid larvae to incorporate the $\delta^{13}\text{C}$ signature of their food.

chapter III



Site-specific methane production and subsequent midge mediation within Esthwaite Water, UK

ABSTRACT

Previous analyses of larval chironomid $\delta^{13}\text{C}$ have suggested that methanotrophic contribution to biomass is site-specific within lakes. We determined larval biomass, larval and methane gas stable carbon isotopes, and potential methane production from the sediments underlying different water column depths in Esthwaite Water, UK. Methane production increased whereas larval $\delta^{13}\text{C}$ values typically became lighter with increasing lake depth. Reduced methane production at 15 m depth and correspondingly less ^{13}C -depleted larvae in the second year of study suggests that the rate of methane production influenced larval assimilation. Larval distribution and other site-specific parameters, combined with two-source mixing models, were used to estimate potential methane-mediation via the abundant chironomid biomass to higher predators.

INTRODUCTION

The widespread occurrence of biogenic methane and substantial biomass of methane oxidising bacteria (MOB, methanotrophs) in freshwater lake sediments (Hanson & Hanson 1996, Segers 1998) could potentially represent a carbon source contributing significantly to benthic freshwater food webs. In marine environments, stable isotope analyses have revealed a considerable contribution of methane-derived carbon to diverse macroinvertebrates in methane seep communities (Levin & Michener 2002). However, until recently, few studies have considered the role of methane as an important source of carbon in freshwater food webs.

The application of stable isotope analysis has led to the hypothesis that methanotrophic biomass is an important dietary component of larval chironomids, frequently the dominant macroinvertebrate group in lake sediments (Bunn & Boon 1993, Kiyashko et al. 2001, Grey 2002, Grey et al. 2004a,b, Kelly et al. 2004). Biogenic methane is ¹³C-depleted (Coplen et al. 2002) compared with the organic matter from which it is produced (Whiticar et al. 1986). As a result, the methanotrophic bacteria that exploit this methane provide an extremely ¹³C-depleted food source (Summons et al. 1994). The extent to which methane-derived carbon is used by chironomids appears to vary with microhabitat conditions (Kiyashko et al. 2001), seasonality (Grey et al. 2004b), chironomid species (Grey et al. 2004a,b), and lake characteristics (Kelly et al. 2004). Grey et al. (2004b) sampled three lakes at two different depths within each lake and found a consistent pattern for chironomid larval $\delta^{13}\text{C}$; chironomids collected from the deeper site were significantly more ¹³C-depleted. Hershey et al. (2005) made a similar observation in arctic lakes and found that offshore chironomids were generally more depleted in ¹³C compared to nearshore chironomids. They speculated that differences in chironomid $\delta^{13}\text{C}$ could be attributed to different conditions within the lake sediment affecting the proportion of methanotrophic bacteria in their diet. However, neither study attempted to collect concurrent data on methane cycling to lend further support to their hypotheses. The one study that has directly compared methane cycling to larval $\delta^{13}\text{C}$, but was limited to one sampling event in two contrasting lakes, found that larvae exhibiting greater ¹³C-depletion were present in the lake with higher methane cycle activity (Eller et al. 2005a).

Methane production in sediments of temperate stratifying lakes tends to peak in late summer by which time anoxia have developed at the sediment water interface and in the overlying water column (Casper et al. 2000). Thus, the deeper parts of the lake will be subjected to a relatively longer period of anoxia resulting in greater methane production compared to the shallower zones, which might ultimately influence chironomid $\delta^{13}\text{C}$. Therefore, the primary objective of the current study was to determine rates of methane production from different water-column depths within a stratifying lake and assess site-specific contributions of biogenic methane to larval biomass. Individual larvae of two closely related chironomid species (*Chironomus anthracinus* and *C. plumosus*) were collected and their $\delta^{13}\text{C}$ values analysed. We additionally determined nitrogen stable isotope ratios, because methanotrophic and chemoautotrophic bacteria often exhibit highly depleted $\delta^{15}\text{N}$ values probably due to the large isotope fractionation during the assimilation of ammonium and nitrate (Hoch et al. 1992, Lee & Childress 1994). Thus, analyses of both stable carbon and nitrogen isotopes are appropriate to assess the contribution of biogenic methane to chironomid larval biomass. We chose to work on Esthwaite Water, UK because of the wealth of background data available and samples were collected in two consecutive years to check for inter-annual variability. In addition, we aimed to estimate the amount of methane-derived carbon potentially available for mediation from the sediments as chironomid biomass immediately prior to larval pupation and emergence.

MATERIALS AND METHODS

Study site

Esthwaite Water, UK (54° 21' N, 2° 59' W) is a highly eutrophic lake (98 ha surface area, 15 m Z_{max} , 1.4 % Z_r), which develops deep-water anoxic conditions soon after the onset of thermal stratification. The lake contains abundant sympatric populations of *Chironomus anthracinus* and *C. plumosus* exhibiting stable carbon isotopic depletion associated with assimilation of methanotrophic biomass (Kelly et al. 2004). Samples were collected on 7 May 2004 and 5 May 2005. Sediment cores and chironomid larvae were collected from three different depth zones in Esthwaite

Water: 6, 10 and 15 m. These depths were selected because the sediment-water interface is rarely subjected to anoxia at 6 m, but endures ~50 and ~130 days of anoxia at 10 and 15 m respectively (Grey et al. 2004a). Vertical profiles of temperature and dissolved oxygen in each water column were measured using a Yellow Springs Instruments probe immediately before taking samples.

Methane incubations

For potential methane production rates (MPR), three sediment cores were collected from each water column depth in 2004 using a Jenkin sediment core-sampler. After taking the cores, the core-tubes were sealed with rubber stoppers and transported directly to the laboratory. Cores were sliced into three sections from the sediment surface: 0-5, 5-10, 10-15 cm. The three depth slices from the three different cores were combined under a flow of N₂ and sediments were converted to slurries by adding autoclaved tap water (1:1, w/w). Three replicates of 50 ml slurry from each section were transferred into sterile serum bottles (total volume 120 ml), closed with butyl rubber stoppers and flushed with N₂ for 30 min to remove residual oxygen. Sediment samples were incubated at 8°C in the dark without agitation. Gas samples from the headspace of the bottles were taken repeatedly (every two to three days) with a pressure lock syringe after shortly shaking the bottles by hand to establish equilibrium between gas phase and slurry. Methane was analysed with a gas chromatograph (GC, HP 5890 series II; Hewlett-Packard, Avondale, PA, USA) equipped with an HP Plot Q capillary column (Agilent Technologies, Omnilab, Germany) and a flame ionisation detector. Potential methane production rates were calculated by linear regression of the methane increase with incubation time and expressed in nmol CH₄ g_{dw}⁻¹h⁻¹ of sediment. The gas produced from these incubations was retained and analysed by gas chromatograph-combustion isotope ratio mass spectrometry to provide stable carbon isotope ratios for the methane. In 2005, cores were collected as in the previous year. However, in the laboratory, a single section representing 0-15 cm sediment depth was removed. The three sections from each water column depth were not pooled, thus providing three replicate sediment samples to assess site heterogeneity. Procedures for determination of MPR were subsequently identical to the previous year.

Chironomid larval biomass

Larval abundance and biomass was estimated by collecting 15 Ekman grabs from each of the three water column depths. Individual grabs were sieved (2 mm mesh) *in situ* and abundances were calculated per m² for each water column depth. Biomass was calculated using elemental composition data derived from the stable isotope analyses.

Stable isotope analyses of chironomid larvae and sediment

Fourth instar chironomid larvae from the abundance estimate grabs (see above) were picked manually and placed into a container with lake water for transport to the laboratory. Larvae were then placed into filtered water to allow gut clearance for 24 h (Feuchtmayr & Grey 2003). Excess faecal material was removed periodically to prevent coprophagy. Individual larvae were then placed into separate wells of cell culture plates, oven-dried at 60°C for 24 h and stored in a dessicator. Lake sediment samples were collected from each depth sampled, acidified to remove the inorganic fraction (cf. Kelly et al. 2004), oven dried at 60°C, homogenised and stored in a dessicator.

For carbon and nitrogen stable isotope analyses, samples were weighed (0.5-0.7 mg for chironomids; 1.5-2.0 mg for sediment) into tin-cups prior to continuous flow isotope ratio mass spectrometry. Stable isotope ratios are given using the δ notation expressed in units per mil as follows: δ (‰) = [(R_{sample}/R_{standard})-1] x 1000, and R = ¹³C/¹²C or ¹⁵N/¹⁴N. The reference materials used were secondary standards of known relation to the international standards of Vienna Pee Dee belemnite for carbon and atmospheric N₂ for nitrogen. Typical precision for a single analysis was ± 0.1 ‰ for $\delta^{13}\text{C}$ and ± 0.3 ‰ for $\delta^{15}\text{N}$.

Modelling and statistical analyses

To estimate the potential methane mediation via chironomid biomass to higher predators, larval distributions and site-specific parameters from Esthwaite Water

were combined with two-source mixing models (Phillips & Gregg 2001). MOB $\delta^{13}\text{C}$ values (derived from site-specific methane $\delta^{13}\text{C}$ with an assumed -16‰ fractionation factor applied, Summons et al. 1994) and sediment $\delta^{13}\text{C}$ were used as end members, while site-specific and species-specific larval $\delta^{13}\text{C}$ values were substituted as the mixture into the mixing model. Individual one-way analysis of variance (ANOVA) was used to test methane production rates (MPR) and larval $\delta^{13}\text{C}$ for significant differences between depth and years. Tukey tests provided post-hoc comparison of means. All statistical tests were performed using STATISTICA, Version 6.0 (StatSoft).

RESULTS

CH₄ production

In incubations from 6 and 10 m methane began to accumulate after an adaptation phase, whereas at 15 m methane production started from the beginning of the incubation, indicating that methanogens utilized easily degradable compounds (Fig. 1). Potential methane production rates (MPR) were determined from the three different sections (0-5, 5-10 and 10-15 cm) from each water column depth in Esthwaite Water in 2004. MPR was typically highest at the sediment surface and decreased with sediment depth, and the same pattern was found in sediments from 6, 10 and 15 m (data not shown). Although we measured potential MPR in all three sections, we calculated mean values representative of 0-15 cm as a more appropriate comparison with larval $\delta^{13}\text{C}$, because chironomid tubes were found to penetrate throughout the top 15 cm sediment depth. Thus in 2005, we did not subsection the cores.

There was an increase in MPR with increasing water column depth in Esthwaite Water in both 2004 and 2005 (Table 1). In 2004, MPR increased from 0.2 to 31.5 nmol CH₄ g_{dw}⁻¹h⁻¹; differences between the three water column depths were significant ($P < 0.001$). However, in 2005 the pattern was similar at 6 and 10 m but mean MPR at 15 m was only 12.4 nmol CH₄ g_{dw}⁻¹h⁻¹, less than 40% of that during the previous year. Again, there was significant difference in MPR between the three depths ($P < 0.001$).

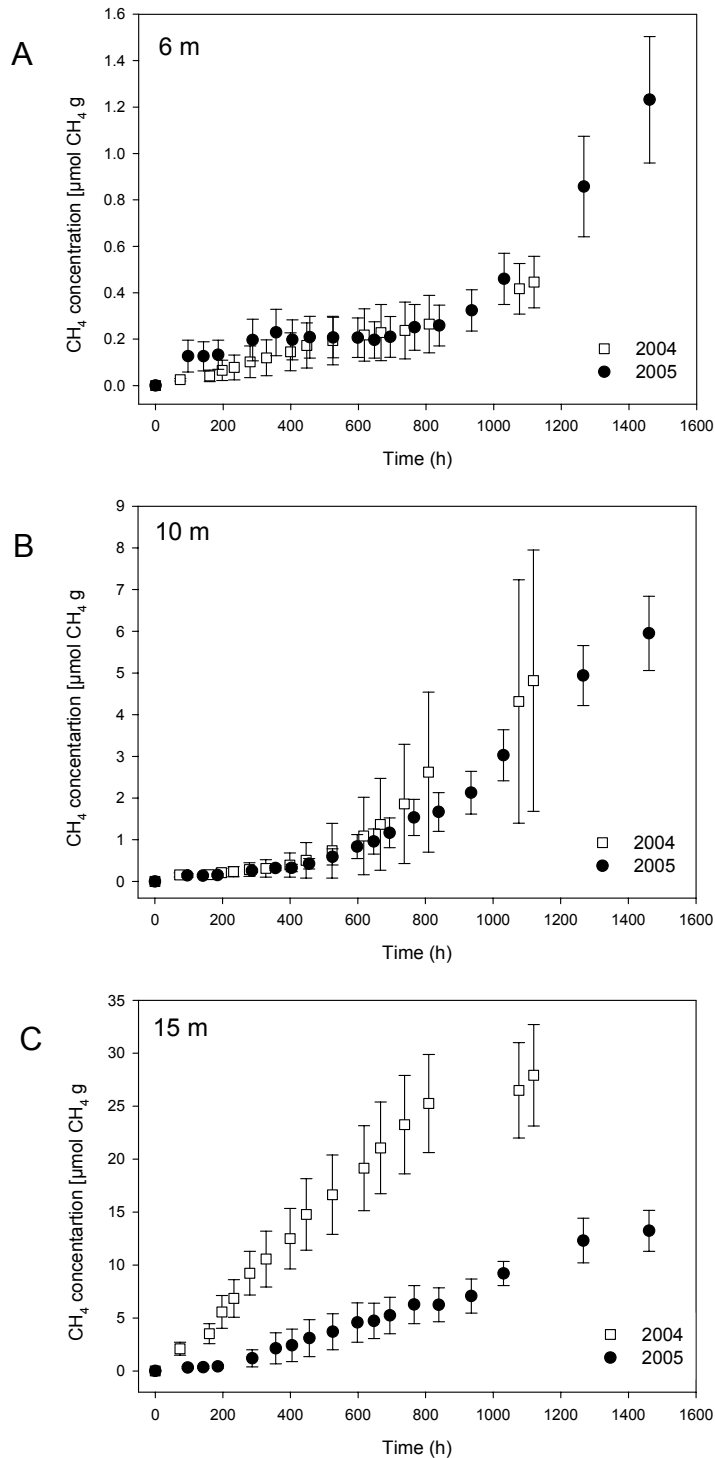


Figure III-1: CH₄ production from sediment collected at (A) 6 m, (B) 10 m and (C) 15 m water column depth, Esthwaite Water in 2004 and 2005 (mean ± SD). Potential methane production rates were calculated by linear regression of CH₄ increase with incubation time. Linear regressions - (A) 2004: $y = 0.0025x - 0.226$ ($r^2 = 0.99$, $P < 0.0001$), 2005: $y = 0.0015x - 1.025$ ($r^2 = 0.95$, $P < 0.001$); (B) 2004: $y = 0.0073x - 3.469$ ($r^2 = 0.99$, $P < 0.0001$), 2005: $y = 0.0066x - 3.67$ ($r^2 = 0.98$, $P < 0.0001$); (C) 2004: $y = 0.0292x + 1.163$ ($r^2 = 0.99$, $P < 0.0001$), 2005: $y = 0.0106x - 1.969$ ($r^2 = 0.98$, $P < 0.0001$). Note the different scales on the y-axes.

Stable isotope values

Sediment samples collected from the lake can be considered isotopically identical ($\delta^{13}\text{C} \sim -29\text{‰}$), both among sites and years (Table 1). Methane collected from laboratory incubations of sediment samples in 2004 exhibited $\delta^{13}\text{C}$ values spanning from -65.0 to -75.0‰ and became more ^{13}C -depleted with increasing water column depth (Table 1).

In 2004, both *Chironomus anthracinus* and *C. plumosus* exhibited increasing stable carbon isotopic depletion with increasing water column depth. There was a 15.6‰ difference in mean *C. anthracinus* $\delta^{13}\text{C}$ between 6 and 15 m sites, whereas the difference was even greater (21.0‰) for *C. plumosus* (Table 1, Fig. 2). Differences in larval $\delta^{13}\text{C}$ between the three depths were significant for both species ($P < 0.05$) with the exception of *C. plumosus* collected in 2004 from 10 and 15 m ($P > 0.1$). In 2005, fourth instar *C. anthracinus* were only collected from 10 and 15 m depth and larval $\delta^{13}\text{C}$ values did not differ significantly between the two sites ($P > 0.1$). There was no significant difference between years for values recorded from 10 m for either species ($P > 0.1$), nor for *C. plumosus* values recorded from 6 m ($P > 0.05$). *Chironomus plumosus* $\delta^{13}\text{C}$ declined with increasing depth from 6 to 10 m in 2005 in a similar pattern to that found in 2004. However, at the 15 m site, mean larval $\delta^{13}\text{C}$ was $-46.1 \pm 6.1\text{‰}$ and therefore relatively ^{13}C -enriched compared to the 10 m site. Differences between the depths were all significant ($P < 0.05$). Thus, in 2004 there was a clear relationship between larval $\delta^{13}\text{C}$ and MPR (and corresponding water column depth) for both chironomid species ($P < 0.0001$, Fig. 2A) but with reduced MPR at 15 m in 2005, the pattern was lost (Fig. 2B).

