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The potential of methanotrophic bacteria to compensate for food quantity or food quality limitations in *Daphnia*

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ABSTRACT: The endpoint of anaerobic degradation of organic compounds in aquatic ecosystems is methane. This methane-carbon is not necessarily lost for ecosystem processes as it can be utilized by methane-oxidizing bacteria (MOB), and possibly recycled into benthic and pelagic food webs. The dominant zooplankton in many lakes are daphnids, which could act as vectors for channeling methane-carbon from methanotrophic bacteria upwards in the food chain. We demonstrate, using ^{13}C-enriched diets in laboratory experiments, that methane-carbon can enter the pelagic food web via filtration of MOB by cladoceran zooplankton. Because carbon use efficiency in *Daphnia* appears to be limited by the availability of dietary sterols on prokaryotic diets, we test the hypothesis that the uptake of MOB, the only prokaryotes possessing sterols and sterol-like compounds, can lead to a quantitative and qualitative upgrading of phytoplankton diets of *Daphnia*. Our results confirm the general superiority of eukaryotic over prokaryotic food sources for *Daphnia* growth and reproduction. Although MOB addition compensated for limited food quantity, we found no evidence for a qualitative upgrading through MOB. Consequently, there was no direct relationship between the quantity of food available and the fitness (somatic growth) of *Daphnia*, but rather a strong food quality effect, independent of MOB addition. Our findings support the view that methane is an important carbon source to pelagic ecosystems and thus have strong implications for qualitative and quantitative assessments of carbon recycling pathways in aquatic ecosystems.

KEY WORDS: *Daphnia* · Methanotrophs · Sterols · Hopanoids · Stable isotopes

INTRODUCTION

In lakes (also 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). Lakes in particular have been found to be 'hot spots' of methane production and thus contribute a large amount (1–16%) to global natural methane emissions (Bastviken et al. 2004). Methanogenesis contributes 30 to 80% of the anaerobic carbon mineralisation in lake waters and sediments (Rudd & Hamilton 1978, Fallon et al. 1980, Bédard & Knowles 1991). The fraction of methane oxidised strongly depends on the type of release — diffusion in small bubbles or ebullition, the latter leading to a higher flux of methane to the atmosphere (Steele et al. 2009). It is also established that methane emissions and oxidation can vary spatially in lakes between profundal sediments, open water and lit-

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toral areas. Nevertheless, a large proportion of the methane produced in profundal sediments is oxidized by methane-oxidizing bacteria (MOB) as it passes into the oxic zones within 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 methane can be converted to MOB biomass. This bacterial biomass is not only accessible to protozooplankton/phagotrophic protozoans (i.e. heterotrophic nanoflagellates and ciliates) but also to metazooplankton, such as cladocerans. Bacteria can be very effectively consumed by zooplankton and can constitute a substantial part of zooplankton diets (e.g. Gophen & Geller 1984, Kankaala 1988, Pace & Cole 1994). Feeding on MOB biomass would be one of the few cases where microbially produced carbon in the form of methane, otherwise unavailable for higher organisms, can enter the zooplankton trophic level via direct transfer from MOB.

Reports from studies of different stratified lakes show that considerable methanotrophic biomass can develop, accounting for up to 3% of total DAPI counts (Eller et al. 2005, Schubert et al. 2010); a study using group-specific phospholipid fatty acid analysis estimated that MOB can account for up to 41% of the total bacterial biomass (Sundh et al. 2005). This indicates 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). Besides a zone of aerobic methane oxidation, an additional zone of anaerobic methane oxidation in the anoxic water body may be present, suggesting that aerobic and anaerobic MOB can be active throughout the water column (Eller et al. 2005). Further, the ability of zooplankton to exploit bacterial resources is not limited to the aerobic parts of lakes. Because of the vertical migration commonly observed in zooplankton (Lampert 1992, 1993), foraging in deeper water layers may be advantageous even if there are high food concentrations in the surface water. Besides reducing the risk of predation by predators such as fish (Lampert 1993), foraging in deeper water layers would also enable exploitation of the potentially dense microbial community in or below the oxycline (e.g. Cole et al. 1993). In particular, methanotrophic bacteria (Jones et al. 1999, Bastviken et al. 2003, Kankaala et al. 2006, Taipale et al. 2008) and perhaps green sulfur bacteria (Kuuppo-Leinikki & Salonen 1992) can be exploited as food by zooplankton able to graze around the oxic–anoxic interface. Jones & Grey (2011) recently reviewed evidence for the transfer of methane-derived carbon to higher trophic levels in freshwater food webs. In addition to field studies (e.g. Bunn & Boon 1993, Jones et al. 1999, Bastviken et al. 2003), laboratory experiments (Kankaala et al. 2006, 2007) and experimental field data by Taipale et al. (2007, 2008) support the hypothesis that *Daphnia* feed on MOB. Generally, MOB consumption was estimated via the characteristically depleted $\delta^{13}C$ values of MOB and, hence, MOB consumers, which is due to the strongly depleted $\delta^{13}C$ signal of methane-carbon. However, there are alternative hypotheses explaining the observed low $\delta^{13}C$ values of zooplankton: for example, Lennon et al. (2006) argued that the low $\delta^{13}C$ values they found in northeastern lakes of the USA could also be attributed to zooplankton feeding on ^{13}C-depleted phytoplankton. Most of the studies on MOB consumption used stable carbon isotope analysis ($\delta^{13}C$), which is now being widely used in studies of the sources and fluxes of organic matter in lake food web analysis (Grey et al. 2001, Pace et al. 2004, Grey 2006). However, there is still limited evidence that MOB provide a suitable food source for zooplankton. In laboratory experiments, Kankaala et al. (2006) found the $\delta^{13}C$ signature of *Daphnia* to be significantly more depleted when feeding on microbial suspensions enriched with biogenic methane compared with non-enriched cultures. The cultures enriched with methane also showed equal or greater *Daphnia* growth rates than the non-enriched cultures.