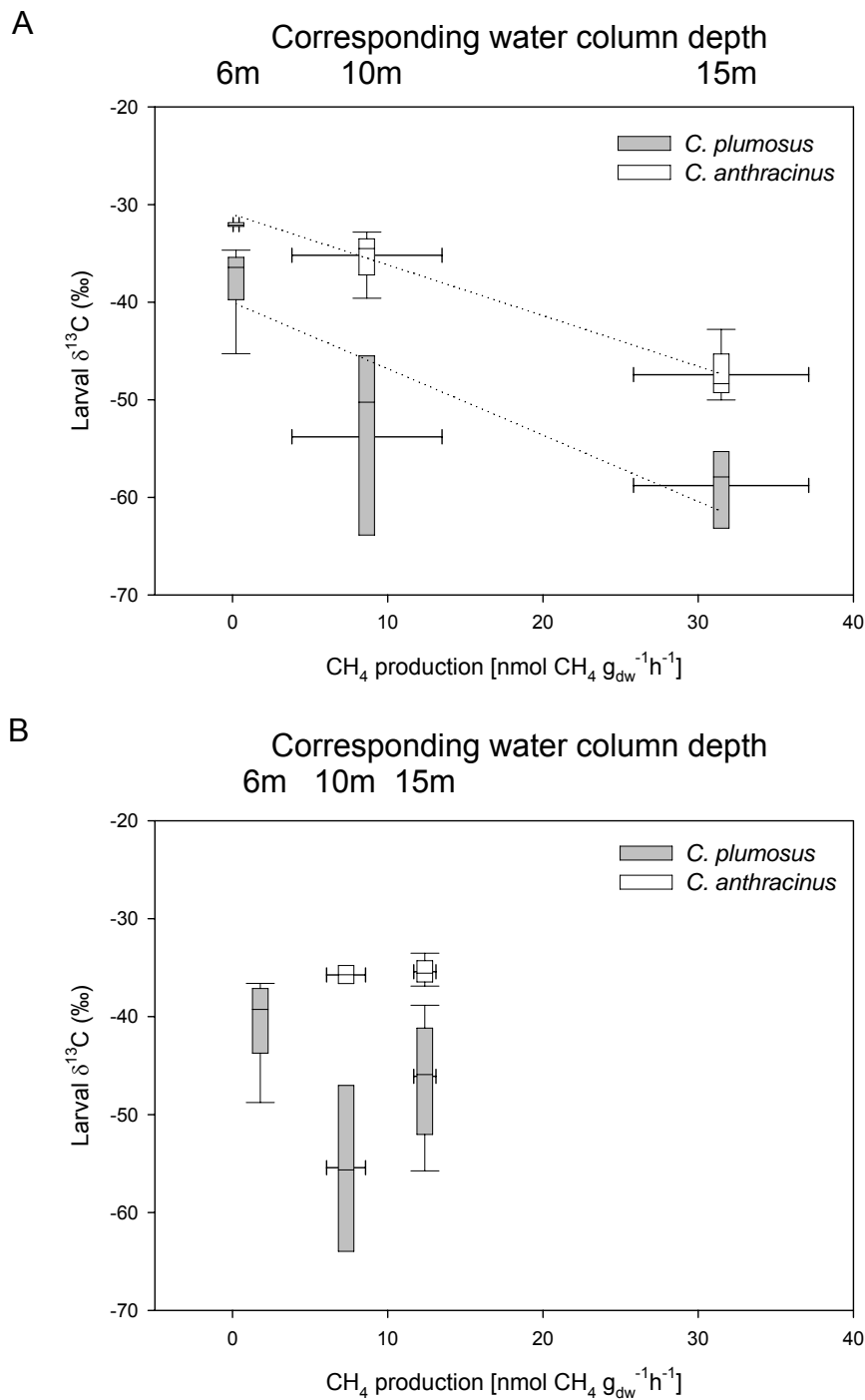


Figure III-2: Larval $\delta^{13}\text{C}$ values (shown as box plots) and site-specific potential methane production rates (means \pm SD) collected from 6, 10 and 15 m depth, Esthwaite Water in (A) 2004; and (B) 2005. The edges of the box plot correspond to the quartiles; the line in the box to the median; and the whiskers to the range. Significant relationships were found in 2004. Linear regressions (dotted lines) - *Chironomus anthracinus*: $y = -0.52x - 31.0$ ($r^2 = 0.89$, $P < 0.0001$); *C. plumosus*: $y = -0.68x - 40.03$ ($r^2 = 0.59$, $P < 0.0001$).

Table III-1: Temperature and oxygen saturation measures immediately above the water-sediment interface, potential methane production rates from sediment slurries (MPR), and stable isotope values of larvae (Ca – *Chironomus anthracinus*; Cp – *C. plumosus*), sediment and CH₄ from 6, 10 and 15 m depth, Esthwaite Water in 2004 and 2005.

Date	Temp (°C)	O ₂ % Sat	MPR		Larval δ ¹³ C (‰)		Larval δ ¹⁵ N (‰)		Sediment δ ¹³ C (‰)	CH ₄ δ ¹³ C (‰)
			(nmol CH ₄ g _{dw} ⁻¹ h ⁻¹)	(μmol CH ₄ m ⁻² d ⁻¹)	Ca	Cp	Ca	Cp		
2004										
6m	10.1	90	0.2 ± 0.2	7.2 ± 7.2	-32.1 ± 0.2	-37.8 ± 3.6	9.8 ± 1.0	8.5 ± 1.1	-29.3	-65.0
10m	9.2	75	8.7 ± 4.8	285.6 ± 158.4	-35.2 ± 2.5	-53.8 ± 9.4	9.5 ± 0.5	2.9 ± 3.3	-29.4	-69.3
15m	8.6	38	31.5 ± 5.6	1022.4 ± 182.4	-47.4 ± 2.6	-58.8 ± 4.3	-0.4 ± 1.7	-1.4 ± 1.5	-29.6	-75.0
2005										
6m	9.6	92	1.8 ± 0.4	62.4 ± 14.4		-40.8 ± 4.3		6.5 ± 2.2	-29.2	
10m	8.7	82	7.3 ± 1.3	249.6 ± 43.2	-35.8 ± 1.4	-55.4 ± 10.9	8.2 ± 0.3	-0.1 ± 4.9	-29.4	
15m	8.1	49	12.4 ± 0.7	415.2 ± 24.0	-35.4 ± 1.2	-46.1 ± 6.1	5.3 ± 0.7	2.0 ± 2.7	-29.5	

Individuals of both chironomid species collected from Esthwaite Water on the same date but from 6, 10 and 15 m depth revealed intraspecific variability in δ¹³C and δ¹⁵N; the degree of isotopic depletion and range of δ¹³C and δ¹⁵N values was typically greater for *C. plumosus* than for *C. anthracinus* (Table 1, Fig. 3). Although *C. anthracinus* larvae exhibited gradual ¹³C-depletion and increased intraspecific variability with increasing water column depth, there was a marked depletion in larval δ¹⁵N (9.9‰) and greater intraspecific variability between 10 and 15 m depths in 2004 (Fig. 3A). In contrast, in 2005, larval δ¹³C did not differ between 10 and 15 m and although there was again some differentiation in δ¹⁵N with associated increased intraspecific variability; the difference between mean larval δ¹⁵N from the two depths was smaller (3.0‰). Inter-individual isotopic variability of *C. plumosus* larvae was most marked at 10 m (2004: 9.4‰; 2005: 10.9‰, Table 1) and was generally greater at each depth in 2005 compared to 2004 (Fig. 3B). Larvae collected from 6 and 10 m exhibited the same relationship between δ¹³C and δ¹⁵N values in both years although mean δ¹⁵N values were lower in 2005 (6 m: 2.0‰; 10 m: 3.7‰). The most marked contrast between 2004 and 2005 data was again found in larvae collected from 15 m; individual *C. plumosus* still exhibited considerable isotopic variability, and although more ¹⁵N-depleted relative to corresponding δ¹³C values in a similar manner to *C. anthracinus* from the same depth, mean δ¹⁵N was 3.4‰ higher compared to 2004 and the range overlapped with both 10 and 6 m.

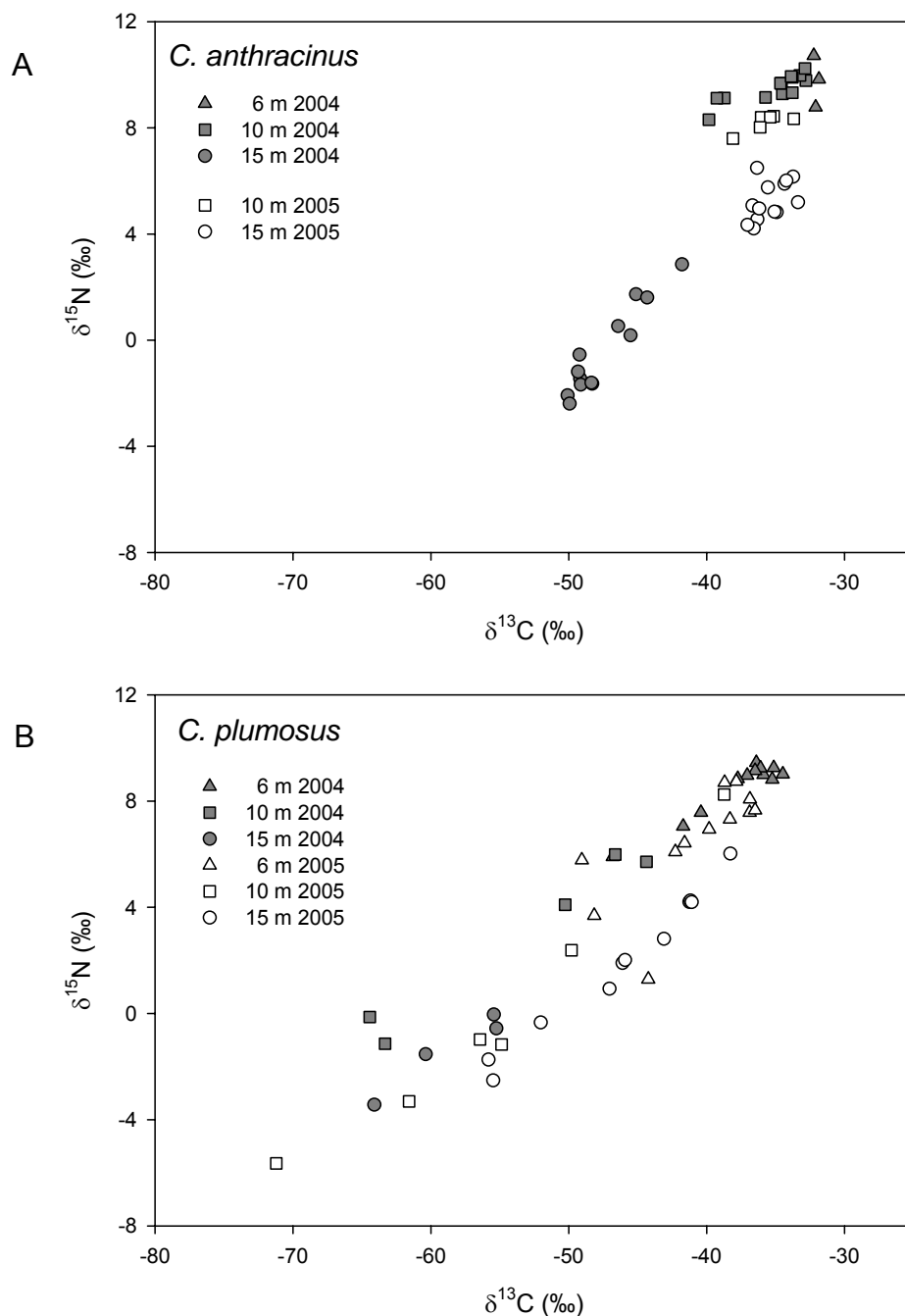


Figure III-3: Relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values plotted for individual (A) *Chironomus anthracinus* and (B) *C. plumosus* from Esthwaite Water in 2004 and 2005. Individuals were collected either from 6, 10 or 15 m depth.

The mixing model estimated that 5 to 29% of *C. anthracinus*, and 16 to 48% of *C. plumosus* biomass was assimilated from methane-derived sources, and that these proportions increased with increasing water column depth (Table 2). The smaller *C. anthracinus* was more abundant at greater depths in Esthwaite, whereas the larger *C. plumosus* became increasingly less so. We calculated the sediment surface area

represented by the three depths from which we sampled (Ramsbottom 1976) and used larval density and dry weights to estimate total larval biomass derived from methane carbon for each depth zone and species; larval density was most influential, resulting in ~89% of *C. anthracinus* methane-derived biomass being found in the 12-15 m zone, whereas ~66% of *C. plumosus* methane-derived biomass was found in the 2-8 m zone. The model estimated that ~280 kg of methane-derived carbon (21.5%) was present as *C. anthracinus* and *C. plumosus* larval biomass in May 2004.

Table III-2: Modelled CH₄-contributions to larval biomass from different depth zones (represented by the three depths: 6, 10 and 15 m) of Esthwaite Water, 2004. Ca – *Chironomus anthracinus*; Cp – *C. plumosus*. Methane oxidising bacteria (MOB) $\delta^{13}\text{C}$ calculated from gas data with an assumed fractionation of -16‰ (Summons et al. 1994). Percentage of larval biomass derived from CH₄ calculated from mixing models using sediment (Sed) and MOB as end-members (Phillips & Gregg 2001).

Depth zone	Lake area	Mean density (m ⁻²)		Mean dry wt (mg)		$\delta^{13}\text{C}$ values used in mixing model (‰)			Larval % CH ₄		Biomass derived from CH ₄ (kg of carbon)	
		Ca	Cp	Ca	Cp	CH ₄	MOB	Sed	Ca	Cp	Ca	Cp
2-8 (6)	34.9	53	777	1.8	6.6	-65.0	-81.0	-29.3	5.4	16.4	0.8	132.1
8-12 (10)	28.7	393	178	1.6	5.6	-69.3	-85.3	-29.4	10.4	43.6	8.4	56.1
12-15 (15)	12.2	2410	57	1.9	7.4	-75.0	-91.0	-29.6	29.0	47.6	72.9	11.0

DISCUSSION

Potential methane production and chironomid $\delta^{13}\text{C}$

For the purposes of the discussion, we largely focus on the $\delta^{13}\text{C}$ values in relation to methane production. Previous studies have shown that depleted macroinvertebrate $\delta^{13}\text{C}$ values were clearly correlated with depleted $\delta^{15}\text{N}$ values (Kiyashko et al. 2001, Grey et al. 2004a, Jones & Grey 2004, Kohzu et al. 2004). Therefore also highly depleted $\delta^{15}\text{N}$ values suggest direct feeding of macroinvertebrates upon ¹⁵N-depleted methanotrophic bacteria as discussed in detail in Grey et al. (2004a) and Kohzu et al. (2004).

We found that methane production increased whereas larval $\delta^{13}\text{C}$ values typically became more depleted with increasing water column depth in 2004. Although the pattern in 2005 appeared similar at 6 and 10 m depth, MPR at 15 m was considerably reduced and there was a corresponding lack of larval isotopic

depletion. This suggests that the isotopic depletion of chironomids is linked to the MPR and not the lake depth *per se*. Thus, our data from 2004 tend to support previous descriptions of larval isotopic depletion with increasing lake depth (e.g. Grey et al. 2004a, Hershey et al. 2005), but further suggest that this phenomenon is actually linked to the site-specific MPR and that it is the quantity of microbial methane turnover influencing larval $\delta^{13}\text{C}$, which previously has only been shown for two different lakes (Eller et al. 2005a). Bulk sediment $\delta^{13}\text{C}$ did not differ significantly between depths and thus appears to have very little influence on larval $\delta^{13}\text{C}$.

The question that needs to be addressed is why MPR at 15 m was so different between years when the rates recorded from 6 and 10 m were comparable, and when we would normally expect MPR to increase with increasing depth in eutrophic lakes (Casper 1996). We cannot rule out sediment heterogeneity as a potential factor, but we combined sediments from three completely separate cores to use in the incubations and the standard deviations from the mean were small, so we assume substrate patchiness as rather unlikely. Potential MPR were only slightly higher at 15 m compared to 10 m in 2005. Studies have shown that methanogenic activity is influenced strongly by the temperature in the sediment (Wagner & Pfeiffer 1997, Van der Nat et al. 1998, Glissmann et al. 2004) and that lake sediments have a tremendous capacity to increase their methanogenic decomposition activities upon increasing temperature (Zeikus & Winfrey 1976, Kelly & Chynoweth 1981, Thebrath et al. 1993, Glissmann et al. 2004). Temperature may not only affect the rate but also the pathway by which organic matter is degraded to CH_4 . There are two major pathways in methane formation; one uses acetate and the second uses H_2/CO_2 as a substrate (Winfrey & Ward 1983, Capone & Kiene 1988, Conrad 1999). Several studies have demonstrated similar temperature-controlled shifts in methanogenic community metabolism (Chin & Conrad 1995, Schulz & Conrad 1996, Chin et al. 1999, Glissmann et al. 2004). These studies showed that the pathway of organic matter degradation changed with increase of temperature from domination by homoacetogenesis and acetotrophic methanogenesis to an increasing contribution of other fermentation pathways and H_2 -dependent methanogenesis. Decreasing temperature resulted in an inhibition of H_2 -dependent methanogenesis and enhanced the contribution of acetate to methanogenesis. Both methane production and methane consumption are controlled by temperature, and additionally by oxygen and

CH₄ (Hanson & Hanson 1996, Börjesson et al. 2004). Temperature profiles from the water column above the sediment showed lower values relative to date in 2005, compared to 2004, and oxygen saturation was elevated; indeed, from water column profile data, it appears that conditions in the hypolimnion in 2005 were lagging behind those in 2004 by ~2 weeks (Centre for Ecology & Hydrology routine monitoring data licensed to Grey). Wagner and Pfeiffer (1997) showed that there was a temperature optimum at 10°C in methane production in freshwater marsh soil, which was 3.4 times higher than at 5°C. Thus although the temperature difference between the two years was small, we suggest that probably it was sufficient to cause the lack of methane production at 15 m in 2005 and the consequently reduced incorporation of methane-derived carbon by chironomid larvae.

The reduction of MPR at 15 m in 2005 appeared to have a disproportionately large influence on *C. anthracinus* isotope values; there was no significant difference in larval $\delta^{13}\text{C}$ and a marked reduction in the difference in larval $\delta^{15}\text{N}$ between 10 and 15 m. Previously, it was proposed that the consistent interspecific differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Grey et al. 2004a) and $\delta^{34}\text{S}$ (Grey & Deines 2005) exhibited by *C. anthracinus* compared to *C. plumosus* (especially with regard to a lack of 'extreme' $\delta^{13}\text{C} < -50\text{‰}$) indicated less reliance of *C. anthracinus* upon MOB and perhaps a heavier reliance on other chemoautotrophic bacteria. The current data would suggest that MOB still play an important role in the nutrition of *C. anthracinus*, and interestingly, further support for the supposition by Grey et al. (2004a), that MOB are also responsible for low larval $\delta^{15}\text{N}$ values by recycling excreted ammonium (Lee & Childress (1994) showed that methanotrophic bacteria oxidise ammonium and exhibit light $\delta^{15}\text{N}$ values).

Inter-individual variability in *C. plumosus* $\delta^{13}\text{C}$ (here represented by the standard deviations) from the three different depths was marked. Our data show that the greatest variability was measured in both years at 10 m (9.4‰ in 2004 and 10.9‰ in 2005). This variability is caused by some individuals exhibiting isotopic signatures consistent with dietary assimilation from phytoplankton, and others exhibiting ^{13}C -depleted signatures associated with incorporation of methanotrophic biomass. We suppose that this transition in reliance upon different food sources is also associated with a change in feeding mode related to water column depth and probably more specifically, oxygen concentration. It has been shown by Hodkinson

and Williams (1980) that *C. plumosus* exhibits great flexibility in its feeding mode. To assimilate settling phytoplankton the larvae must be filter-feeding, as classically described for *C. plumosus*; but to assimilate MOB, larvae must switch to deposit-feeding. Filter-feeding is more energetically costly relative to deposit-feeding and thus more likely to occur in shallower waters with an ample supply of oxygen. Our stable carbon isotope data support this hypothesis; that *C. plumosus* is filter-feeding in the shallower waters and deposit-feeding in the deepest zone. At 10 m it appears that chironomids filter- or deposit-feed, or switch between both feeding modes, accounting for the large inter-individual differences found in larval $\delta^{13}\text{C}$. Bastviken et al. (2003) demonstrated that even a low percentage of an isotopically light food source in the diet from methane-derived carbon can have a marked effect on the consumer signature. The greater range in our carbon isotope data collected in 2005 indicated that some individual *C. plumosus* at 15 m were less reliant on MOB biomass relative to the previous year (corresponding to the reduced MPR), and to a greater extent on phytoplankton biomass. Since oxygen depletion at 15 m in 2005 was not as marked as in 2004, potentially larvae could spend a longer time filter-feeding.

Inter-individual variability in larval *C. anthracinus* $\delta^{13}\text{C}$ did increase with depth in 2004 but standard deviations never exceeded 2.5‰ (only one fourth of the variability found for *C. plumosus*). *C. anthracinus* larvae are deposit-feeders on the sediments surrounding their tube mouths and have not been reported to switch feeding modes (Jónasson 2003). Thus the lower isotopic variability suggests that all larvae are feeding in a similar manner at all three depths.

Modelling

We acknowledge the assumptions and limitations associated with two-source mixing models, especially as our data from 2004 were collected only on a single sampling event. However, we chose May as the sampling event specifically to determine the isotopic signature of the larvae immediately prior to their departure from the sediments and their migration as pupae through the water column to emerge as imagos on the water surface (Grey et al. 2004b). Effectively then, changes to the isotopic signature of the chironomids during the pupal and flight period are

physiological due to the use of lipid and glycogen storage products because feeding ceases prior to pupation. Isotopic shifts between larval and imago stages are relatively small (<1‰) compared to those induced by a diet switch (Grey, pers.comm.). Thus, the isotopic signature at this time period should reflect the proportion of methane-derived carbon that potentially can be mediated from the sediments via the chironomids, and hence used as a tracer. Because of their high densities, chironomids form an important constituent of the diet of benthic (Fox 1989), pelagic (Winfield et al. 1992) and terrestrial predators (Collier et al. 2002). We estimate that in Esthwaite Water sediments below 2 m water column depth, the total biomass for the two *Chironomus* species was ~1305 kg, of which 21.5 % was methane-derived. The potential for mediation of this carbon into benthic, pelagic, or terrestrial predators clearly varies with chironomid species, timing of emergence (emergence of *C. anthracinus* usually occurs prior to *C. plumosus* in Esthwaite, Grey et al. 2004b), and origin within the lake (Table 2). The majority of methane-derived carbon was found in *C. plumosus* biomass in the shallower waters simply reflecting the density and size of larvae throughout this depth zone. However, the second largest contribution comprised profundal *C. anthracinus*, despite the relatively small area of sediment at this depth. In stratifying lakes such as Esthwaite Water, we assume that predation pressure within the benthos reduces with increasing depth because of decreasing temperatures, and more importantly, oxygen concentrations preventing permanent foraging by fish (Headrick & Carline 1993, Wanink et al. 2001). In the shallower regions of the lake, which occupy a greater proportion of the lake sediment area, oxygen conditions are higher at the sediment-water interface for the majority of the year and predation pressure in the benthos will be relatively higher. We speculate then, that a greater proportion of the methane-derived carbon from the profundal is exported from the sediments and will become available to pelagic and terrestrial predators, relative to that from the littoral, which may be preyed upon by benthic invertebrate or fish predators.

chapter IV



Differential assimilation of methanotrophic and chemoautotrophic bacteria by lake chironomid larvae

ABSTRACT

Bacteria play an important role in the nutrition of many lake-dwelling detritivorous macroinvertebrates, yet few studies have investigated the roles of differing groups of bacteria in freshwater systems. Recent stable carbon isotope analyses have revealed that methanotrophic bacteria help fuel lake food webs. We analysed individual larvae of co-existing *Chironomus plumosus* and *C. anthracinus* for stable sulphur isotopes as an alternative tracer for bacterial assimilation, and compared them with existing stable carbon isotope data. The combination of these two isotopes suggests there are large inter- and intraspecific differences in the incorporation of bacteria rather than algae in the diet of both species. *Chironomus anthracinus* appears to assimilate a greater proportion of chemoautotrophs relative to its congener, which is consistent with classical descriptions of *C. anthracinus* feeding mode relative to bacterial stratigraphy in the sediments. The higher intraspecific isotope variability of *C. plumosus* indicates variable proportions of methanotrophs, chemoautotrophs and phytoplankton to the diets of individuals.

INTRODUCTION

Chironomid larvae often dominate the littoral and profundal benthos of lakes. As such, their ecology, and in particular, their trophic role within food webs has been intensively studied (see Berg 1995 and refs therein). The food most commonly identified from gut content analyses of chironomids is detritus, typically constituting 50-90% (Johnson 1987). However, the nutritional value of detritus is questionable because much is refractory, and detritivorous macroinvertebrates typically lack the required enzymatic capacity to breakdown cellulose and other complex plant polysaccharides. Thus, the associated microbial flora is likely of greater nutritional importance, either directly as a food source, or via mineralisation of the detritus into more readily digestible forms (Johannsson & Beaver 1983). In fact, *Chironomus plumosus* and *C. riparius* are both capable of completing larval development on diets consisting solely of bacteria (Rodina 1971, Baker & Bradnam 1976).

The importance of bacteria in chironomid diets differs both between lakes of differing trophy, and seasonally within lakes. Goedkoop & Johnson (1992) found that in more productive lakes, bacterial carbon accounted for only 2% of total carbon demand, whereas this proportion increased to around 47% in oligotrophic systems. Seasonality and inter-annual variability of plankton primary productivity makes such a basal resource rather unpredictable for benthic invertebrates (Johnson et al. 1989), whereas sediment bacterial biomass is relatively constant (Boström & Törnblom 1990, Brunberg & Boström 1992). Thus, *C. plumosus* switched from a detrital dominated diet in February to a more algal biased diet, associated with the spring bloom, in Lake Erken (Johnson 1987). Conventional techniques such as gut content analyses reveal little information regarding the types of bacteria constituting the diet and, although specific fatty acid or amino acid biomarkers can be used to differentiate between bacterial groups (e.g. Boon et al. 1996), a chironomid dietary study that incorporated fatty acid analyses only differentiated between bacterial and algal components (Goedkoop et al. 1998). Molecular methods could provide information on the different phylotypes of the microbial community in the gut, but the effort and costs involved can be prohibitive. In comparison, rapid stable isotope analysis may provide additional insight into chironomid feeding behaviour.

Stable isotope analyses have recently indicated that species commonly assumed to be filter feeders of algal production in productive shallow lakes (e.g. *Chironomus plumosus*) might be heavily reliant upon methanotrophic bacterial biomass (Grey et al. 2004a, Kelly et al. 2004). Biogenic methane exhibits a markedly ^{13}C -depleted isotopic signature which is distinct from the majority of other basal resources in freshwater systems (-35 to -20‰; del Giorgio & France, 1996), and thus, can be used with confidence as a natural tracer. Chironomids, and zooplankton, which exhibited $\delta^{13}\text{C} < -40\text{‰}$ have been assumed to have had their isotopic signatures lowered by a contribution from methane, mediated by methane oxidising bacteria either in the plankton or in the sediment (Bastviken et al. 2003; Kelly et al. 2004).

However, rather than assimilating carbon partly from a methanotrophic source and partly from an algal or detrital source, as assumed in the mixing models used by Kelly et al. (2004), chironomids and zooplankton could exhibit isotopic signatures between -50 and -40‰ via consumption of chemoautotrophs that have assimilated respired carbon, as outlined by Kohzu et al. (2004). Briefly, this results either from the oxidation of methane (Hollander & Smith 2001), or indeed, in high respiration dominated systems, where $\delta^{13}\text{C}$ values of dissolved inorganic carbon may approach -20‰, then further fractionation by chemoautotrophs such as sulphur oxidising bacteria results in bacterial $\delta^{13}\text{C}$ between -50 and -40‰ (e.g. Ruby et al. 1987). A further alternative is that lipid synthesis by chemoautotrophs under anoxic conditions can produce an isotopically light carbon pool (c.f. Teece et al. 1999, Londry et al. 2004). These two alternatives are not mutually exclusive.

We hypothesised that analyses of sulphur stable isotopes could provide further insight into linkages between sediment microbial flora and macroinvertebrate consumers in freshwater lakes, as in marine systems (e.g. Brooks et al. 1987, Cary et al. 1989). Incorporation of elemental sulphur into chemoautotrophs results in lower $\delta^{34}\text{S}$ values, because ^{34}S -depleted sulphides are the likely source (Fry 1986). Also, sulphur is an excellent source indicator because trophic fractionation is negligible (Peterson & Fry 1987). In previous isotopic studies of chironomid larvae, *Chironomus plumosus* typically had lower $\delta^{13}\text{C}$ than *C. anthracinus*, and was assumed to be relatively more trophically reliant upon methanotrophic biomass. *Chironomus*

anthracinus more often exhibited $\delta^{13}\text{C}$ values within the -50 to -40‰ range (e.g. Grey et al. 2004a). Allowing for a displacement of ~10‰ because of differing carbon sources between fresh and marine waters (Peterson & Fry 1987), this corresponds to the $\delta^{13}\text{C}$ range (-42 to -30‰) reported by Brooks et al. (1987) to represent use of sulphur-based energy sources, as opposed to more ^{13}C -depleted methane-based sources (<-40‰) in marine systems. Consequently, we expected *C. anthracinus* to exhibit lower $\delta^{34}\text{S}$ relative to its congener. We analysed the remaining material (when sufficient) used for the intraspecific stable carbon and nitrogen isotope study of Grey et al. (2004a), to provide direct comparison between $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ data.

MATERIALS AND METHODS

In June 2002, sediment and associated chironomid larvae were collected by Ekman grab from 15m depth in Esthwaite Water (54° 22'N, 2° 59'W), and 3m depth in Wyresdale Park Lake (53° 56'N, 2° 44'W). These two small (<100 ha), productive, meromictic lakes have been described by Kelly et al. (2004). Surficial sediment samples (50ml) were removed directly from the grab; the remainder was sieved (2mm mesh) *in situ*, and 4th instar chironomid larvae placed into containers with lake water. In the laboratory, larvae were placed into filtered water and left over night for gut clearance (*cf* Feuchtmayr & Grey 2003). Faecal material was removed periodically to prevent coprophagy. Individual larvae were placed into separate wells of cell culture plates and dried at 60°C for 24 h and stored in a dessicator. Further details of larval and sediment preparation procedures are outlined in Grey et al. (2004a) and Kelly et al. (2004). Prior to analysis, elemental sulphur content of sediment samples (0.55 to 0.83%) was determined to calculate an appropriate sample weight. Sufficient sediment was weighed to yield approximately 40 µg of elemental sulphur. Whenever larval size allowed, 2-5 mg of chironomid tissue was weighed into tin cups. Sulphur sample preparation involved adding ~2x the sample weight of vanadium pentoxide catalyst to promote combustion. Stable sulphur isotopes were analysed by continuous flow isotope ratio mass spectrometry (ANCA-GSL elemental analyser coupled to a Geo20-20 isotope ratio mass spectrometer, Europa Scientific Ltd, Crewe, UK) at Iso-Analytical, UK. Stable isotope ratios are expressed using the δ notation as per mil difference from the standard:

$$\delta^{34}\text{S} = \left(\frac{{}^{34}\text{S}/{}^{32}\text{S}_{\text{sample}}}{{}^{34}\text{S}/{}^{32}\text{S}_{\text{standard}}} - 1 \right) \times 1000$$

The reference material against which all samples were measured was barium sulphate (NBS-127, $\delta^{34}\text{S}_{\text{V-CDT}} = +20.3\text{‰}$) distributed by the IAEA, Vienna. NBS-127, IAEA-S-1 (silver sulphide, $\delta^{34}\text{S}_{\text{V-CDT}} = -0.3\text{‰}$) and Iso-Analytical OP-7 (barium sulphate, $\delta^{34}\text{S}_{\text{V-CDT}} = +11.0\text{‰}$) were used for calibration and correction.

Stable isotope data were checked for normality using Lilliefors tests. The slope and intercept of $\delta^{13}\text{C} / \delta^{34}\text{S}$ relationships for each species in each lake were checked using least-squares linear regression. Since all but one of the latter relationships were non-significant, it was not possible to draw comparisons using ANCOVA, so we compared $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ between species and lakes using individual ANOVAs. All statistical analyses were conducted using SYSTAT v.8 (SPSS 1998).

RESULTS

Bulk sediment $\delta^{13}\text{C}$ values exhibited little variability between replicates within lakes, or between lakes (Table 1). Wyresdale sediments were slightly ^{34}S -enriched ($\sim 1\text{‰}$) relative to those from Esthwaite, again with low variability between replicates (max: 1.3‰ , Esthwaite). Chironomid larvae of both species were consistently ^{13}C -depleted relative to bulk sediments in both lakes (Fig. 1); *Chironomus plumosus* tended to be more ^{13}C -depleted compared to its congener but only significantly so in Esthwaite ($F_{1,24} = 9.17$, $P=0.006$). Chironomid $\delta^{34}\text{S}$ ranged over 6‰ in both lakes, although species-specific ranges were typically smaller. There was overlap between *C. plumosus* $\delta^{34}\text{S}$ values and those of sediment in Wyresdale, both with maximum recorded $\delta^{34}\text{S}$ of 1.4‰ . The remaining chironomid individuals were all ^{34}S -depleted relative to the corresponding sediment (by up to 8‰ in Esthwaite). *Chironomus anthracinus* was typically ^{34}S -depleted compared to its congener: mean *C. plumosus* $\delta^{34}\text{S}$ (Table 1) was significantly heavier than that of *C. anthracinus* in both Wyresdale ($F_{1,29} = 37.51$, $P<0.001$) and Esthwaite ($F_{1,24} = 38.18$, $P<0.001$).

Interspecific isotope variability was marked, and exhibited a similar pattern in both lakes (Fig. 1). There were no significant differences in mean $\delta^{13}\text{C}$ for either species between lakes, or in mean $\delta^{34}\text{S}$ of *C. anthracinus* between lakes. Only *C. plumosus* $\delta^{34}\text{S}$ varied significantly between lakes ($F_{1,38} = 18.18$, $P<0.001$).

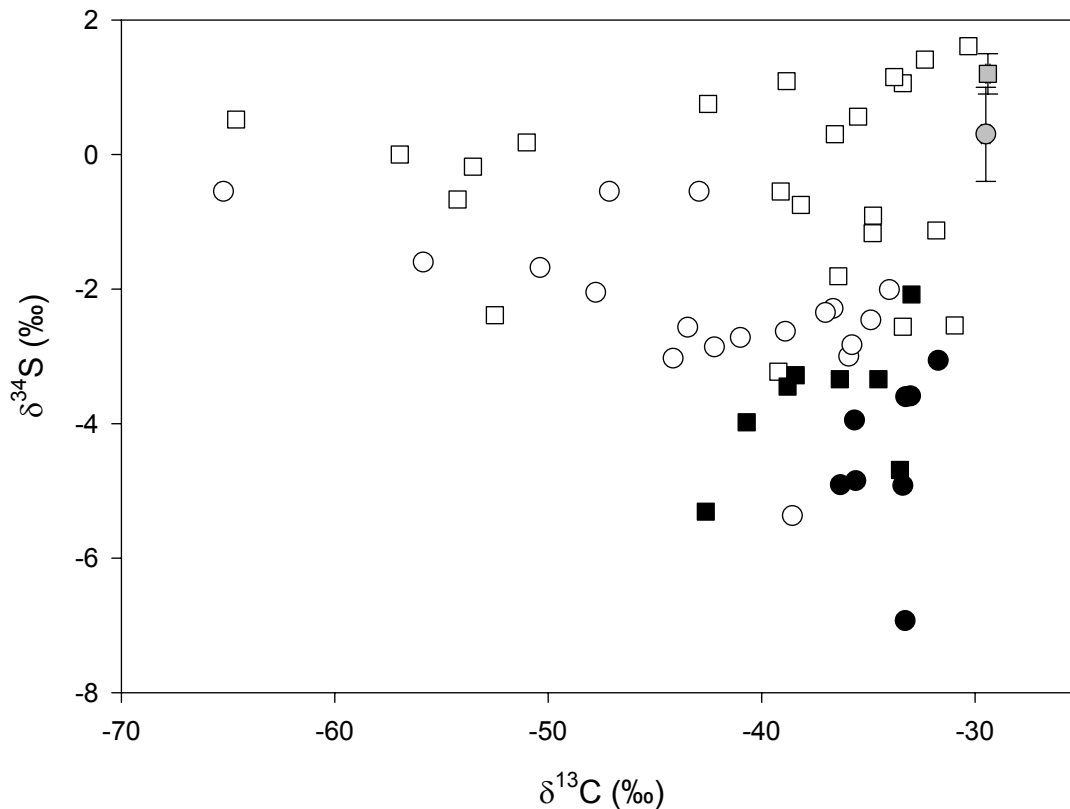


Figure IV-1: Carbon and sulphur isotope signatures of chironomid larvae (each symbol corresponds to one individual larva) and bulk sediments (mean \pm 1 SD) collected from two UK lakes. Squares – Wyresdale Park Lake; circles – Esthwaite Water; grey – sediment; white – *Chironomus plumosus*; black – *C. anthracinus*.

Table IV-1: Summary of stable carbon and sulphur isotope data for two congeneric tubicolous chironomid species (*Chironomus plumosus* & *C. anthracinus*) and bulk sediment from Esthwaite Water and Wyresdale Park Lake, UK.