Taking recent work into account, which has identified dietary sterol content as a crucial factor in determining food quality for *Daphnia* (von Elert et al. 2003, Martin-Creuzburg et al. 2005b, 2008, 2009), MOB could play an important role not only in terms of quantity, but also in terms of dietary quality. MOB are unique among prokaryotes in possessing relatively large amounts of sterols or sterol-like compounds, which are absent in most other prokaryotes (Bird et al. 1971, Cvejic et al. 2000, Volkman 2003). Experimental evidence suggests that sterol-like compounds, such as hopanoids, can fulfill functions equivalent to those of sterols (Ourisson et al. 1987, Raederstorff & Rohmer 1988, Cvejic et al. 2000). Because sterols are essential for multiple physiological processes and arthropods are incapable of the de novo synthesis of sterols, these compounds must be obtained from the diet (Grieneisen 1994). Sterols are precursors of steroid hormones, such as ecdysteroids, which are involved in the process of molting and the manufacture of membrane constituents (Grieneisen 1994).

Here, we test the hypotheses that methane via MOB can serve as a supplementary carbon source for zooplankton and that MOB could potentially be a dietary source for sterols or hopanoids, thus biochemically upgrading sterol-free diets. By using ^{13}C-

enriched methane, carbon isotope values of MOB can be manipulated, allowing the flow of methane-carbon into zooplankton to be tracked. When this enriched methane is utilized by MOB, their carbon isotope value will become correspondingly enriched. In standardized growth experiments, a eukaryotic green alga rich in phytosterols and a sterol-free cyanobacterium were supplied to *Daphnia magna* to simulate a diet with high and low dietary sterol availability. By adding high and low amounts of additional MOB, we simulated spring conditions with a low proportion of MOB in the seston and autumn conditions with a higher proportion of MOB in the edible size fraction of the plankton. In this way, we tested the relative importance of food quantity and food quality (i.e. sterol/hopanoid availability) on growth and reproduction of the keystone herbivore *Daphnia*.

MATERIALS AND METHODS

Cultivation and preparation of food organisms

The green alga *Scenedesmus obliquus* (Kützing), strain SAG 276.3a, was used as food for the stock culture of daphnids and the experiments. It was grown in a chemostat culture in Chu12-medium (Müller 1972) at a dilution rate of 0.5 d^{-1} at 20°C at approximately 100 µmol photons m^{-2} s^{-1} light intensity with a 12 h light:12 h dark photoperiod. *S. obliquus* is rich in phytosterols, which daphnids can convert into the major crustacean sterol, cholesterol (Martin-Creuzburg & von Elert 2004). The sterol-free cyanobacterium *Synechococcus elongatus* (Nägeli) was grown in semi-continuous batch culture in WC medium (Guillard 1975) at a dilution rate of 0.2 d^{-1} under the same light and temperature conditions as *S. obliquus*. Although the large-celled *S. obliquus* was concentrated by sedimentation, the cyanobacterium *S. elongatus* was concentrated by centrifugation. Subsequently, both phytoplankton species were resuspended with filtered lake water and the carbon concentrations of the food suspensions were estimated from photometric light extinction (800 nm) and from carbon extinction equations determined previously. The MOB strain *Methylosinus trichosporium* (NCIMB strain number 11131) was grown in nitrate mineral salt (NMS) medium with 1/10 of the standard nitrate concentration (modified from Whittenbury et al. 1970) and supplemented with ^{13}C-labelled methane. Two 1 l screw cap glass bottles were filled with 0.5 l NMS medium, inoculated with *M. trichosporium* and closed with a rubber stopper. From the headspace, 180 ml air were removed and replaced by 200 ml $^{12/13}CH_4$ at a mixing ratio of 70/30 ([v/v], $^{13}CH_4$ 99 atom percentage; Isotec). Pre-incubation was carried out at 20°C in the dark on a rotary shaker (120 rpm) for 2 d prior to use in the experiment. Before the feeding experiments, bacteria were harvested by centrifugation, washed and re-suspended in filtered lake water. Cell numbers of MOB were determined from samples fixed with 2% formaldehyde. MOB cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and counted by epifluorescence microscopy (Porter & Feig 1980) and volumes were adjusted. An effect of the culture medium on the quality of MOB as food seems unlikely, as a study by Bodelier et al. (2009) showed that growth on different substrates did not affect the biochemical composition (fatty acid profiles) of the investigated MOB strains.

Experimental design

Laboratory growth experiments were conducted with fourth clutch juveniles (birth ± 6 h) of a clone of *Daphnia magna*, which was originally isolated from Großer Binnensee, Germany (Lampert 1991). The newborn experimental animals were cultured to the age of 48 h on *Scenedesmus obliquus* (2 mg particulate organic carbon [POC] l^{-1}) as described in von Elert et al. (2003). The growth experiment was carried out in 1.5 l glass beakers filled with 600 ml of filtered Lake Schöhsee water (0.4 µm pore-sized membrane filter) and 6 *D. magna* at 20°C and a constant 16 h light:8 h dark cycle.

The experiment was designed to test whether methanotrophic bacteria can provide a significant food source for lake zooplankton and whether their sterols can increase the carbon transfer efficiency of cyanobacterial carbon known for its low content of sterols. Six treatments with 6 replicates each were produced by factorial combination of 3 food suspensions containing *Scenedesmus obliquus* (SCE) or *Synechococcus elongatus* (SYN) with 3 different amounts of MOB. The carbon concentration of phytoplankton added per day was 0.1 mg POC l^{-1}. This non-saturating amount was chosen because it is intermediate between the food threshold concentration of *Daphnia magna* for somatic growth (approximately 0.017 mg POC l^{-1}, Gliwicz 1990) and the incipient limiting level of approximately 0.25 mg POC l^{-1} (Lampert & Muck 1985). The 3 MOB additions were as follows: no MOB added (NO-MOB); 1×10^8 *Methylosinus trichosporium* cells l^{-1} added (LO-MOB), representing approximately 50% of the

phytoplankton (*S. obliquus* or *S. elongatus*) carbon added; and 1×10^9 *M. trichosporium* cells l^{-1} added (HI-MOB), representing approximately 500% of the phytoplankton carbon added. This particular MOB concentration (LO-MOB) was chosen because MOB can represent up to 2 to 3% of all DAPI-stained cells in lake water (Eller et al. 2005, Schubert et al. 2010). In contrast, the HI-MOB treatment simulates a situation where all lake bacteria would be MOB. Every day the animals were transferred into new jars with fresh food suspensions. The experiment lasted until animals from one treatment reached maturity and made investments into their first clutch, which was at Day 7 at saturating food quantities of *S. obliquus* and at Day 8 in all other treatments. After counting clutch sizes (numbers of neonates per individual for each replicate and treatment) under a dissecting microscope, the animals were dried in an oven for 24 h at 60°C, and somatic growth rates and stable carbon isotopes determined from the dried samples. Somatic growth rates (g) were determined as the increase in dry mass (M) during the experiment using the equation $g = (\ln M_t - \ln M_0)/t$. Samples of the experimental animals were taken at the beginning (M_0) and at the end (M_t) of the experiment. The samples consisting of 4 to 10 animals were dried for 24 h at 60°C and weighed on an electronic balance for n = 6 replicates.

Analyses of POC and stable carbon isotopes

Algal and MOB samples were sampled each day during the experiment by filtration on pre-combusted Whatman GF/F glass fiber filters, dried at 60°C over night and stored in an exsiccator until analysis using a FISONS® NA2000 elemental analyzer. Cladocerans from the growth measurements were transferred into tin cups and stored in an exsiccator for subsequent analysis of stable carbon isotopes. Algal and MOB samples were sampled by filtration every day using pre-combusted Whatman GF/F glass fiber filters. After drying for 24 h at 60°C, filters were stored in an exsiccator prior to analysis. Isotope analyses were performed using a Micromass IsoPrime continuous flow isotope ratio mass spectrometer interfaced with a Carlo-Erba NA1500 elemental analyzer. Isotope ratios are expressed using the δ notation in units per mil (‰) as follows: $\delta^{13}C$ (‰) = $[(R_{sample}/R_{standard}) - 1] \times 1000$ with $R = {}^{13}C/{}^{12}C$. The reference material used was a secondary standard of known relation to the international standard of Vienna Pee Dee belemnite. Typical precision for a single analysis was ±0.1 ‰ for $\delta^{13}C$.