Species/sediment	n	$\delta^{13}\text{C}$ (‰)			$\delta^{34}\text{S}$ (‰)		
		mean \pm SD	max	min	mean \pm SD	max	min
Esthwaite Water							
<i>Chironomus plumosus</i>	17	-41.4 \pm 7.7	-33.2	-65.2	-2.1 \pm 0.9	-0.6	-3.0
<i>Chironomus anthracinus</i>	9	-34.6 \pm 2.0	-31.7	-38.6	-4.6 \pm 1.2	-3.1	-6.9
Sediment	6	-29.5 \pm 0.2	-29.0	-29.7	0.3 \pm 0.7	1.1	-0.2
Wyresdale Park Lake							
<i>Chironomus plumosus</i>	23	-40.7 \pm 9.5	-30.3	-64.6	-0.5 \pm 1.4	1.4	-3.2
<i>Chironomus anthracinus</i>	8	-38.2 \pm 3.1	-32.8	-44.0	-3.7 \pm 1.0	-2.1	-5.3
Sediment	3	-29.4 \pm 0.1	-29.4	-29.5	1.2 \pm 0.3	1.4	1.0

DISCUSSION

Grey et al. (2004a) previously found that chironomid larvae exhibited considerable (~30‰) intraspecific variability in $\delta^{13}\text{C}$. The present study showed that these same chironomid larvae collected at a single location within a lake on a specific date also showed large variability in $\delta^{34}\text{S}$ values (up to 4.6‰ for *Chironomus plumosus* in Wyresdale Park Lake). Together, these isotopic data suggest alternative dietary intake between individual larvae collected from the same environs. Larvae assimilating settling phytoplankton should exhibit relatively ^{13}C - and ^{34}S -enriched signatures, reflecting phytoplankton use of carbon dioxide, bicarbonate and sulphate in the water column (Fry 1986). Conversely, larvae using primarily methanotrophic biomass in the sediments should exhibit $\delta^{13}\text{C} < -50\text{‰}$ (Grey et al. 2004a) and although there are no data available for the $\delta^{34}\text{S}$ values of methanotrophs *per se*, they are more likely to reflect sulphide rather than sulphate use, and exhibit $\delta^{34}\text{S}$ tending to 0‰ or lower (Fry 1986). Thus, there should be a positive relationship between larval $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ if larvae are assimilating differing proportions from these two dietary sources. However, we found no significant, positive relationship in either species from either lake. Indeed, the degree of ^{34}S -depletion exhibited by both species indicates primarily bacterial, rather than algal, sources, despite there being considerable primary productivity (June chlorophyll *a* values: Esthwaite, 29.0 $\mu\text{g/L}$; Wyresdale, 60.5 $\mu\text{g/L}$; Kelly et al. 2004) available in the water column. This is at odds with the findings of Goedkoop & Johnson (1992) that suggested the percentage contribution derived from bacterial carbon to larval carbon demand is generally low in productive lakes.

Moreover, in accordance with our expectation, *Chironomus anthracinus* was typically ^{34}S -depleted relative to its congener in both lakes, and thus in each lake, the combination of stable carbon and sulphur isotopes qualitatively separated the two species (Fig. 1). The stable sulphur isotope data suggest that *C. anthracinus* may actually be rather reliant on chemoautotrophic bacteria, reflected in the lighter $\delta^{34}\text{S}$ values exhibited by this species. In that case, light $\delta^{13}\text{C}$ values may not represent such an important dietary contribution from methanotrophic bacterial biomass, as previously suggested by Kelly et al. (2004), but simply reflect assimilation of respired carbon *sensu* Kohzu et al. (2004). The isotope data do not preclude assimilation from

methanotrophs, rather, that relative to its congener, *C. anthracinus* assimilates a greater dietary proportion from chemoautotrophs. *Chironomus plumosus* in Wyresdale Park Lake exhibited the greatest intraspecific isotopic variability, in both carbon and sulphur, and the greatest range in $\delta^{34}\text{S}$ (4.6‰) corresponding to $\delta^{13}\text{C}$ values between -40 and -30‰. This increased variability suggests that some *C. plumosus* individuals utilise more methanotrophic bacterial biomass, some more chemoautotrophic bacterial biomass, and others, a relatively larger phytoplankton diet proportion.

The two species found in our study lakes are classically described as having different modes of feeding. *Chironomus plumosus* is typically a filter feeder within its tube, although it can switch to deposit feeding under periods of low plankton availability; *C. anthracinus* is a deposit feeder of detritus around its tube mouth. The diagrams of tube morphology and feeding mode, elegantly presented in Jónasson (2003), suggest that the chironomid larvae may be exploiting different communities of chemoautotrophic bacteria within the sediment strata. *Chironomus anthracinus* sweep the sediment surface surrounding their tubes using the anterior part of their body, and generally feed down to the depth between oxidised and reduced sediment. These bioturbated sediment strata contain microsites with high concentrations of sulphur-oxidising chemoautotrophs compared to non-bioturbated sediments (Goni-Urriza et al. 1999), which we suppose are likely to be ingested by foraging larvae. Indeed, when Jónasson & Thorhauge conducted feeding experiments with *C. anthracinus* from Lake Esrom in aquaria, removal of the top 2cm of the L. Esrom mud (i.e. that layer with highest percentage organic matter and associated bacteria) resulted in larval starvation, indicating its nutritional importance (P.M. Jónasson, personal communication). *Chironomus plumosus* in Esthwaite was significantly ^{34}S -depleted, and by inference, more reliant upon bacterial sources than in Wyresdale, which may reflect the deeper water column in Esthwaite (15m compared to 3m) and associated recycling of nutrients therein.

Microbial chemosynthesis has been implicated as the primary source of reduced carbon for organisms inhabiting the environs of marine hydrocarbon vents and seeps (e.g. Rau 1981, MacAvoy et al. 2002). It may occur via symbiotic relationships, such as those between lucinid bivalves or marine nematodes and sulphur-oxidising bacteria, in sub-oxic, anoxic and sulphidic benthic habitats (Spiro et

al. 1986, Dando et al. 1994, Ott et al. 2004). Multiple trophic resources for marine chemoautotrophic communities have been demonstrated using multiple stable isotopes (Brooks et al. 1987, Cary et al. 1989) but comparatively little work has been conducted regarding the importance of these alternative trophic pathways in freshwater systems. Perhaps this is because of the perceived lack of basal resource (<1% elemental sulphur in our freshwater sediments). Cary et al. (1989) described a cold water brine seep that was dominated by two macrofauna: an un-described mytilid mussel reliant on methane oxidation; and a vestimentiferan worm, *Escarpia laminata*, reliant on sulphide oxidation for growth. The result of our stable isotope study of the two congeneric chironomids is similar to that of Cary et al. (1989): *Chironomus plumosus* appearing more reliant upon methanotrophic biomass; and *C. anthracinus* more reliant upon chemoautotrophic biomass. However, the intraspecific isotope variability indicates that they are not as exclusively reliant upon each source as animals in the marine system. Thus, we have shown that complementing stable carbon with stable sulphur isotope analyses can potentially identify such trophic pathways in freshwaters.

chapter V



Can stable hydrogen and carbon isotope analyses be used to unravel complexities in benthic food webs?

ABSTRACT

Stable carbon isotope techniques have been an essential component of research regarding the contribution of methane-derived carbon to freshwater food webs. Previous $\delta^{13}\text{C}$ studies have suggested that benthic macroinvertebrates in billabongs, streams and lakes are partially 'fuelled' by methanotrophic biomass. However, the singular use of carbon isotopes can lead to ambiguous interpretations concerning the question of carbon origin in food webs. These uncertainties can be further resolved by the inclusion of additional isotopic data. By using hydrogen as a second biogeochemical tracer we could provide further evidence for the assimilation of methane oxidising bacteria by chironomid larvae. Moreover, by combining hydrogen and carbon isotope data we were able to distinguish between the methane formation pathways, which subsequently fuel the methanotrophic biomass.

INTRODUCTION

Stable isotope studies are used to unravel food web structure and function since their composition in animal body tissues corresponds closely to that of their diets (with modifications according to the way food components are partitioned and fractionated during metabolism: Gannes et al. 1998). Stable carbon and nitrogen isotope ratios commonly are used in combination because $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of organisms potentially can provide an indication of the carbon and nitrogen sources and fixation pathways, the trophic level and the nutritional mode, provided that different basal resources are isotopically distinct.

Stable carbon isotope ratios have been particularly useful for elucidating nutritional modes of organisms. Typically, phytoplankton-derived organic matter in freshwater systems has $\delta^{13}\text{C}$ signatures of -30‰ to -20‰ (Fry & Sherr 1984, del Giorgio & France 1996), whereas very negative tissue carbon signatures ($\delta^{13}\text{C} \leq -30\text{‰}$) have been associated with respired carbon and in particular, methane-derived carbon, since biogenic methane is known to be extremely ^{13}C -depleted (Woltemate et al. 1984, Whiticar 1999, Coplen et al. 2002). Thus, ^{13}C -signatures can be confidently used as a natural tracer to track the flow of methane-derived carbon through biological communities (e.g. Childress et al. 1986, Fisher 1990). Zooplankton $\delta^{13}\text{C}$ from some lakes has indicated selective feeding upon prey which was isotopically lighter than bulk particulate organic matter (POM), suggesting that methane oxidising bacteria (MOB, methanotrophs) can provide a significant food source for zooplankton (Bunn & Boon 1993, Jones et al. 1999, Bastviken et al. 2003). More recently, there has been further evidence for the importance of methane as a carbon source especially for macroinvertebrates in freshwater food webs (Kiyashko et al. 2001, Grey et al. 2004b, Kelly et al. 2004, Kohzu et al. 2004, Eller et al. 2005a). Grey et al. (2004b) and Kelly et al. (2004) demonstrated that species commonly assumed to be filter-feeders of algal production in productive shallow lakes (e.g. *Chironomus plumosus*) might be heavily reliant upon methanotrophic bacterial biomass. However, the analysis of carbon isotopes alone often fails to provide definitive answers to questions of carbon origin in food webs. Therefore, in a previous study, we used a multiple stable isotope approach where in addition to carbon, stable sulphur isotopes were analysed as an alternative tracer of bacterial

assimilation by individual larvae of co-existing *Chironomus plumosus* and *C. anthracinus* (Grey & Deines 2005). The result of that study indicated that *C. plumosus* relies more on methanotrophic while *C. anthracinus* relies more on chemoautotrophic biomass.

Since one of the key processes in the benthic-pelagic coupling story is the degradation of organic matter to methane (CH₄), it may be intuitive to use not only carbon but also hydrogen (²H/¹H ≡ D/H) isotopes as a biochemical tracer to characterise the basis of the food web and the primary methanogenic pathways. Previous studies already have shown that stable isotope ratios of hydrogen in various animal tissues can be used in food web delineation (Smith & Epstein 1970, Estep & Dabrowski 1980, Schimmelmann & DeNiro 1986); especially in aquatic environments, the application of δ²H signatures could help to distinguish methane oxidising bacteria versus phytoplankton as the basis of lake food webs, e.g. for chironomids. Since methane has low δ²H values, methane oxidising bacteria also are likely to have low δ²H values (Schoell 1982).

Biogenic methane has a wide range of carbon and hydrogen isotopes varying in δ¹³C_{CH₄} from -110‰ to -50‰, and in δ²H_{CH₄} from -400‰ to -150‰ (Whiticar et al. 1986, Whiticar 1999). The combination of δ¹³C and δ²H (δD) values defining the various natural sources of CH₄ is demonstrated in the CD-diagram (Fig. 1). In Fig. 1, two biogenic CH₄ fields (methyl-type (acetate) fermentation (acetogenic methanogenesis) and carbonate (H₂/CO₂) reduction (hydrogenotrophic methanogenesis)) can be separated at approximate boundaries of δ¹³C_{CH₄} of -60‰ and δ²H_{CH₄} of -250‰ (Whiticar 1999). These two pathways in methane formation are commonly classified with respect to the type of carbon precursor: acetate (i.e. the methyl group) or H₂/CO₂ (Winfrey & Ward 1983, Capone & Kiene 1988, Conrad 1999). The carbon kinetic isotope effect (KIE) associated with fermentation of methylated substrates gives δ¹³C_{CH₄} values of -60‰ to -50‰ whereas the reduction of CO₂ to CH₄ results in more depleted δ¹³C_{CH₄} values of -110‰ to -80‰ (Whiticar 1999). Hydrogen isotope effects during methanogenesis of methylated substrates can lead to deuterium depletions as large as -531‰ in δ²H_{CH₄} whereas ²H/¹H discrimination for the CO₂-reduction pathway is significantly less pronounced (δ²H_{CH₄} ca. -170‰ to -250‰, Whiticar 1999). Therefore, we propose that the stable carbon

and hydrogen isotope ratios of methane, and their subsequent trophic transfer and assimilation into chironomids, could be particularly helpful in defining the dominant methanogenic pathways.

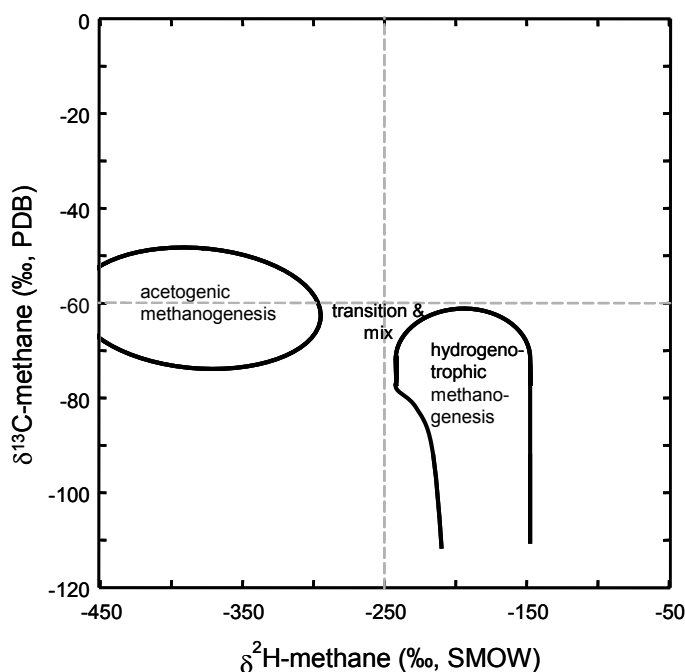


Figure V-1: CD-diagram for classification of biogenic methane by the combination of $\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^2\text{H}_{\text{CH}_4}$ ($\delta\text{D}_{\text{CH}_4}$) information. The biogenic methane strongly depleted in deuterium through acetate fermentation (acetogenic methanogenesis) can be distinguished from methanogenesis by H_2/CO_2 reduction (hydrogenotrophic methanogenesis) (modified after Whiticar 1999).

By comparing chironomids inhabiting different lake types, different depths within one lake, and two related and co-occurring chironomid species, we examined whether the analyses of $\delta^2\text{H}$ isotopes can be used (as a tool) to unravel the complex linkages between sediment microbial flora and macroinvertebrate consumers. The following specific questions were addressed: (a) Can $\delta^2\text{H}$ values be used to augment $\delta^{13}\text{C}$ in detecting contributions of methanotrophic biomass to chironomid diet? (b) Can $\delta^{13}\text{C}$ and $\delta^2\text{H}$ analyses of animal tissue be informative on the pathway of methane formation which subsequently fuels the methanotrophic biomass?

MATERIALS AND METHODS

Sampling sites

Three lakes were selected as sampling sites and all lakes contain abundant populations of *Chironomus plumosus* exhibiting stable carbon isotopic depletion probably associated with the assimilation of methanotrophic biomass (Kelly et al. 2004, Grey et al. 2004a,b, Eller et al. 2005a). Two of these lakes, Großer Binnensee and Holzsee, were sampled for analysing differences in $\delta^2\text{H}$ and $\delta^{13}\text{C}$ composition of chironomids between lakes. Both are located in northern Germany and were selected because of differences in their morphology and hence the degree to which they are 'fuelled' by methane-derived carbon (Eller et al. 2005a, chapter II). Großer Binnensee (N54°20' E10°37') is a shallow, exposed and polymictic lake, whereas Holzsee (N54°10' E10°11'), although shallow, has a smaller surface area, stratifies and exhibits a regular oxygen depletion in the hypolimnion.

The third lake, Esthwaite Water, UK (N54°22' W2°59'), was sampled to analyse variation in $\delta^2\text{H}$ and $\delta^{13}\text{C}$ composition of two co-existing chironomid species within and between different lake depths. The lake also develops deep-water anoxic conditions soon after the onset of thermal stratification. We chose to work on this lake because previous studies showed interspecific and within lake variation in chironomid larval $\delta^{13}\text{C}$ values (Grey et al. 2004a, chapter III).

Samples were collected in Großer Binnensee and Holzsee between August 2003 and August 2004. Esthwaite Water was sampled in 2002 and in addition to *C. plumosus* co-existing *C. anthracinus* were collected for analyses.

Chironomid sampling

Sediment samples and associated fourth instar chironomid larvae were collected by Ekman grab from 3 m depth in Großer Binnensee, 6.5 m depth in Holzsee and from two different depth zones in Esthwaite Water: 10 m and 15 m. Sediment was sieved (2 mm mesh) *in situ* and chironomid larvae were placed into a container with lake water and transported to the laboratory. Larvae were then placed into filtered water to allow gut clearance for 24 h (Feuchtmayr & Grey 2003). Excess faecal material was removed periodically to prevent coprophagy. Individual larvae were then placed into

separate wells of cell culture plates, oven-dried at 60°C for 24 h and stored in a dessicator.

Stable isotope analyses of chironomid larvae

Animal tissue of each chironomid was divided into two sub-samples for separate analysis of carbon ($^{13}\text{C}/^{12}\text{C}$) and hydrogen ($^2\text{H}/^1\text{H} \equiv \text{D}/\text{H}$) isotopes. For carbon stable isotope analysis, 0.5-0.7 mg of animal tissue was weighed into tin-cups prior to combustion in a Carlo-Erba NA1500 elemental analyser coupled to a Micromass Isoprime continuous flow isotope ratio mass spectrometer at the Max Planck Institute for Limnology, Germany. The reference material used was a secondary standard of known relation to the international standard of Vienna Pee Dee belemnite. Repeated analyses of an internal reference resulted in typical precision of $\pm 0.1\%$ for carbon.