Statistics and modelling

Somatic growth rates, survival as well as $\delta^{13}C$ values of daphnids using food treatment as the effect variable were compared using 2-way ANOVA and Tukey's honestly significant difference (HSD) post hoc test. Incorporation of methane carbon by daphnids was tested with a one-way ANOVA. Data were tested with a Tukey–Kramer post hoc test for significant differences between the groups. Statistical analyses were performed either with the SigmaStat module of SigmaPlot v.11 (Systat) or JMP 5.0.1.2. To estimate the percent contribution of MOB to *Daphnia*, a 2-source isotope mixing model (Phillips & Gregg 2001) was applied, using MOB and phytoplankton as alternative dietary carbon sources.

RESULTS

Food biomass in experimental treatments

Food concentrations of *Scenedesmus obliquus* and *Synechococcus elongatus* (±SD) were 0.072 ± 0.005 and 0.086 ± 0.027, respectively, and thus only slightly lower than the planned 0.1 mg POC l^{-1}. The intention of the high and low MOB addition was to add 50% (0.05 mg POC l^{-1}) or 500% (0.5 mg POC l^{-1}) to the approximately 0.1 mg phytoplankton carbon l^{-1}. The actual values of MOB carbon determined in the added cultures were close to the target concentrations with 0.042 ± 0.011 mg POC l^{-1} for the LO-MOB treatment and 0.423 ± 0.114 mg POC l^{-1} for the HI-MOB treatment.

Daphnia magna growth and egg production rates

Saturating amounts of the high-quality food alga *Scenedesmus obliquus* allowed for high growth rates (0.35 d^{-1}) of *Daphnia magna*, whereas all treatments with food concentrations below the incipient limiting level (approximately 0.25 mg POC l^{-1}; Lampert & Muck 1985) yielded somatic growth rates below 0.2 d^{-1} (Fig. 1). Both the phytoplankton organism offered as food to *D. magna* and the addition of MOB had a significant influence on the somatic growth of the daphnids, as indicated by a 2-way ANOVA and post hoc comparisons with Tukey's HSD tests (PHYTO $F_{1,35} = 557.2$, $p < 0.001$, MOB $F_{2,35} = 71.0$, $p < 0.001$, PHYTO × MOB $F_{2,35} = 25.8$, $p < 0.001$). Growth rates with or without addition of MOB were always higher on the green

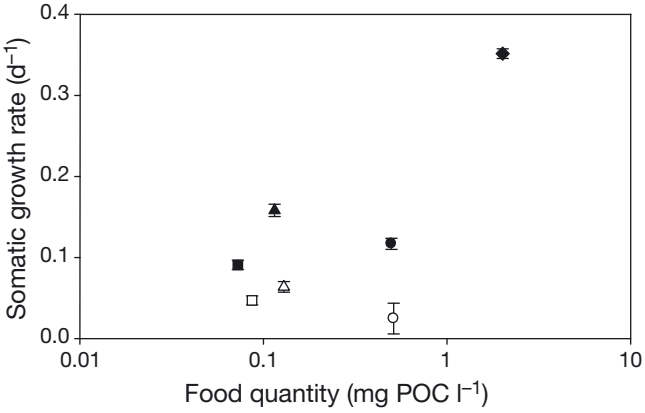

Fig. 1. Relationship between somatic growth rate (means ± 1 SD, n = 6) of *Daphnia magna* fed either *Scenedesmus obliquus* (filled symbols) or *Synechococcus elongatus* (open symbols) and available food quantity (particulate organic carbon [POC] l^{-1}, note log scale). Squares: limiting quantities of phytoplankton without addition of methane-oxidizing bacteria (MOB); triangles: addition of MOB in low concentration; circles: addition of MOB in high concentration; diamond: saturating quantity of algae (*S. obliquus*, 2 mg POC l^{-1}) (phytoplankton food quantities in the other treatments were limiting, at 0.1 mg POC l^{-1}). All growth rates — except for those on a diet of *S. elongatus* without MOB (open square) or *S. elongatus* with MOB in high concentration; (open circle) — were found to be significantly different (2-way ANOVA, Tukey's honestly significant difference, $p < 0.05$)

alga *S. obliquus* than the respective MOB treatment on the cyanobacterium *Synechococcus elongatus* (Fig. 1), thus confirming the higher food quality of the eukaryotic versus the prokaryotic phytoplankton organism. For both phytoplankton organisms, addition of low amounts of MOB (equivalent to 0.042 mg POC l^{-1}) to approximately 0.1 mg l^{-1} of phytoplankton POC (see above) significantly increased the somatic growth rates of the daphnids compared with the treatments without addition of MOB (Fig. 1). Addition of high amounts of MOB (equivalent to 0.423 mg POC l^{-1}) to approximately 0.1 mg l^{-1} of phytoplankton POC (see above) yielded somatic growth rates intermediate to those with and without the addition of low amounts of MOB on a basic diet of *S. obliquus* (Fig. 1). On a basic diet of *S. elongatus*, addition of high amounts of MOB decreased the growth rates to the same level (no significantly difference) as that on *S. elongatus* without addition of MOB, but to a significantly lower level than with the addition of low amounts of MOB (Fig. 1).

Overall, an increase in carbon availability through the addition of low amounts of MOB to both *Scenedesmus obliquus* and *Synechococcus elongatus* led to an increase in somatic growth that is consistent with other food quantity limitation studies with *Daphnia* feeding on *S. obliquus* (e.g. Wacker & von Elert 2001). However, a further increase in the availability of MOB-derived carbon (i.e. in the high-MOB addition treatments) led to a decoupling of the relationship between carbon availability and somatic growth, as can clearly been seen in the comparison with the treatment in which saturating amounts of *S. obliquus* (2 mg POC l^{-1}) were offered to *D. magna* (Fig. 1). The mortality of *D. magna* did not differ between treatments (2-way ANOVA).

Similar to the growth rates, saturating amounts of the high-quality reference food *Scenedesmus obliquus* led to the production of high egg numbers (mean ± SD of 9.62 ± 0.44) (Fig. 2). When the food quantity (on the high-quality organism *S. obliquus*) was limiting (0.1 instead of 2.0 mg POC l^{-1}), lower numbers of eggs per mother were produced. This food quantity effect could be partly ameliorated by the addition of MOB at a carbon concentration equivalent to 0.042 mg POC l^{-1}, which led to an increase in egg number per female from 0.06 ± 0.14 with low amounts of *S. obliquus* only to 1.59 ± 0.34 in the treatment where low amounts of MOB (LO-MOB) were added to the same amount of *S. obliquus* (Fig. 2).

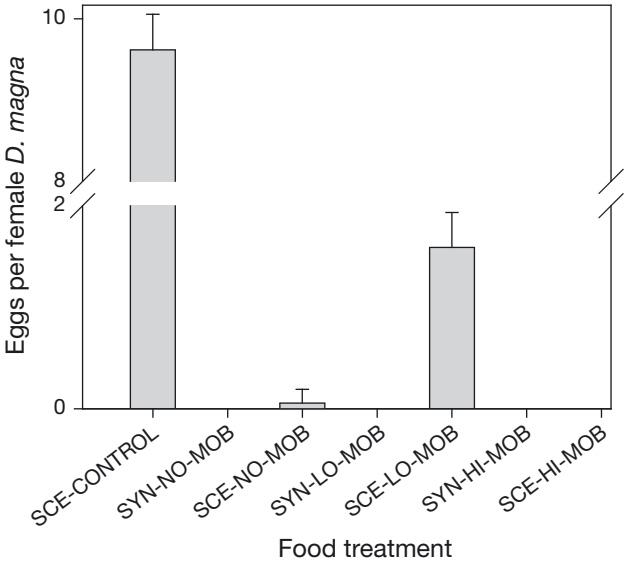

Fig. 2. Egg numbers (means ± 1 SD, n = 6) in the first brood of *Daphnia magna* fed *Synechococcus elongatus* without methane-oxidizing bacteria (MOB) (SYN-NO-MOB), *Scenedesmus obliquus* without MOB (SCE-NO-MOB), *S. elongatus* with a low concentration of MOB (SYN-LO-MOB), *S. obliquus* with a low concentration of MOB (SCE-LO-MOB), *S. elongatus* with a high concentration of MOB (SYN-HI-MOB) or *S. obliquus* with a high concentration of MOB (SCE-HI-MOB). The control treatment for food-saturated growth (SCE-CONTROL) was *S. obliquus* at a saturating food quantity of 2 mg POC l^{-1}; phytoplankton food quantities in the other treatments were limiting (0.1 mg POC l^{-1})

However, when MOB at a high carbon concentration equivalent to 0.42 mg POC l^{-1} (HI-MOB) was added to a diet of *S. obliquus*, no eggs were observed in the maternal brood chambers, indicating an inhibitory effect of high amounts of dietary MOB on the reproduction of *D. magna* (Fig. 2). None of the treatments with *Sycnechococcus elongatus* as the basic diet produced eggs independent of the addition of MOB (Fig. 2).