For hydrogen stable isotope analysis, animal tissue and keratin check samples (see below) were weighed (~ 1 mg) into silver capsules. Filled capsules were left open for a period of not less than 4 days to allow the exchangeable hydrogen in the tissue to fully equilibrate with moisture in the laboratory air. Then, the capsules were sealed immediately prior to analysis on an ANCA-GSL elemental analyser coupled to a Geo20-20 continuous flow isotope ratio mass spectrometer at Iso-Analytical, UK. Any traces of water produced were removed by magnesium perchlorate, while any traces of CO_2 formed were removed via a Carbosorb™ trap. Samples of IAEA-CH-7 (polyethylene foil, $\delta^2\text{H}_{\text{V-SMOW}} = +100.3\%$) were analysed along with the tissue samples as quality control checks. In addition, samples of BWB-II (whale baleen, non-exchangeable $\delta^2\text{H}_{\text{V-SMOW}} = -108 \pm 4\%$) and RSPB egg shell membrane standard were analysed, provided by Geoff Hilton of the RSPB and independently measured by Len Wassenaar, NWRI, Saskatoon, Canada (non-exchangeable $\delta^2\text{H}_{\text{V-SMOW}} = -93.8 \pm 2.3\%$), with the tissue samples. By using the measured $\delta^2\text{H}_{\text{V-SMOW}}$ value (non-exchangeable plus exchangeable hydrogen) for BWB-II we were able to apply a simple correction for exchangeable hydrogen to the $\delta^2\text{H}_{\text{V-SMOW}}$ data for the keratin and egg shell membrane samples. The reference material used was IA-R002 (mineral oil, $\delta^2\text{H}_{\text{V-SMOW}} = -111.2\%$). IA-R002 is traceable

to NBS-22 (mineral oil, $\delta^2\text{H}_{\text{V-SMOW}} = -118.5\text{‰}$). All stable isotope ratios are reported using the δ notation expressed in units per mil.

For Esthwaite Water we analysed the remaining material for $\delta^2\text{H}$ (when sufficient) used for a stable carbon and nitrogen isotope study of Grey et al. (2004a). For $\delta^2\text{H}$ analyses of chironomid larvae from Großer Binnensee and Holzsee partly remaining material (when sufficient) was used from a study of Deines et al. (chapter II).

Data analysis

To check whether $\delta^{13}\text{C}$ and $\delta^2\text{H}$ data of chironomid larvae were different between species, lakes and depth we calculated individual analyses of variances (ANOVA) with $\delta^{13}\text{C}$ and $\delta^2\text{H}$ signatures as the response variable and species, lake and depth as factors. Tukey tests provided post-hoc comparison of means. Effects were considered significant at a level of $P < 0.05$. All statistical analyses were performed using JMP™ Statistics and Graphics Guide, Version 5.0.1a (SAS Institute Inc.).

RESULTS

Variability between lakes

Individual chironomids exhibited considerable differences in their larval $\delta^{13}\text{C}$ and $\delta^2\text{H}$ signatures between Großer Binnensee and Holzsee and formed two distinct clusters (Fig. 2). Larval $\delta^{13}\text{C}$ values from Großer Binnensee were consistently more enriched compared to Holzsee ($F_{1,55}=717.60$, $P < 0.0001$). For Großer Binnensee we measured a mean larval $\delta^{13}\text{C}$ of $-32.0 \pm 2.3\text{‰}$ and for Holzsee $-60.1 \pm 5.2\text{‰}$, a difference of $\sim 28\text{‰}$ (Table 1). In contrast, we found that chironomid $\delta^2\text{H}$ values were significantly more depleted in Großer Binnensee compared to Holzsee ($F_{1,55}=118.70$, $P < 0.0001$). Mean larval $\delta^2\text{H}$ was $-119.5 \pm 12.4\text{‰}$ for Großer Binnensee and $-88.8 \pm 8.5\text{‰}$ for Holzsee, a difference of $\sim 31\text{‰}$.

Table V-1: Stable carbon and hydrogen isotope data for two congeneric tubicolous chironomid species (*Chironomus plumosus* and *C. anthracinus*) from Großer Binnensee and Holzsee, Germany, and Esthwaite Water, UK.

Lake / species	n	$\delta^{13}\text{C}$ (‰)			$\delta^2\text{H}$ (‰)		
		Mean \pm SD	Max.	Min.	Mean \pm SD	Max.	Min.
Großer Binnensee							
<i>C. plumosus</i>	29	-32.0 \pm 2.3	-28.5	-39.2	-119.5 \pm 12.4	-101.4	-140.2
Holzsee							
<i>C. plumosus</i>	28	-60.1 \pm 5.2	-51.9	-70.3	-88.8 \pm 8.5	-74.9	-111.8
Esthwaite Water							
<i>C. anthracinus</i> 10 m	9	-32.1 \pm 1.1	-30.5	-34.5	-124.9 \pm 8.9	-109.1	-138.0
<i>C. anthracinus</i> 15 m	10	-38.3 \pm 3.2	-32.7	-43.6	-132.3 \pm 5.3	-127.5	-141.9
<i>C. plumosus</i> 10 m	11	-37.4 \pm 2.0	-33.3	-39.3	-144.5 \pm 9.5	-123.5	-155.0
<i>C. plumosus</i> 15 m	10	-52.9 \pm 5.1	-44.0	-58.9	-123.8 \pm 7.8	-110.6	-136.9

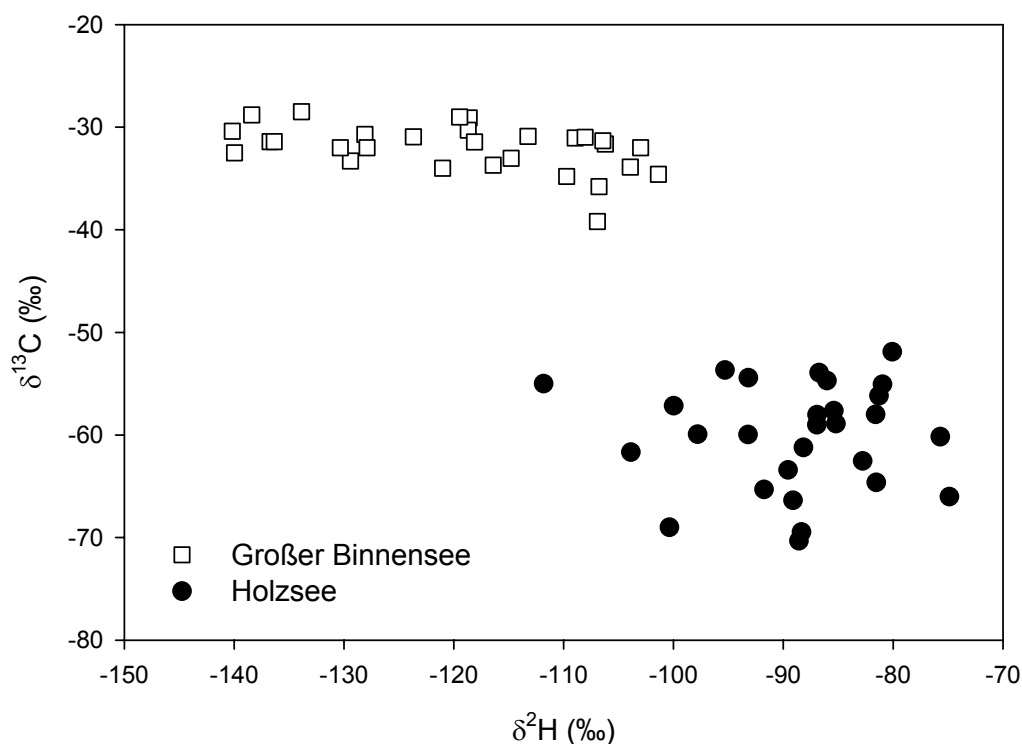


Figure V-2: Differences between lakes: relationship between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values (‰) plotted for individual *Chironomus plumosus* from Großer Binnensee and Holzsee.

Intra- and interspecific variability within one lake

In Esthwaite Water, we determined the intraspecific variability in $\delta^{13}\text{C}$ and $\delta^2\text{H}$ for both *Chironomus* species between depths (Fig. 3). In general, the degree of depletion of $\delta^{13}\text{C}$ was greater in *C. plumosus* than *C. anthracinus*, but for $\delta^2\text{H}$ no general trend was visible (Table 1). We found significant differences in $\delta^{13}\text{C}$ ($F_{1,17}=30.15$, $P<0.0001$) and $\delta^2\text{H}$ ($F_{1,17}=4.96$, $P<0.05$) between 10 and 15 m for *C. anthracinus*; larval $\delta^{13}\text{C}$ and $\delta^2\text{H}$ became more depleted with increasing depth. Similarly, *C. plumosus* larvae were significantly more ^{13}C -depleted at 15 m compared to 10 m ($F_{1,19}=88.68$, $P<0.0001$) but in contrast to *C. anthracinus*, *C. plumosus* $\delta^2\text{H}$ was significantly more enriched at 15 m compared to 10 m ($F_{1,19}=29.12$, $P<0.0001$).

We also noted significant interspecific isotopic variability between *C. anthracinus* and *C. plumosus* collected from the same depth (Table 1, Fig. 3). For both $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values at 10 m *C. plumosus* was always significantly more depleted in carbon ($F_{1,18}=50.88$, $P<0.0001$) and hydrogen ($F_{1,18}=22.04$, $P<0.001$) compared to *C. anthracinus*. At 15 m both species also showed significant differences in their stable isotope values, with *C. plumosus* being more depleted in carbon ($F_{1,18}=58.69$, $P<0.0001$) but more enriched in hydrogen ($F_{1,18}=8.11$, $P<0.05$) compared to *C. anthracinus*.

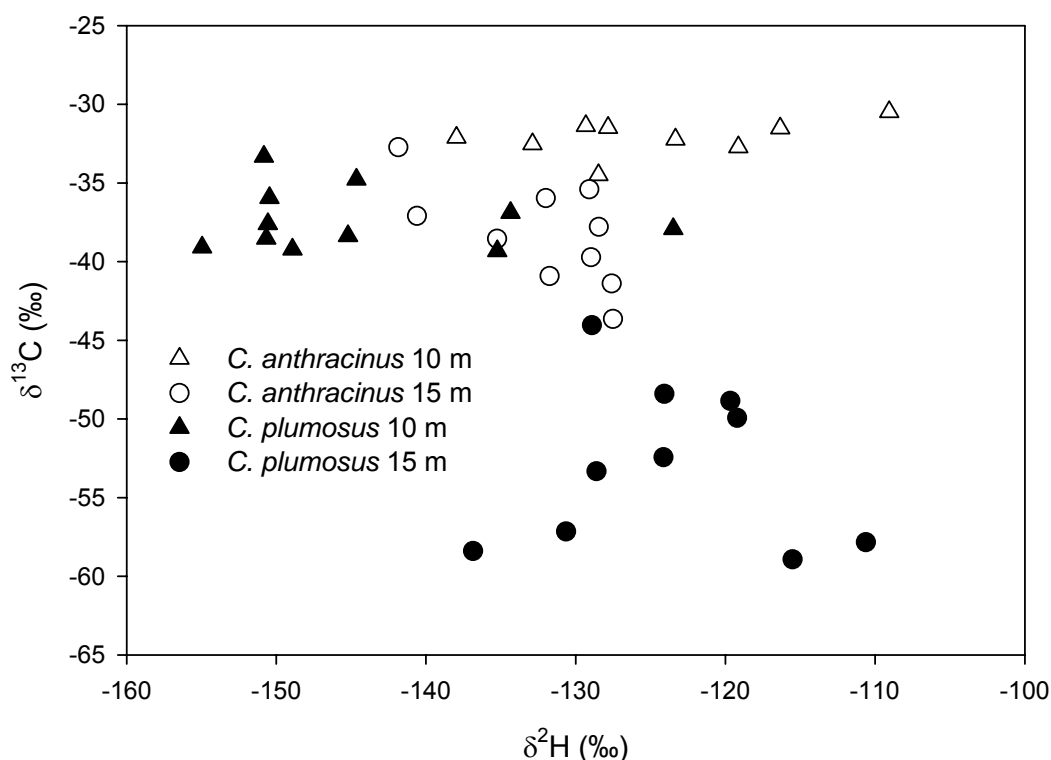


Figure V-3: Differences between depth and between species: relationship between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values (‰) plotted for individual *Chironomus anthracinus* and *C. plumosus* from Esthwaite Water. Individuals collected from either 10 m or 15 m depth.

DISCUSSION

As far as the authors are aware, this is the first study to incorporate hydrogen as a second biochemical marker in combination with carbon to unravel linkages between microbial fauna in the sediment and macroinvertebrate consumers. With this isotope combination, we could not only provide further strong evidence that chironomids are most likely feeding on MOB (Kiyashko et al. 2001, Grey et al. 2004b, Kelly et al. 2004, Eller et al. 2005a) but also differentiate which of the two CH_4 pathways was being used during methane formation by methanogens and hence, providing the energy base for the food chain.

Our $\delta^{13}\text{C}$ and $\delta^2\text{H}$ analyses of chironomid tissue from Großer Binnensee and Holzsee formed two distinct groups. Chironomids from Großer Binnensee were more enriched in $\delta^{13}\text{C}$ and more depleted in $\delta^2\text{H}$, whereas chironomids from Holzsee were more depleted in $\delta^{13}\text{C}$ and more enriched in their $\delta^2\text{H}$ values. After accounting for the kinetic isotope effects (KIEs) for carbon and hydrogen isotopes, the results fit

to the two CH₄ clusters with separate $\delta^{13}\text{C}$ and $\delta^2\text{H}$ ranges in the CD-diagram of biogenic methane (Fig. 1). Acetogenic methanogenesis (precursor acetate) is characterized by enriched $\delta^{13}\text{C}$ and depleted $\delta^2\text{H}$ values while hydrogenotrophic methanogenesis (precursor H₂/CO₂) shows values typically depleted in $\delta^{13}\text{C}$ and more enriched in $\delta^2\text{H}$ (Whiticar et al. 1986, Whiticar 1999, Conrad 2005). This suggests that acetate was the prevailing substrate in Großer Binnensee and thus the methanotrophic biomass was therefore fuelled by CH₄ produced by acetate-dependent methanogenesis. In the case of light $\delta^{13}\text{C}$ and more enriched $\delta^2\text{H}$ signatures, as found in Holzsee, chironomids most probably assimilated methanotrophic biomass fuelled by CH₄ formed via H₂/CO₂-dependent methanogenesis. Our results suggest that we are able to differentiate between the two CH₄ production pathways using the second biochemical marker $\delta^2\text{H}$. We propose the transition from acetate- to H₂/CO₂-dependent methanogenesis to lie roughly between the boundaries of $\delta^2\text{H}$ of $\sim -90\text{‰}$ to -120‰ . This interpretation is consistent with findings of Eller et al. (2005a). They found acetate-dependent methanogenesis dominating in Großer Binnensee and H₂/CO₂-dependent methanogenesis dominating in Holzsee by molecular community analysis of methanogens and $\delta^{13}\text{C}$ analysis of CH₄ produced from sediment incubations.

In addition to the differentiation of the two methanogenic pathways, predictions can be made concerning the importance of methanotrophic biomass for chironomids. Compared to the CD-diagram (Fig. 1) where the two biogenic CH₄ fields are not well separated by their $\delta^{13}\text{C}$ signature, we found a $\sim 10\text{‰}$ difference between the two data clusters for larvae sampled in the two lakes. This indicates that the more $\delta^{13}\text{C}$ depleted chironomid larvae collected from Holzsee probably assimilate more methane-derived carbon compared to chironomids collected from Großer Binnensee; and further supports the suppositions of Eller et al. (2005) of a higher contribution of CH₄-derived carbon to larval biomass in Holzsee compared to Großer Binnensee based upon a two-source mixing model (for August 2003).

In Esthwaite Water it was tested if two co-occurring chironomid species showed depth dependent differences in stable carbon and hydrogen isotope signatures. At first glance, both species showed different isotope signatures. While *C. plumosus* showed great variability in its $\delta^{13}\text{C}$ ($\sim 25\text{‰}$) and $\delta^2\text{H}$ ($\sim 45\text{‰}$) isotope

signatures, the variability in *C. anthracinus* was much lower: ~15‰ in $\delta^{13}\text{C}$; and ~30‰ in $\delta^2\text{H}$. These differences can most probably be attributed to different feeding modes described for both chironomid species. *Chironomus anthracinus* is a surface deposit feeder of detritus around its tube mouth while *C. plumosus* is typically a filter feeder within its tube, although it can switch to deposit feeding under periods of low plankton availability (Jónasson 2003, Monakov 2003). The diagrams of tube morphology and feeding mode, presented in Jónasson (2003), suggest that the chironomid larvae are likely to exploit different communities of methanotrophic bacteria within the sediment strata.

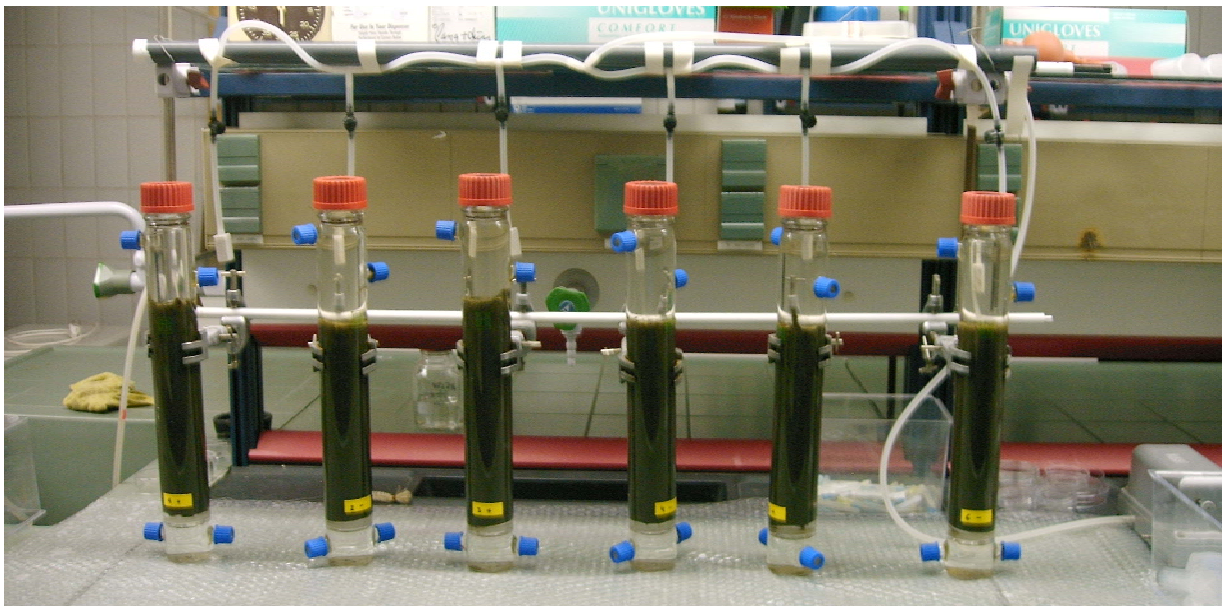
However, the isotopic differences expressed in macroinvertebrate consumers could also be a function of differing methanogenic pathways operating in different sediment strata. It has been shown that in lake sediments, CH_4 production by acetotrophic methanogens prevails in the top sediment layers, but decreases with depth, whereas hydrogenotrophic methanogenesis increases with depth (Whiticar 1999, Chan et al. 2005). This suggests that *C. anthracinus*, which sweep the sediment surface surrounding their tubes using the anterior part of their body, and generally feed down to the depth between oxidised and reduced sediment, ingest methane oxidising bacteria supplied by CH_4 production from acetate; our *C. anthracinus* $\delta^2\text{H}$ data is consistent with this hypothesis. For *C. plumosus*, we propose that CH_4 -derived carbon is consumed by 'microbial gardening' of methanotrophs within their tubes (Deines et al. chapter II). Because *C. plumosus* tubes penetrate deep into the sediment we suggest that methanotroph biomass is fuelled by the more prevalent H_2/CO_2 -dependent methanogenesis (Whiticar 1999, Chan et al. 2005); at least, this appears to be the case at 15 m depth in Esthwaite Water.