Daphnia magna stable isotope patterns

Daphnia magna collected at the end of the experiment exhibited significant differences in their mean $\delta^{13}C$ values depending on the amount of MOB biomass added. At the start of the experiment, $\delta^{13}C$ values of *D. magna* were −16.6 ± 0.1‰. At the end of the experiment, mean $\delta^{13}C$ values for *D. magna* fed *Scenedesmus obliquus* were −10.9‰; for animals fed *Synechococcus elongates*, this value was −20.3‰, and for those fed labelled MOBs it was 10 293.9‰. The $\delta^{13}C$ values of *D. magna* differed significantly depending on the food treatment ($F_{5, 30}$ = 1495.48, p < 0.0001) (Fig. 3). $\delta^{13}C$ values of daphnids in the NO-MOB treatments were −14.0 ± 0.7‰ with *S. obliquus* and −17.8 ± 2.0‰ with *S. elongatus* as the food organism. Increasing proportions of MOB led to a significant enrichment in $\delta^{13}C$ of *D. magna* in all treatments (*S. obliquus*: LO-MOB 3589.0 ± 297.3‰, HI-MOB 6980.9 ± 268.7‰; *S. elongatus*: LO-MOB 4551.3 ± 120.4‰, HI-MOB 9580.8 ± 418.1‰; Fig. 3). LO- and HI-MOB treatments significantly differed between both algal treatments, with daphnids feeding on *S. elongatus* and MOB being more enriched.

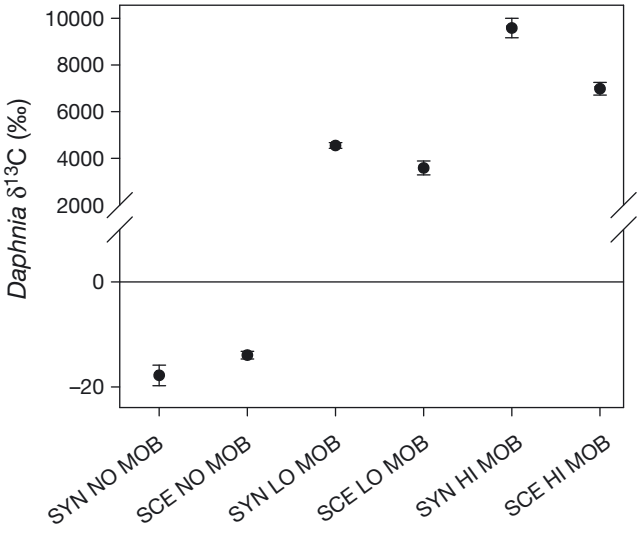

Fig. 3. $\delta^{13}C$ (‰, means ± 1 SD, n = 6) of *Daphnia magna* fed *Synechococcus elongatus* without methane-oxidizing bacteria (MOB) (SYN-NO-MOB), *Scenedesmus obliquus* without MOB (SCE-NO-MOB), *S. elongatus* with a low concentration of MOB (SYN-LO-MOB), *S. obliquus* with a low concentration of MOB (SCE-LO-MOB), *S. elongatus* with a high concentration of MOB (SYN-HI-MOB) or *S. obliquus* with a high concentration of MOB (SCE-HI-MOB)

The mixing model estimated that under LO-MOB conditions and *Scenedesmus obliquus* as the algal food source, 35% of *Daphnia magna* biomass was assimilated from methane-derived carbon; this increased to 68% under HI-MOB conditions. For daphnids grown on *Synechococcus elongatus* and MOB, a similar trend was found with 44% (LO-MOB) and 93% (HI-MOB) of methane-derived carbon contributing to *Daphnia* biomass.

DISCUSSION

Although methane cycling in lakes has been studied for more than 40 yr, it was not until recently that methane has gained interest as an alternative carbon and energy source in freshwater ecosystems. Compared with benthic food webs in lakes where the contributions of methane-carbon have been intensively studied, there is less evidence for a methane-carbon contribution to the carbon cycling in planktonic food webs of lakes (Jones & Grey 2011). Field data suggest that the exploitation of methane-carbon by zooplankton can be quite variable and that lake morphology, trophic status and seasonality have a profound impact on how much methane-carbon can be utilized. Low $\delta^{13}C$ values in zooplankton are usually indicative of the uptake of MOB via grazing (e.g. Jones et al. 1999, Taipale et al. 2007). Nevertheless, it is important to keep in mind that there are other potential pathways that could result in low $\delta^{13}C$ values of zooplankton without the consumption of MOB, such as selective feeding on ^{13}C-depleted phytoplankton (Lennon et al. 2006, Jones & Grey 2011). Another aspect that has not yet been investigated in more detail is the potential of MOB to upgrade the food quality of cyanobacterial carbon or to serve as a sole carbon source for daphnids, by supplying essential sterols or sterol-like compounds.

Our results support recent findings that methane-carbon can re-enter freshwater food webs by the direct consumption of MOB by cladoceran zooplankton (Kankaala et al. 2007, Taipale et al. 2007). The addition of MOB pre-cultured with ^{13}C labelled methane strongly affected the $\delta^{13}C$ signatures of

Daphnia magna. Increasing proportions of MOB in the diet led to an increased enrichment (label incorporation) in *D. magna*. This suggests that *D. magna* would also utilize this resource in nature where MOB can be suspended in the water column.

As predicted by our hypothesis, the nutritional quality of the supplied phytoplankton species strongly affected daphnids' fitness (i.e. somatic growth and reproduction). High rates of growth and reproduction of *Daphnia magna* were found on the high-quality food *Scenedesmus obliquus*. Limiting quantities of this high-quality food organism obviously reduced growth and reproduction as compared with saturating food quantities. In all treatments, *D. magna* growth was higher on the eukaryotic *S. obliquus* as the base food compared with the prokaryotic *Synechococcus elongatus* as base food. This supports the view that *Daphnia* growth on cyanobacteria is limited by the prokaryotes' lack of essential lipids such as PUFAs and sterols (DeMott & Müller-Navarra 1997, von Elert et al. 2003, Martin-Creuzburg et al. 2005b). Addition of MOB in low abundance increased the somatic growth of *D. magna* on both phytoplankton diets. This can be interpreted as a partial compensation of the food quantity limitation in the experiment. Addition of MOB in high abundance to a (quantity-limited) diet of *S. obliquus* resulted in higher *D. magna* growth rates than without MOB, but lower growth rates than with the addition of MOB in low abundance, whereas an addition of MOB in high abundance to a (quantity-limited) diet of *S. elongatus* yielded the same *D. magna* growth as a diet of pure *S. elongatus* without addition of MOB. Kankaala et al. (2006) found a linear relationship between the natural logarithm of food concentration and *Daphnia* growth rates, irrespective of food type (algae with or without MOB). In contrast to this, our results suggest that MOB as an almost sole food source (HI-MOB treatment) is detrimental for *D. magna* growth and cannot compensate for a limitation by food quantity. This becomes particularly apparent when comparing the somatic growth rates on phytoplankton plus high availability of MOB-derived carbon with the treatment in which saturating quantities of *S. obliquus* were fed to *D. magna*: although the high growth rates on saturating quantities of *S. obliquus* are consistent with other experiments on the relationship between food quantity and somatic growth (e.g. Wacker & von Elert 2001), the growth yield on both phytoplankton species with the addition of MOB in high (albeit different) abundance is much lower than would be expected by a compensation of food quantity limitation through the supplementation of MOB-carbon. This strongly indicates that MOB are qualitatively inferior to (eukaryotic) phytoplankton, although the mechanisms underlying this observation remain unclear. Despite the notion that bacteria are generally poorly assimilated, glucosaminidase enzymes digesting peptidoglycans in bacterial cell walls can be found in heterotrophic bacteria, protists and metazoa (Zubkov & Sleigh 1998). Recent studies have further confirmed the nutritional value of bacterial food such as methane-utilizing bacteria as a source of protein. This was based on criteria such as chemical composition, effects on energy metabolism, digestibility, growth performance and animal health (Øverland et al. 2010).

Daphnia magna reproduction is highly dependent on both resource quantity and quality, as shown in previous studies (e.g. Urabe & Sterner 2001). Saturating quantities of the high-quality food organism *Scenedesmus obliquus* yielded high egg numbers. Limiting food quantities caused a reduction in egg numbers even on the high-quality food organism. This apparent food quantity limitation could be ameliorated by the addition of (low amounts of) MOB. Addition of MOB in high abundance suppressed egg formation on a *S. obliquus* diet, which indicates that MOB alone are insufficient for a successful population growth of daphnids. MOB might even contain constituents that inhibit *D. magna* reproduction at high concentrations. Aquatic bacteria, with their remarkable biosynthetic versatility, produce a vast range of secondary metabolites that are biologically active towards protozoan and metazoan grazers, functioning as antipredator compounds (Jensen & Fenical 1994, Matz & Kjelleberg 2005, Deines et al. 2009). As shown in previous investigations, freshwater proto- and metazooplankton appear to have well-defined sensitivity levels to bacterial metabolites such as microcystins and violacein (Christoffersen 1996, Deines et al. 2009). It has been estimated that *Daphnia* species need at least 50% of the green algae *S. obliquus* in their diet to compensate for a dietary sterol deficiency when consuming a sterol-free diet such as cyanobacteria (Martin-Creuzburg et al. 2005b). This suggests that we exceeded this boundary with our HI-MOB treatment, as shown by the isotope mixing data, where 68% (*S. obliquus*) and 93% (*Synechococcus elongatus*) of *Daphnia* carbon was contributed by MOB. No reproduction was observed in any of the treatments where *D. magna* was fed *Synechococcus*, independent of MOB addition. This corroborates the strong impact of dietary quality on *Daphnia* reproduction (e.g. Martin-