$\delta^{13}\text{C}$ values of *C. anthracinus* only decrease slightly with increasing depth from -32.1‰ to -38.3‰, suggesting that *C. anthracinus* is less reliant on methanotrophic biomass at every depth compared to *C. plumosus*. In a previous study, we could show by the analyses of stable sulphur isotopes that *C. anthracinus* indeed appears to be assimilating more chemoautotrophic biomass (Grey & Deines 2005). In contrast, we found that *C. plumosus* was consistently ^{13}C -depleted relative to *C. anthracinus*; we reported that this reflects a higher reliance on methanotrophic biomass (Grey & Deines 2005) and different feeding behaviour. H^2 -signatures of *C.*

plumosus collected at 10 m infer that ingested methanotroph biomass was fuelled by acetotrophic methanogenesis and that with increasing depth the contribution of hydrogenotrophic methanogenesis increased. Our interpretation is supported by ^{13}C analyses of CH_4 suggesting a slight shift from acetate- to H_2/CO_2 -dependent methanogenesis with increasing depth (Grey, pers.comm.).

By directly comparing our results to data on metabolic pathways used for methane production and the analyses of the methanogenic community structure in Großer Binnensee and Holzsee collected by Eller et al. (2005a), we hereby promote $\delta^2\text{H}$ signatures of consumer tissues as a promising biochemical marker for the assimilation of methane oxidising bacteria. In addition we can provide inferences on the dominant methanogenic pathway at the base of the food web.

chapter VI



Experimental evidence of chironomid larvae feeding on benthic as well as pelagic methane-derived bacterial carbon

ABSTRACT

It is now recognized that biogenic methane can also function as a potential carbon and energy source for macroinvertebrates in freshwater food webs. Stable carbon isotopes, used to infer an organism's food source, indicated that methane can play a major role in the nutrition of chironomid larvae, but the methanotrophic nature of this diet is still not confirmed. Although interactions between chironomid larvae and methanotrophs have been proposed, none have experimentally been demonstrated. Using ^{13}C labelled methane we could experimentally show for the first time that chironomid larvae assimilate methane-derived carbon through methanotrophic bacteria. Chironomid larval biomass was significantly ^{13}C enriched after dwelling for 10 days in lake sediment supplemented with labelled methane. Specific phospholipid fatty acids (PLFAs), diagnostic for methanotrophic bacteria, were detected in larval tissues. Additionally, stable carbon isotope values of methanotrophic biomarkers (PLFAs C16:1w5t, C16:1w7c, C16:1w8c and C18:1w7c) were significantly ^{13}C enriched, which encompasses the ^{13}C -uptake predicted for a methane-based nutrition.

INTRODUCTION

It is now recognized that macroinvertebrate communities sustained by energy from the oxidation of methane are not only restricted to specific anoxic environments such as deep-sea hydrothermal vents and cold seeps (Paul et al. 1985, Childress et al. 1986, Page et al. 1990). Despite the broad distribution of anoxic environments in freshwater ecosystems and the occurrence of biogenic methane (Hanson & Hanson 1996), the role of methane oxidising bacteria (MOB, methanotrophs) as a carbon and energy source in freshwater is not as well studied as in marine food webs. Until recently, only a few studies have considered methane as an important carbon source contributing to freshwater food webs (e.g. Bunn & Boon 1993, Kiyashko et al. 2001, Bastviken et al. 2003, Kelly et al. 2004, Kohzu et al. 2004).

In marine environments it has been the application of stable isotope analyses that revealed a considerable contribution of methane-derived carbon to diverse macroinvertebrates in methane seep communities (e.g. Levin & Michener 2002). Also in freshwater, several studies (Bunn & Boon 1993, Kiyashko et al. 2001, Grey et al. 2004a,b, Kelly et al. 2004, Eller et al. 2005a) used stable carbon isotope analyses and found an extensive use of methane-derived carbon by macroinvertebrates e.g. chironomid larvae. These larvae often dominate the benthic fauna of lakes (Coffman 1995) and showed $\delta^{13}\text{C}$ values in the range from -40 to -75‰ (Kiyashko et al. 2001, Grey et al. 2004a,b, Kelly et al. 2004, chapter II). The degree of chironomid larval ^{13}C depletion led to the hypothesis that larvae are feeding on MOB. While $\delta^{13}\text{C}$ data can provide evidence of assimilation of isotopically light methane-derived carbon in animal tissues, the exact route of this carbon to animals remains obscure. Therefore, other biomarkers are necessary to confirm the trophic association of macroinvertebrates and MOB. Certain fatty acids from dietary sources are conservatively transferred into lipids of animals and can thus be used as biomarkers in studies of trophic relationships (Sargent 1976). Lately, bacterial biomarkers were used in marine environments to confirm predator-prey (Ederington et al. 1995, Dalsgaard et al. 2003 and references therein) and host-symbiont relationships (e.g. Jahnke et al. 1995, Pranal et al. 1996, Pond et al. 1998).

In freshwater and terrestrial ecosystems methanotrophic fatty acid biomarkers have been used so far to study activity and diversity of methanotrophs in freshwater

lake sediments and soils (Boschker et al. 1998, Knief et al. 2006, Mohanty et al. 2006). Methanotrophic bacteria can be separated into two groups, type I and II (Hanson & Hanson 1996). Both groups occur in the sediment and the water column of freshwater lakes. They therefore provide a source of carbon and energy not only for benthic, but also for pelagic animals. It has been proposed that for low ^{13}C values found in zooplankton (selective) consumption of MOB from the water column could be responsible (Bunn & Boon 1993, Jones et al. 1999). A very distinct characteristic of the two MOB families is the presence of specific phospholipid fatty acids (PLFAs), which differentiate them from each other and also from all other organisms. PLFAs C16:1w8c and C16:1w5t are characteristic of type I (Nichols et al. 1985, Bowman et al. 1993) and C18:1w8c and C18:1w7c are typical of type II methanotrophs (Bowman et al. 1993, Dedysh et al. 2004).

To the best of our knowledge there is no study so far that could provide direct evidence for the assimilation of methanotrophic bacteria by chironomid larvae. Only Kiyashko et al. (2004) demonstrated that methanotrophs can be ingested by detecting group specific fatty acids of type I methanotrophs in tissues of field derived chironomid larvae *Stictochironomus pictulus*. To confirm the results from the field and test whether chironomid larvae feed on MOB and assimilate methane-derived carbon, we used a combination of stable carbon isotope and phospholipid fatty acid analyses. We experimentally changed the isotopic signature of methane, which in natural environments is ^{13}C -depleted (Coplen et al. 2002) to a ^{13}C -enriched signature. Consequently, methanotrophic bacteria that exploit this methane should provide an extremely ^{13}C -enriched food source. If this energy source (MOB) is really assimilated by chironomid larvae they should show a ^{13}C enriched isotopic signature. Furthermore, larvae should also contain PLFAs that are characteristic for methanotrophs and are enriched in ^{13}C . With two different experimental setups we addressed the questions whether chironomid larvae are able to generally access MOB as a carbon and energy source and additionally if they are able to feed upon benthic as well as pelagic methane-derived bacterial biomass.

MATERIALS AND METHODS

Sediment sampling, pre-incubation and MOB cultivation

The sediment was collected by Ekman grab from 3 m depth on August 2nd 2005 at lake Großer Binnensee (N54°20' E10°37'), a lake in northern Germany. In the laboratory the sediment was sieved with a 2 mm mesh to remove macroinvertebrates and debris. Sediment was pre-incubated at 20°C on a plankton wheel in the dark for 17 days. For that, 0.5 l of sieved sediment was filled in each of the five 1 L Schott flasks and moistened by adding 50 ml of autoclaved tap water. After closing the flasks with butyl rubber stoppers, 180 ml air were removed with a syringe from the headspace and replaced by 200 ml of ¹²CH₄ spiked with ¹³CH₄ (99 atom %; Isotec, Miamisburg, Ohio, USA; supplied by Campro Scientific, Berlin, Germany) at a mixing ratio of 70% ¹²CH₄ to 30% ¹³CH₄.

Parallel to sediment pre-incubations, a pure culture of a type II MOB strain (*Methylosinus trichosporium*, NCIMB strain number 11131) was grown in 500 ml nitrate mineral salt medium (in a 1 L Schott flask) with 1/10 of the standard nitrate concentration (modified after Whittenbury et al. 1970). This culture was supplemented with the same amount of ¹³C-labelled methane as described above for sediment pre-incubations and incubated at 20°C in the dark on a rotary shaker at 120 rpm for 17 days prior to the use in experiment 2.

Chironomid larvae

On August 20th 2005 Großer Binnensee sediment samples with associated chironomid larvae were collected by Ekman grab. In the field, sediment was sieved through a metal sieve (mesh size 2 mm) from which retained chironomid larvae were picked manually. Fourth instar *Chironomus plumosus* larvae were placed into a container with lake water and returned to the laboratory for the experiments. Larvae were used in the experiments without previous gut clearance.

Experimental set-up

The experimental tubes (diameter: 4.5 cm, height: 38 cm; Ochs, Germany; Fig. 1) are divided into two parts by a glass-sintered separation (si, poresize 100 µm), which

was covered with a Whatman GF/F filter. The top part (height: 33 cm) is open and has a side-port 5 cm below the top. The bottom part of the tube represents the injection reservoir (rv), which was filled with degassed and N₂ saturated autoclaved tap water and where ¹³C-labelled methane was injected. The separation allowed ¹³CH₄ diffusion from the reservoir into the top part of the tube.

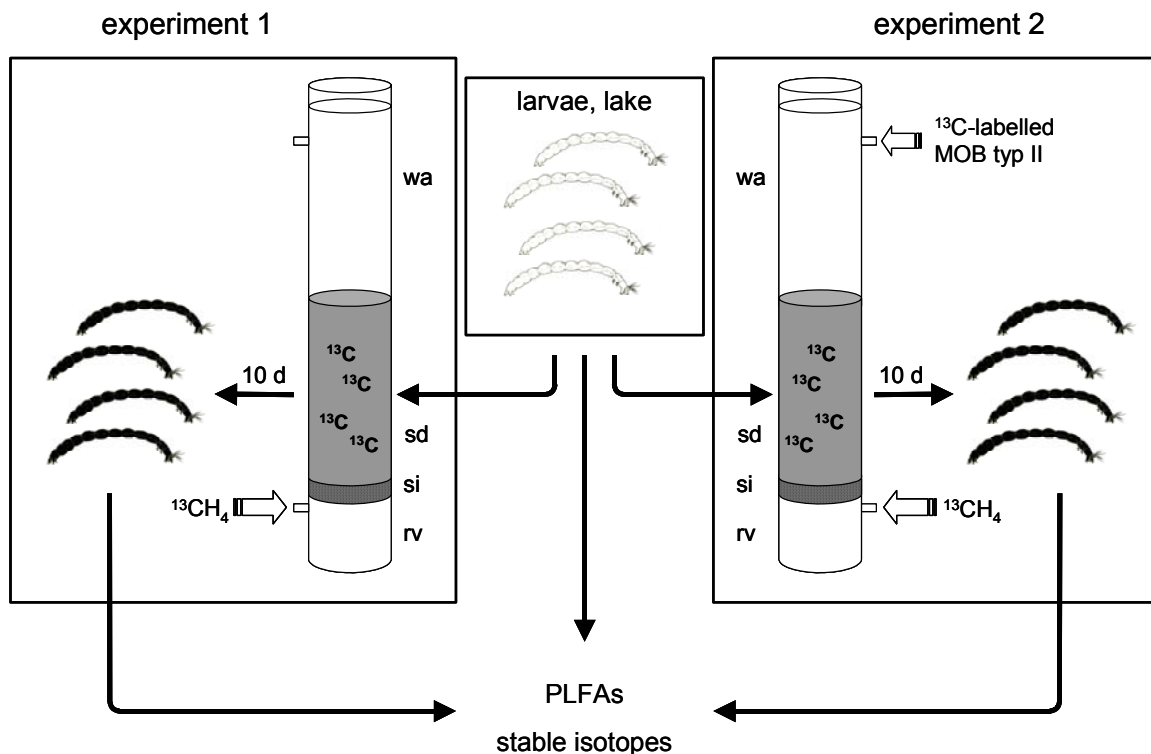


Figure VI-1: Experimental chironomid-sediment system. The tubes were half-filled with pre-incubated sediment (sd). The lower part of the tube consists of a reservoir (rv). The reservoir is separated from the upper part by a “sieve” (si) and was filled with degassed water to which ¹³C-labelled methane is added which then can diffuse into the sediment. After tubes were filled with lake water (wa) the sediment was allowed to settle for 24 h before chironomid larvae were added.

After pre-incubation with labelled methane for 17 days, sediment from all five flasks was mixed and used to fill six experimental tubes one half each. The remaining volume of the tubes was filled up with filtered lake water (250 ml, 100 μm). In each reservoir, 20 ml of the ¹³CH₄ mixture (described above) was injected to additionally supply the pre-incubated sediment with ¹³C-labelled methane during the experiment. The tubes were left for 24 h in the dark for the sediment to settle, before both experiments were started on August 20th 2005. For experiment 1 and 2, 10

chironomid larvae were placed into each of the three tubes (Fig. 1). In contrast to experiment 1, in experiment 2 10 ml of ^{13}C labelled type II MOB culture were injected daily through the upper side-port into the water column of each tube (Fig. 1). Oxygen supply from the water column to the sediment was ensured in both experiments by aerating the water using an air stone at the water surface. The experiments were running for 10 days at 20°C in the dark and harvested on August 30th 2005.

To retrieve the chironomid larvae the sediment of each tube was sieved and larvae collected were placed into filtered tap water for 24 h to allow gut clearance (Feuchtmayr & Grey 2003). Excess faecal material was removed periodically to prevent coprophagy. For each tube, half of the chironomid larvae retrieved were used for stable isotope and the other half for PLFA analyses, resulting in typically 5 larvae per method. Sediment samples of each tube were analysed by the same methods. As a reference, chironomids and sediment directly from the lake (Fig. 1) as well as pre-incubated sediment were analysed, too.

Stable carbon isotope analyses of chironomid larvae and sediment

Gut evacuated chironomid larvae were placed into separate wells of cell culture plates, dried at 60°C for 24 h and stored in a dessicator. Sediment samples from each tube were also dried at 60°C for 72 h and stored in a dessicator. Prior to analysis larvae and sediment were homogenized and samples were weighed into tin-cups (0.5-0.7 mg for chironomids; 1.5-2.0 mg for sediment). Isotope analyses were undertaken using a Micromass Isoprime continuous flow isotope ratio mass spectrometer interfaced with a Carlo-Erba NA1500 elemental analyser. Isotope ratios are given conventionally using the δ notation expressed in units per mil as follows: $\delta^{13}\text{C}$ (‰) = $10^3 (R_{\text{sa}}/R_{\text{st}} - 1)$ with $R = ^{13}\text{C}/^{12}\text{C}$ of sample (sa) and standard (st), respectively. The reference material used was a secondary standard of known relation to the international standard of Vienna Pee Dee belemnite. Repeated analyses of an internal reference ($n = 50$) resulted in typical precision of $\pm 0.1\text{‰}$ for $\delta^{13}\text{C}$.

Lipid analyses and stable isotope probing of PLFAs (SIP-PLFA)

Chironomid larvae and sediment were stored at -80°C until samples were freeze-dried. Lipids were extracted from 5 pooled chironomid larvae retrieved from each experimental tube or the lake and from 4 g of sediment. A modified Bligh & Dyer extraction procedure was used as described previously (Boschker et al. 1998, 2001, Mohanty et al. 2006). The lipid extract was fractionated on silicic acid into different polarity classes by sequential elution with chloroform, acetone, and methanol. The methanol fraction containing the PLFAs was derivatised using mild-alkaline methanolysis to yield fatty acid methylesters (FAME).

FAME concentrations were determined using a GC-FID system (Thermo Finnagan TRACE GC) equipped with a polar capillary column (SGE, BPX-70; 50 m by 0.32 mm by 0.25 μm), using the following oven conditions: initial temperature of 50°C for 1 min, followed by a temperature increase to 130°C (ramp of $40^{\circ}\text{C min}^{-1}$) and subsequently to 230°C (ramp of $3^{\circ}\text{C min}^{-1}$).

FAME standards of both $\text{C}_{12:0}$ and $\text{C}_{19:0}$ were used for calculating retention indices and for FAME quantification. Identification of FAMEs was based on retention time data with known standards. Additional identification was gained by GC-mass spectrometry (GC-MS) using a Thermo Finnagan TRACE GC-MS system. For identification of methanotroph-specific PLFA, extracts of cultures of *Methylomonas methanica* S1 NCIMB 11130, *Methylomicrobium album* NCIMB 11123, *Methylobacter luteus* NCIMB 11914, *Methylocystis parvus* NCIMB 11129, *Methylosinus trichosporium* NCIMB 11131, *Methylosinus sporium* NCIMB 11126 were used as a reference. PLFA nomenclature used is described by Guckert et al. (1985). Fatty acids are designated by the number of carbon atoms. The degree of unsaturation is indicated by a number separated from the chain length by a colon and is followed by ωxc , where x indicates the position of the double bond nearest to the aliphatic end (ω) of the molecule and c indicates a *cis* stereoisomeric position of the double bond on the molecule.

Stable carbon isotope ratios for individual FAMEs were determined using a Varian 3400 GC equipped with an ATAS Optic 2 programmable direct thermal desorption injection system. The GC was coupled to a Finnagan Delta S isotope ratio mass spectrometer via a type II combustion interface. The same polar capillary column was used as for FAME identification and quantification on the GC-FID and

GC-MS system. The oven temperature for the GC-isotope ratio mass spectrometry analyses was as follows: initial temperature of 50°C for 4 min, followed by subsequent temperature increase to 130°C (ramp of 30°C min⁻¹), 200°C (ramp of 6°C min⁻¹), 220°C (ramp of 5°C min⁻¹) and finally 250°C (ramp of 20°C min⁻¹). The sample was injected directly into the thermal desorption system at 50°C, in which the temperature was raised to 260°C (ramp of 10°C s⁻¹). The absolute amount of label incorporated into every separate PLFA was calculated as described previously (Boschker & Middelburg 2002).

Multivariate analyses of SIP-profiles

Stable Isotope Probing (SIP) ¹³C-PLFA profiles of incubated sediment were compared to profiles of methanotrophic cultures to identify active MOB in these samples as defined by the most similar profile available from cultivated MOB, as described by Mohanty et al. (2006). A prerequisite to make these comparisons, however, is that only label incorporation in type I and type II related PLFAs separately can be determined. Otherwise a mixed profile of type I and II species would be compared to profiles from separate cultures. Since in a soil SIP profiles there are PLFA that both occur in type I and type II MOB a selection of PLFA was made that were regarded as being predominantly occurring in type II (>0.1%) but not in type I (<0.1%) and vice versa. The selected PLFAs for type I MOB were C14:0, iC15:0, C16:0, C16:1w9t, C16:1w5t, C16:1w8c, C16:1w7c, C16:1w6c, C16:1w5, cyC17:0, cyC19:0. Since label uptake by type II PLFA in incubated sediment was very low compared to type I PLFA and the fact that we could detect the type I signature lipid (C18:1w8c) no SIP profiles could be made for the active type II MOB in the sediment incubations. The sum of ¹³C taken up in these selected PLFA was used to calculate the relative uptake in all individual PLFA. These relative PLFA concentration profiles of soil samples and known cultures formed the matrix which was used for cluster analyses and non-metric Multidimensional Scaling (MDS) analyses. Input of cluster analyses as well as MDS were Bray-Curtis similarity matrices which were log(x+1) transformed to even out the contribution of very rare and very dominant PLFAs. Clustering was made using the Group-Average linking routine. The MDS analyses results in a 2-dimensional plot were the distance between

samples indicates the similarity of these samples relative to other samples in the plot. The accuracy of the 2-dimensional representation is indicated by the “stress” value (Kruskall’s stress formula). Stress values <0.1 indicate a good ordination with no prospect of misleading interpretation. Stress values <0.2 still give a good 2-dimensional representation where not too much reliance should be put on the detail. In this case other methods of representation should be used in parallel, like clustering analyses. All clustering and MDS analyses were performed using the Primer-E software (Plymouth Marine Laboratory, Plymouth, UK). All theoretical aspects of the used cluster and MDS analyses are described by Clarke and Warwick (2001).

Data analysis

To check whether $\delta^{13}\text{C}$ data of chironomid larvae and sediment were different between the different treatments, individual analyses of variances (ANOVA) were calculated with the stable isotope signature of carbon as the response variable and different experiments as factors. Tukey tests provided post-hoc comparison of means. All test statistics refer to two-tailed tests. Effects were considered significant at a level of $P<0.05$. All statistical analyses were performed using JMP™ Statistics and Graphics Guide, Version 5.0.1a (SAS Institute Inc.).

RESULTS

Sediment and chironomid larvae $\delta^{13}\text{C}$ values

Stable carbon isotope signatures were compared between sediment samples from both experiments and the natural lake sediment. Sediments from both experiments exhibited significant differences in their $\delta^{13}\text{C}$ signatures after 10 days (Table 1). Sediment isotope values from experiment 1 were significantly enriched compared to lake sediment by 15‰ ($F_{1,2}=206.92$, $P<0.01$). Likewise, sediment from experiment 2 was significantly enriched compared to sediment from the lake by over 22‰ ($F_{1,2}=70.52$, $P<0.05$).

Table VI-1: Stable carbon isotope data for chironomid larvae (*Chironomus plumosus*) and bulk sediment collected from Großer Binnensee before and after incubation under experiment 1 and experiment 2 conditions.

	Larval $\delta^{13}\text{C}$ (‰) Mean \pm SD	n	Sediment $\delta^{13}\text{C}$ (‰) Mean \pm SD	n
lake	-29.3 \pm 0.4	10	-17.6	1
experiment 1	22.3 \pm 19.4	9	-2.6 \pm 0.9	3
experiment 2	225.9 \pm 170.9	8	5.0 \pm 2.3	3

The chironomid larval $\delta^{13}\text{C}$ signatures showed in general the same trend, but the degree of $\delta^{13}\text{C}$ enrichment was much higher (Fig. 2, Table 1). Larvae from experiment 1 ($F_{1,17}=70.91$, $P<0.0001$) and experiment 2 ($F_{1,16}=22.65$, $P<0.001$) were significantly enriched compared to larvae from the lake. Additionally, the inter-individual variability of larval $\delta^{13}\text{C}$ increased during the experiments. In chironomids from the lake, $\delta^{13}\text{C}$ signatures clustered tightly together (range -29.1 to -29.7‰), whereas larvae from experiment 1 and 2 exhibited values from 3.7 to 62.6‰ and 58.3 to 505.6‰, respectively (Fig. 2). Nevertheless, the majority of chironomid larvae from experiment 2 exhibited ^{13}C values between 130‰ and 230‰. Only two larvae were more depleted (~60‰) and two more enriched (~490‰) than the average (Fig. 2). An experimental artefact can probably be excluded as neither of the two larvae from each pair originated from the same tube. A few chironomid larvae pupated during the experiment probably triggered by temperature, accounting for the varying numbers of chironomids analysed for stable carbon isotopes.

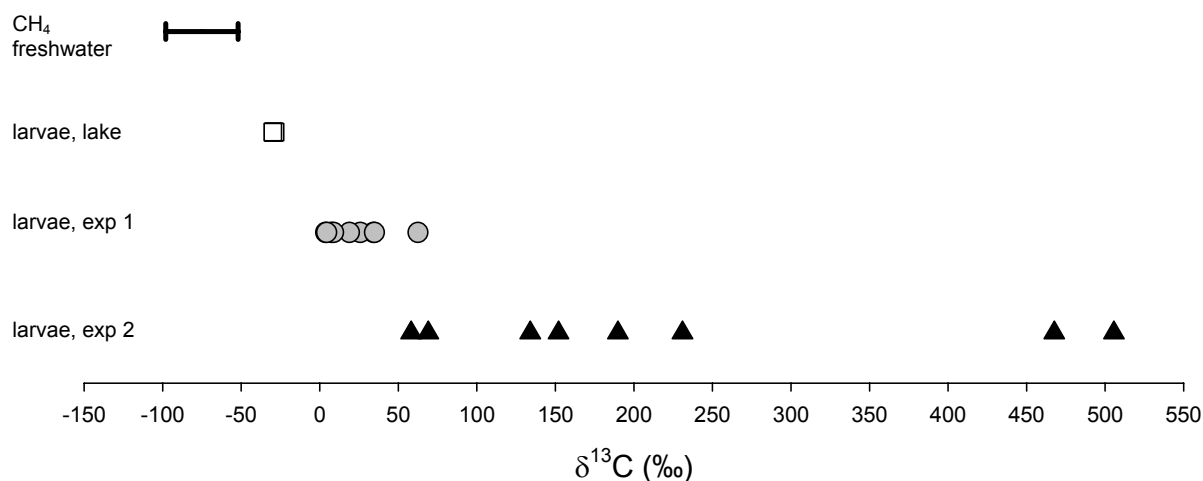


Figure VI-2: Carbon isotope ratios of CH₄ from freshwater sources (Coplen et al. 2002) and chironomid larvae. Symbols indicate individual larval values: larvae, lake = signatures of larvae at the beginning of the experiments (n=10); larvae, exp 1 = signatures of larvae grown under experiment 1 conditions (n=9) and larvae, exp 2 = signatures of larvae grown under experiment 2 conditions (n=8).

Incorporation of ¹³C into individual PLFAs and their $\delta^{13}\text{C}$ signature in samples of sediment and chironomid larvae

The incorporation of labelled ¹³C into different PLFAs retrieved from sediment samples was similar in both experiments. The dominant PLFAs C14:0, C16:0, C16:1w7c and C16:1w8c in the pre-incubated sediment showed the highest incorporation of ¹³C under both experimental conditions (Fig. 3). All these PLFAs are present in type I MOB (Nichols et al. 1985, Bowman et al. 1993), but not diagnostic for this family. Nevertheless, the type I specific PLFA C16:1w5t was ¹³C-labelled, too, but the uptake was less pronounced than in the aforementioned PLFAs. The major difference between the sediments from the two experiments is PLFA C18:1w7c, which is present in all known type II MOB (Bowman et al. 1993, Dedysh et al. 2004). This PLFA was only weakly labelled in the sediment after pre-incubation and in experiment 1 but intensively labelled in the sediment of experiment 2, in which labelled type II MOB were injected into the water column (Fig. 3). The majority of the label in C18:1w7c originates from the type II specific lipid C18:1w8c. However, this lipid was not detected by GC-FID because it could not be separated from the very large neighbouring peaks (C18:1w9c and C18:w7c). However, from then GC-IRMS

signal it could be derived that the majority of the ^{13}C signal could be assigned to a peak in between C18:1w9c and C18:w7c, which is C18:1w8c (data not shown). This peak is the major lipid in *Methylocystis* and *Methylosinus trichosporium* species (Bowman et al. 1993).

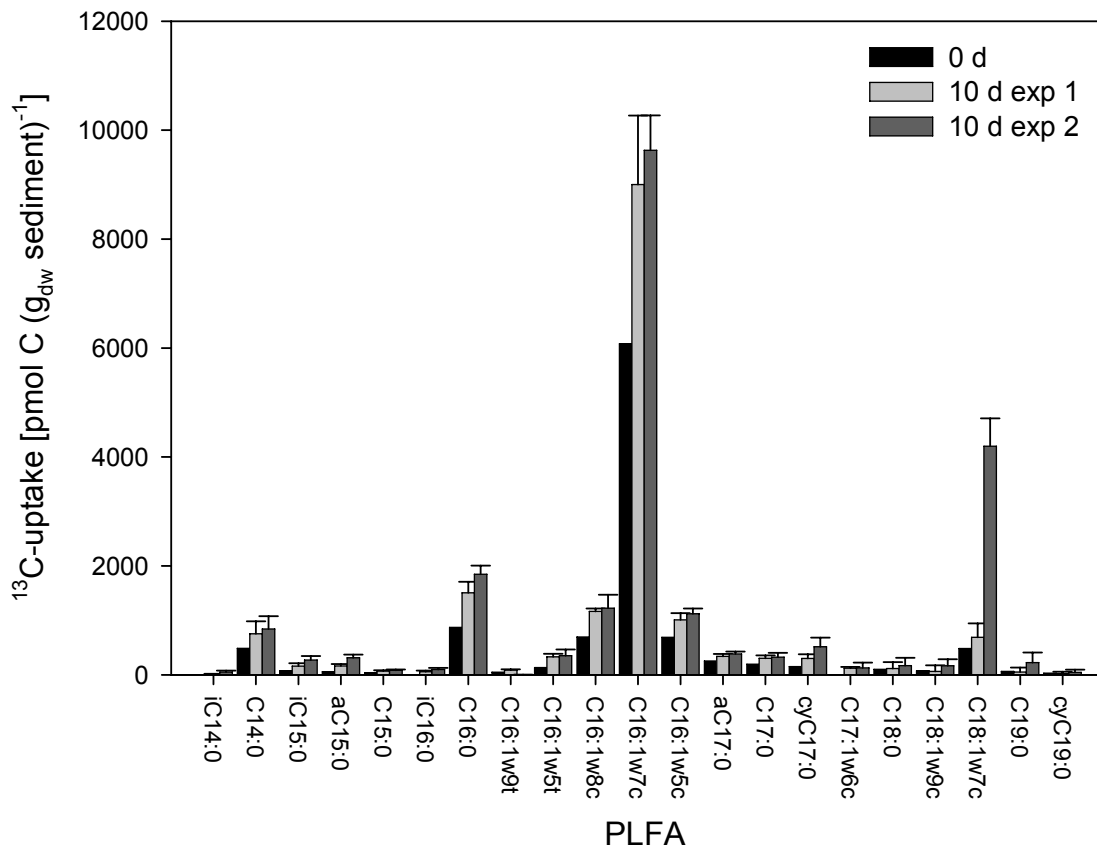


Figure VI-3: ^{13}C -uptake into different PLFAs in sediments incubated 10 days under experiment 1 and experiment 2 conditions in comparison to pre-incubated sediment (0 d). Data for the labelling patterns are means of triplicate samples (tubes) \pm standard deviation.

MOB specific PLFAs were found in tissue of chironomid larvae in both experiments. Furthermore, incorporation of ^{13}C was detectable in these MOB specific PLFAs retrieved from larval biomass. The amounts of label incorporated into chironomids varied between the two experiments and was type-specific. High amounts of label incorporated into C16:0, C16:1w5t, C16:1w7c and C16:1w8c suggests that the dominant organisms assimilated by chironomid larvae in experiment 1 belong to type I MOB (Fig. 4A). However, in experiment 2 the type II

MOB diagnostic PLFA C18:1w7c (+C18:1w8c) was stronger labelled than the PLFAs of type I MOB, even though uptake of ^{13}C was also detected for type I MOB (Fig. 4B).

The overall pattern of ^{13}C -labelled PLFAs in larval biomass was different between the two experiments and compared to larvae directly sampled in the lake. $\delta^{13}\text{C}$ values of all individual PLFAs from lake chironomids that do not occur in methanotrophs (i.e. C18:26c, C18:9c, C18:3(n6), C20:4, C20:5(n3)) were ^{13}C depleted and ranged from -25 to -30‰. In contrast PLFAs C16:1w7c, C16:1w8c and C16:1w5t, present in type I MOB, were strongly labelled with ^{13}C in chironomids of experiment 1 (Fig. 4C). Mean type I PLFA $\delta^{13}\text{C}$ values ranged from 75 to 90‰ in experiment 1. In experiment 2 the incorporation of label into PLFAs C18:1w7c (+C18:1w8c) reflects the presence of type II MOB biomarkers in chironomid larval biomass (Fig. 4D). The incorporation of label into 16:1 PLFAs, present in type I MOB, was much weaker than for type II MOB specific PLFAs.

Linking identity and activity by using SIP-PLFA profiles

From Fig. 5 it is obvious that the active type I MOB in the incubated sediment are closely related to the genus *Methylobacter*. Multivariate analyses of SIP-profiles clearly separated the active type I MOB from all other known genera. This finding is in agreement with community composition assessment using DGGE and FISH analyses (Eller et al. 2005a) of the same sediment, which demonstrated *Methylobacter* species as the dominant strains in these sediments. The SIP profiles also indicated that incorporation of label into type II MOB was negligible. Due to the latter SIP profiles of the active type II MOB could not be made.

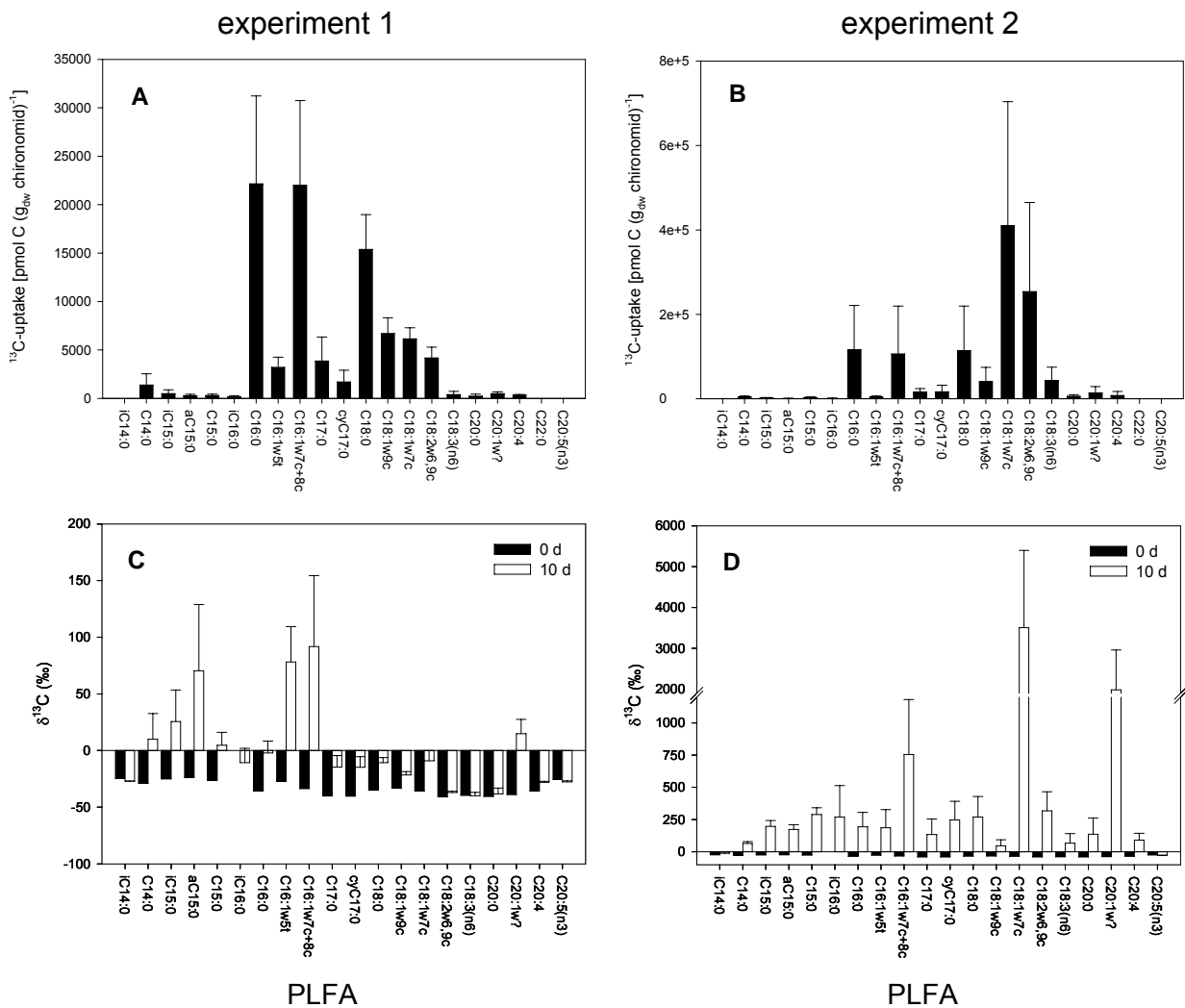


Figure VI-4: Amounts of ^{13}C incorporated into the different PLFAs (A, B) and comparison between carbon isotope ratios of PLFA (C, D) extracted from chironomids incubated in sediment supplied with labelled CH_4 under experiment 1 (left panels) or under experiment 2 conditions (right panels). For comparison $\delta^{13}\text{C}$ values of PLFAs of lake chironomid larvae (0 d) were used. Data for the labelling patterns are means of triplicate samples (\pm standard deviation) consisting of five chironomid larvae from each tube.