Creuzburg et al. 2008, 2009). In a recent study by Martin-Creuzburg et al. (2011), 6 different bacterial isolates from 5 phylogenetically distinct groups were tested for their food quality, one of them being a methanotrophic bacterium. By supplementing bacterial food suspensions with sterols and/or polyunsaturated fatty acids, the sterol limitation of *Daphnia* on prokaryotic food organisms was confirmed. Interestingly, *D. magna* did not produce eggs in any of the different treatments of Martin-Creuzburg et al. (2011) within the experimental period (6 d), except for a single animal (on *Flavobacterium* sp. supplement with cholesterol and eicosapentaenoic acid). Our data are in line with these findings, supporting the hypothesis that a bacterial or bacterial-dominated diet can lead to sterol limitation of reproduction in daphnids.

We have demonstrated here that the addition of MOB to limiting quantities of phytoplankton carbon is able to (at least partially) compensate for growth limitation by food quantity. However, we found no evidence for a similar compensatory mechanism on the level of food quality (no sterol-effect on *Synechococcus elongatus* vs. *Scenedesmus obliquus*). This could suggest that plant/phytoplankton and prokaryote (MOB) sterols are not substitutable. An overlap in the occurrence of certain sterols between microalgae and bacteria has been reported (Volkman 2003), but comparisons between phytoplankton and bacteria are lacking. Martin-Creuzburg et al. (2011) tested the food quality of different heterotrophic bacteria and demonstrated that somatic growth rates in *Daphnia magna* increased significantly upon supplementation with cholesterol. Their study thus demonstrates that the major food quality constraint in bacteria is the lack of sterols, owing to the fact that sterols are vital components of eukaryotic cell membranes (Martin-Creuzburg et al. 2011). However, sterols could potentially be replaced by other, functionally equivalent compounds, such as tetrahymanol, hopanoids and other polyterpenoids (Ourisson et al. 1987, Raederstorff & Rohmer 1988, Martin-Creuzburg et al. 2006). To date, almost nothing is known about sterol or hopanoid compositions and concentrations from prokaryotes in lakes. Only a few studies have looked at depth distributions of phytosterols (such as sitosterol) and zoosterols (such as cholesterol) in lakes (e.g. Bechtel & Schubert 2009). Likewise, information on sterol and hopanoid biosynthesis in MOB are scarce; a few species, such as *Methylococcus capsulatus* (Jahnke & Nichols 1986), however, have been studied in more detail. Literature on biochemical differences between type I and type II MOB in relation to sterol and hopanoid composition is not available at this point. In *Methylosinus trichosporium*, hopanoids have been detected and isolated (Neunlist & Rohmer 1985, Cvejic et al. 2000) that are functionally equivalent to sterols as structural components of cell membranes (Conner et al. 1968, Harvey & McManus 1991). Further, hopanoids have been shown to be incorporated into the membranes of eukaryotes as sterol supplements. This can explain the high nutritional value of hopanoid-rich flagellates and ciliates for herbivorous zooplankton, which can at least partly release this zooplankton from sterol limitation (Martin-Creuzburg et al. 2005a, 2006, Bec et al. 2006). It remains to be tested whether the hopanoid content of *M. trichosporium* used in the present study was too low to release *Daphnia* from sterol limitation. It is known that the composition and amount of hopanoids varies strongly between different strains of MOB (Cvejic et al. 2000). A naturally diverse community of MOB with quantitative and qualitative differences in sterol and hopanoid biosynthesis might provide a better resource than a single strain as used here. In addition to direct grazing on MOB, methane-derived carbon could also enter higher trophic levels (e.g. *Daphnia*) via intermediate consumers (i.e. heterotrophic nanoflagellates and ciliates; Jones & Lennon 2009) which could synthesize sterols and hopanoids that subsequently release zooplankton feeding on these intermediate consumers from sterol limitation (Martin-Creuzburg et al. 2005a, 2006, Bec et al. 2006).

To gain a better understanding of the benefits and limitations of bacteria as a carbon source for daphnids, further studies are needed to elucidate how different mixtures of algae and MOB (using a variety of strains) support growth and reproduction in *Daphnia*. Although there is general evidence for methane-derived carbon to be considered as a potential important carbon source for lake zooplankton (see review by Jones & Grey 2011), the extent to which zooplankton exploit methane-carbon through grazing (direct), an intermediate consumer pathway, or an indirect pathway via phytoplankton remains to be explored, taking various environmental conditions and lake characteristics into account.

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