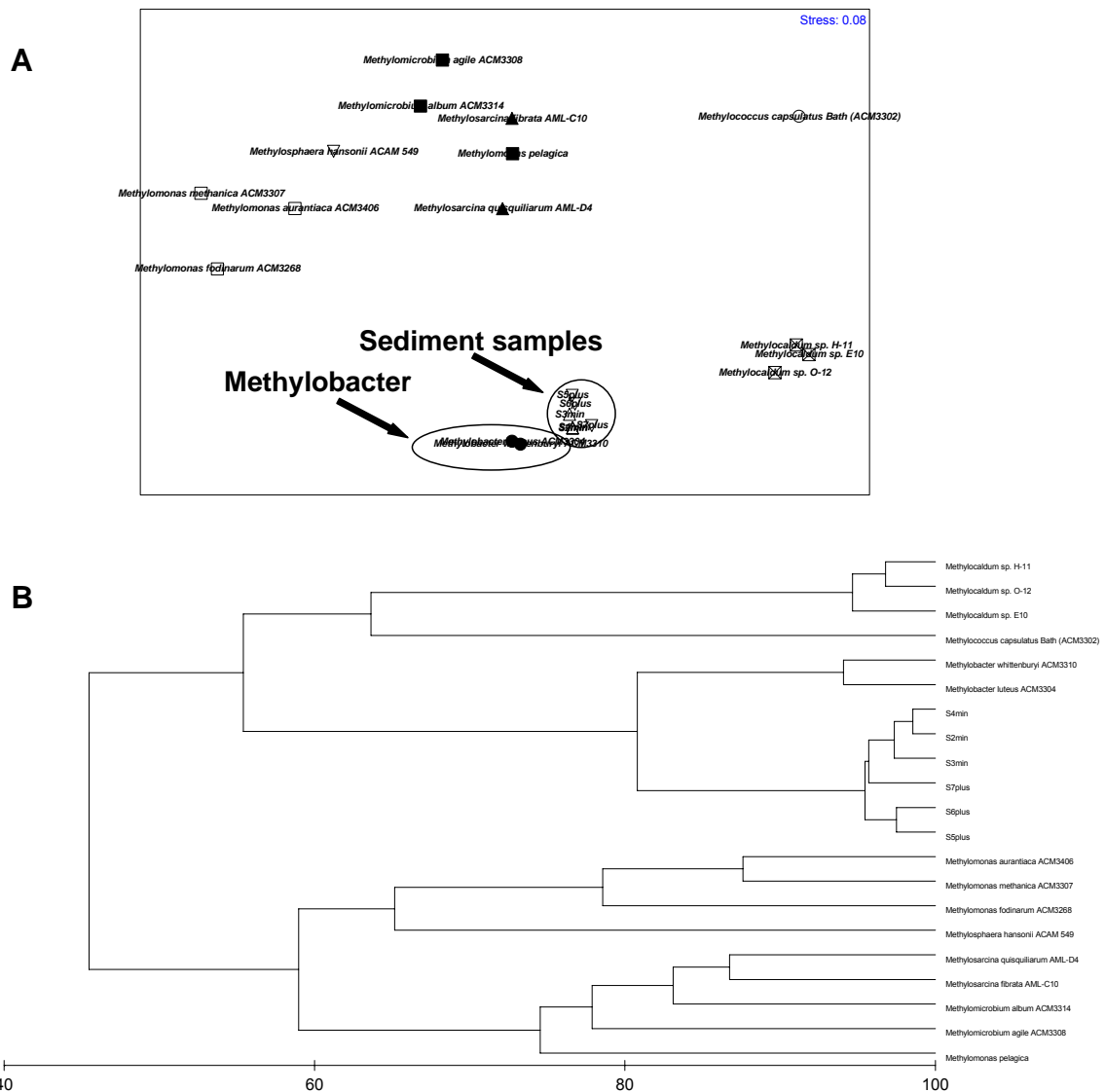


Figure VI-5: Multivariate statistical analyses of SIP ^{13}C -PLFA (type I) profiles of the MOB community in sediment samples. The inputs of the MDS (A) and the cluster analyses (B) were PLFA profiles of MOB cultures (expressed as percentage of total PLFA content) and of SIP profiles of sediment samples (expressed as percentage of ^{13}C incorporated in separate PLFAs of the total PLFA ^{13}C -uptake). The two-dimensional distances between samples in the MDS plot show the relative similarity between samples. For the cluster analyses, the profiles were transformed $[\log(x+1)]$ before the Bray-Curtis similarity matrix was established. The clustering was done using the Group-Average linking method.

DISCUSSION

For the first time we could experimentally show that type I and II MOB were assimilated by chironomid larvae. Although interactions between chironomid larvae and methanotrophs have been proposed (Bunn & Boon 1993, Kiyashko et al. 2001,

Grey et al. 2004a, Kelly et al. 2004, Eller et al. 2005a), none have experimentally been demonstrated. In our experiments, the combination of stable carbon isotope and PLFA analysis of larval tissue allowed not only the detection of an overall ^{13}C carbon uptake by the larvae, but additionally also the specific detection of labelled MOB biomarkers in their tissue.

Both detection methods used in this study are well established: stable carbon isotope analyses to examine trophic relationships between organisms and to establish the dependence of animals on organic material produced via methanotrophy (for a review, see Fisher 1990, Conway et al. 1994). Besides the analysis of soils and cultures (e.g. Boschker et al. 1998, Mohanty et al. 2006, Knief et al. 2006) PLFA analysis has been used also in marine environments. There the detection of PLFA C16:1w8 in deep-sea marine macroinvertebrates confirmed the presence of endosymbiotic MOB in cold seep mussels (Jahnke et al. 1995) and in mussels and gastropods, inhabiting hydrothermal vents (Pranal et al. 1996, Pond et al. 1998). For freshwater macroinvertebrates to our knowledge only one study demonstrated the presence of type I MOB biomarker PLFAs in the larvae of chironomid *Stictochironomus pictulus* collected from Lake Biwa (Kiyashko et al. 2004).

In our study, the $\delta^{13}\text{C}$ pattern of PLFA extracted from the sediment samples nicely illustrated the successful labelling of indigenous type I MOB in the lake sediment used for the experiments. This exclusive labelling was due to the use of methane as labelled substrate, which can only be assimilated by MOB (Hanson & Hanson 1996) and to the strong dominance of the active *Methylobacter* (type I MOB) over type II in the experimental sediment (Fig. 5). This strong dominance of *Methylobacter* in the sediment of Großer Binnensee (proved by independent methods: Eller et al. 2005a) enabled us in experiment 2 to discriminate between MOB from the sediment (type I) and MOB supplied via the water column (type II). Thus a differentiation between feeding of chironomid larvae in the sediment (benthic, type I MOB) and directly from the water column (pelagic, type II MOB) was possible.

In experiment 1, already after 10 days larval biomass was significantly enriched in ^{13}C compared to larvae from the lake, thus larvae must have been feeding on labelled benthic bacterial biomass. This result was further supported by the PLFA data: all labelled PLFAs (C16:1w8c, C16:1w7c, C16:1w5t) are present in

type I MOB (Nichols et al. 1985, Bowman et al. 1993). Even though we are aware that C16:1w8c cannot be separated from the huge C16:1w7c peak, which is also a major component of sulphur oxidising bacteria (McCaffrey et al. 1989, Conway & McDowell Capuzzo 1990), we nevertheless think that the argument that chironomids feed upon type I MOB still holds. First, methanotrophic (and not sulphur oxidising) bacteria are the primary consumers of the labelled substrate CH₄. Second, PLFA C16:1w5t, which is diagnostic for type I MOB (Nichols et al. 1985, Bowman et al. 1993), was also labelled and detected in the larvae. Further, the high amount of label incorporated into 16:1 PLFAs suggests that the dominant organisms assimilated by chironomids belong to type I MOB.

In experiment 2, besides the use of labelled sediment, a suspension of ¹³C-labelled type II MOB was injected daily into the water column. In contrast to experiment 1, the larvae from experiment 2 were not only significantly ¹³C-enriched compared to larvae from the lake, but showed also a surprisingly high inter-individual variability in their δ¹³C signatures. Because the only difference between the two experiments was the addition of type II MOB to the water column in experiment 2, this increased variability should be due to the assimilation of bacterial carbon from the water column (pelagic) in addition to the sediment derived (benthic) carbon. This hypothesis was supported by the presence of the C18:1w7c PLFA, which is present in all known type II MOB (Bowman et al. 1993, Dedysh et al. 2004). Again, this PLFA is not only a substantial component of type II MOB but has been reported as indicative for the presence of sulphur oxidising bacteria (McCaffrey et al. 1989, Conway & McDowell Capuzzo 1990). Despite the fact that we could not detect the highly type II MOB specific lipid C18:1w8c by GC-FID the ¹³C chromatograms indicated that most of the label was associated with a peak in between C18:1w9c and C18:1w7c, being C18:1w8c. This fact combined with the highly labelled C18:1w7c gives clear evidence for a type II MOB labelling pattern.

This high inter-individual variability of larval δ¹³C signatures found in experiment 2 might be explained by the different feeding modes reported for *Chironomus plumosus* (Jónasson 2003). Hodkinson and Williams (1980) suggested, that *C. plumosus* larvae probably show trophic plasticity in their modes of feeding and switch between deposit- and filter-feeding. Thus, they can explore different food sources and thereby cope with seasonal changes in food availability (McLachlan et

al. 1978, Ward & Williams 1986). Experiment 2 supports this hypothesis of high feeding mode flexibility. Three types of feeding behaviour could be identified: Chironomid larvae exhibiting $\delta^{13}\text{C}$ values as found in experiment 1 (and less enriched ones in experiment 2) were deposit-feeding animals because the only labelled food source available in experiment 1 were benthic methanotrophs (type I MOB). In contrast, larvae with the most positive $\delta^{13}\text{C}$ values in experiment 2 most probably selectively ingested labelled type II MOB from the water column by filter-feeding. Thus, intermediate $\delta^{13}\text{C}$ values should be achieved by chironomids when switching between deposit- and filter-feeding, which seemed to be the case for most larvae in the experiment. These conclusions are supported by the $\delta^{13}\text{C}$ values of PLFA C18:1w7c, which was present in high concentrations and strongly ^{13}C enriched in some larvae from experiment 2. This indicates that these larvae were indeed feeding to a high extent from the water column. High incorporation of ^{13}C into PLFAs 16:0 and C18:1w7c (present in type II MOB, Bowman et al. 1993, Dedysh et al. 2004) and 16:1 PLFAs (all present in type I MOB, Nichols et al. 1985, Bowman et al. 1993) extracted from larval tissue provide strong evidence that the larvae were utilizing both, benthic and pelagic MOB biomass.

Another interesting aspect in this study is that the pre-incubated sediment without the addition of chironomid larvae showed lower ^{13}C -uptake rates for all 16:1 PLFAs present in type I MOB than the sediment in the two experimental treatments with chironomids. Thus, the methane incorporation into MOB must have been generally stimulated in the experiments. This could be due to bioturbation activity of chironomid larvae, leading to increased oxic-anoxic interfaces, where both essential sources for MOB, oxygen and methane, are available (e.g. Frenzel et al. 1990, Damgaard et al. 1997). This phenomenon has been reported for several marine habitats, where bioturbation by benthic macroinvertebrates promoted the development of a rich microbial community with increased microbial activity and biomass relative to the surrounding sediment (e.g. Aller & Yingst 1978, Dobbs & Guckert 1988a,b). For chironomid larvae, Kajan and Frenzel (1999) have shown in lab experiments that not only methane oxidation rates, but also the cell numbers of MOB were significantly higher in the larval tubes than in bulk sediment. Already Yingst and Rhoads (1980) showed that feeding of bioturbating invertebrates on the

microbial community in their close vicinity keeps these bacteria in an active physiological state. Accordingly, the increased ^{13}C -uptake by MOB in our experiments could be due to the effect of bioturbation and 'microbial gardening' by chironomids.

In this paper we present unambiguous experimental evidence for the assimilation of methane-derived carbon by chironomid larvae. The strong enrichment of larval biomass in ^{13}C and the amount of labelled PLFAs specific for MOB detected in larval tissue in our experiments suggest that methane-derived carbon has to be considered as an important carbon and energy source for benthic macroinvertebrates in freshwater food webs. Additionally, our study showed that chironomid larvae probably feed on benthic and pelagic MOB and by bioturbation potentially stimulate MOB growth.



Conclusion

In recent years it has become increasingly apparent that the microbial loop and microbial production play an important role in many aquatic ecosystems (Shively et al. 2001, Thorp & Delong 2002, Ventela et al. 2002). The knowledge gathered from these investigations has changed our perception of the ecology of benthic macroinvertebrates in general. Still, there is a significant gap in knowledge with respect to linkages between aquatic sediment biota, e.g. sediment-dwelling organisms, and how that relates to the decomposer microbial loop. The microbial loop is fundamental for most aquatic ecosystems because microorganisms decompose and thereby recycle organic material in the water column and the sediment (Thorp & Delong 2002), which would otherwise be lost from the food web - biologically unavailable. Decomposition of organic matter takes place in aerobic and anaerobic sediments of lakes and wetlands, with anaerobic regions representing the sites for methane production (e.g. Segers 1998). It is estimated that methane oxidising bacteria (MOB, methanotrophs) consume more than 90% of the methane available (Oremland et al. 1993, Utsumi et al. 1998). Thus, MOB represent a significant pathway for the recycling of methane carbon back into aquatic food webs. It has been proposed that anoxic carbon metabolism may be important for the whole pelagic food web of lakes via methane oxidation in the water column (Bunn & Boon 1993, Jones et al. 1999, Bastviken et al. 2003). Another likely site of trophic transfer is at the oxic-anoxic boundary layers in the sediment or at the water-sediment interfaces. The results presented in this thesis looked at this specific habitat in lakes. For the first time it was shown experimentally that sediment-dwelling chironomid larvae can feed directly on MOB biomass and thus include methane-derived carbon in high amounts into their diet (chapter VI). This thesis also emphasises that the contribution of methane carbon to larvae can vary during the year and is influenced strongly by lake characteristics (chapter I & II). Further, results from chapter III highlight that the methanotrophic contribution to larval biomass is site-specific within lakes.

Methane-derived carbon will be partially recycled in the aquatic food web, since chironomid larvae are heavily preyed upon by both invertebrate and vertebrate predators in the sediments (Mackey 1979, Fox 1989, Baxter et al. 2005), as well as

in the pelagic when the pupal stages migrate through the water column to emerge as imagos (Winfield et al. 2002). When leaving the water, the adults become an energy source for terrestrial and avian predators and so chironomid mediation of methane-derived carbon can extend across both benthic-pelagic and aquatic-terrestrial boundaries. In fact the flow of nutrients from aquatic to terrestrial systems has received surprisingly little attention so far. However, it is clear that emerging aquatic insects do provide a substantial energy subsidy for spiders, lizards, bats and birds in terrestrial and riparian food webs (Nakano & Murakami 2001, Baxter et al. 2005, Paetzold et al. 2005). For instance, web-building spiders living in riparian areas can obtain around 60% of their body carbon from aquatic insects (Collier et al. 2002). Moreover at certain times of the year, aquatic insects may account for up to 90% of the diet of some terrestrial bird species (Nakano & Murakami 2001).

The results of this thesis show that methane is not only an important carbon and energy source for aquatic benthic macroinvertebrates but could also substantially fuel terrestrial and riparian food webs. Future work should investigate the magnitude and importance of methane-derived carbon for terrestrial and riparian consumers and how that affects individual consumer fitness and population demographics. Studying and resolving connectivity among food webs will have implications for our understanding of many ecosystem processes such as population/metapopulation dynamics, trophic cascades and stability (Polis et al. 1997).

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J.G. & P.D. fishing for data...

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eingereicht wurden:

Cordes, E.E., Predmore, B.L., Jones, C., Deines, P. & Fisher, C.R. Impact of an ecosystem engineering tubeworm on the habitat characteristics and community structure of Gulf of Mexico hydrocarbon seeps. eingereicht bei *Ecology*.

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Deines, P., Grey, J., Richnow, H.-H. & Eller, G. Are seasonal changes of $\delta^{13}\text{C}$ signatures in chironomid larval biomass related to changes in the microbial methane cycle? eingereicht bei *Aquatic Microbial Ecology*.

Erklärung

Hiermit versichere ich, dass diese Abhandlung - abgesehen von der Beratung durch meine akademischen Lehrer - nach Inhalt und Form meine eigene Arbeit ist und dass ich keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Die Arbeit hat bisher weder ganz noch zum Teil schon an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Desweiteren ist die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis entstanden.

Teile dieser Arbeit wurden als Manuskripte bei Zeitschriften eingereicht und/oder veröffentlicht: Kapitel I wurde mit Gundula Eller als Erstautor, Jonathan Grey, Hans-Hermann Richnow und Martin Krüger als Koautoren bei *FEMS Microbiology Ecology* veröffentlicht. Kapitel II wurde mit Jonathan Grey, Hans-Hermann Richnow und Gundula Eller als Koautoren bei *Aquatic Microbial Ecology* eingereicht. Kapitel IV wurde mit Jonathan Grey als Erstautor bei *Aquatic Microbial Ecology* veröffentlicht. Kapitel III und V befinden sich in Vorbereitung für eine Publikation mit Jonathan Grey als Koautor, genauso wie Kapitel VI mit Paul L. E. Bodelier und Gundula Eller als Koautoren.

Plön, den 22.05.2006

Peter Deines