The incredible lightness of being methane-fuelled: stable isotopes reveal alternative energy pathways in aquatic ecosystems and beyond

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Abstract

We have known about the processes of methanogenesis and methanotrophy for over 100 years, since the days of Winogradsky, yet their contributions to the carbon cycle were deemed to be of negligible importance for the majority of that period. It is only in the last two decades that methane has been appreciated for its role in the global carbon cycle, and stable isotopes have come to the forefront as tools for identifying and tracking the fate of methane-derived carbon within food webs, especially within aquatic ecosystems. While it is not surprising that chemosynthetic processes dominate and contribute almost 100% to the biomass of organisms residing within extreme habitats like deep ocean hydrothermal vents and seeps, way below the reach of photosynthetically active radiation, it is perhaps counterintuitive to find reliance upon methane-derived carbon in shallow, well-lit, well-oxygenated streams. Yet, apparently, methane-derived carbon contributes to varying degrees across the spectrum from point sources to extremely diffuse sources. Certainly a good proportion of the evidence for methane-derived carbon contributing to freshwater food webs comes from somewhere in the middle of that spectrum; from studies of seasonally stratifying lakes (mono- or dimictic) wherein, there is a defined gradient or boundary at which anoxic meet oxic conditions and consequently allows for close coupling of methanogenesis and methanotrophy. However, even seemingly well-mixed (polymictic) lakes have a contribution of methane-derived carbon contributing to the benthic biomass, despite an almost continual supply of photosynthetic carbon being delivered from the surface.

Aside from the fundamental importance of identifying the carbon sources fuelling biomass production, stable isotopes have been integral in the tool box of palaeolimnologists seeking to identify how contributions from methane have waxed and waned over time. Here, we synthesise the current state of knowledge in the use of stable isotopes to trace methane-derived carbon in primarily freshwater ecosystems.

Running head:

Identifying and tracking methane in food webs

Keywords:

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A brief synopsis on the global importance of methane in aquatic systems, and particularly in freshwaters

The global carbon cycle was considered, until relatively recently, to be solely the flux and storage of carbon between the atmosphere, and terrestrial and oceanic pools. Within the total carbon budget, it has been noted that despite their relatively small area, inland freshwaters make a considerable contribution to the global methane (CH_4) budget with emissions of CH_4 from freshwaters being at least comparable to the terrestrial CH_4 sink (Battin *et al.*, 2009). However, there is a considerable bias toward data from lakes and other wetlands, and the role of rivers remains poorly defined (Bastviken *et al*., 2011). Emissions of CH_4 may be small in terms of carbon, but one must consider that CH_4 is a more potent greenhouse gas than CO_2 over century time scales; (Bastviken *et al*., 2011) estimated that global CH_4 emissions expressed as CO_2 equivalents correspond to at least 25% of the estimated terrestrial greenhouse gas sink. Our understanding of the global carbon cycle will only be complete if we include the flux of carbon through inland freshwaters (Battin et al., 2009); (Cole et al., 2007); Trimmer *et al*., 2012); getting to grips with methane-fuelling of food webs is an interesting and important component of this. Indeed, (Cole, 2013) noted that “the role of methane in supporting food webs in lakes, and perhaps even beyond their shores, has come as a surprise” and that “the notion that lake methane partially supports higher organisms in surrounding terrestrial environments fundamentally changes our understanding of how aquatic food webs work”.

Methanogenesis is a universal terminal degradation process of organic matter in anoxic aquatic sediments when inorganic oxidants such as nitrate, ferric iron or sulphate are depleted (Conrad, 2005). Hence, in marine systems where there is typically a high concentration of sulphate, the sulphur cycle tends to dominate chemosynthesis, but in freshwaters where sulphate concentrations are typically lower (Hobbie, 1988) then methanogenesis dominates. Stable isotopes have been an incredibly useful tool in the identification and quantification of methanogenic and methanotrophic pathways (Conrad, 2005) and further identifying the constituents of the complex microbial community that is actively involved via stable isotope probing (SIP; e.g. (He et al., 2012), but those aspects are not the focus of this review. Methane may be lost directly from the system via ebullition or the recently hypothesised micro-bubble pathway, stochastic processes notoriously difficult to quantify (Prairie and del Giorgio, 2013) or be effectively ‘piped’ to the surface via plants (Bergstrom et al., 2007); (Sanders et al., 2007). Alternatively, or in addition, it may subsequently serve as an energy and C source for methanotrophs (methane oxidising bacteria; MOB), typically at oxic-anoxic boundaries (if anaerobic CH_4 oxidation is excluded) in the sediment, or in the water column (Rudd and Taylor, 1980; (Kankaala et al., 2007). It is essentially from this point in the cycle that stable isotopes have been key in tracing the use of methane-derived carbon (MDC) into, and through, food webs, particularly in freshwaters (Jones and Grey, 2011). A schematic of potential routes by which CH_4 produced in anoxic freshwater sediments may either by-pass or become incorporated into food webs is shown in Figure 1.

Could a methane pathway be important to secondary production in food webs?

Anoxic water and sediments are typically rich in organic matter compared to the 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 and Nygaard, 1992). Methanogenesis in lakes has been reported corresponding to 30-80% of the anaerobic mineralisation in waters and sediments (Bastviken, 2009). While seasonal variability in CH_4 oxidation is known to be considerable, especially in dimictic lakes, between 30-94% of the CH_4 reaching oxygenated layers is reputedly oxidised (Casper et al., 2000); (Morana *et al.*, 2015). In essence then, CH_4 is a major product of the C mineralisation in lakes, and a large proportion may be converted to microbial biomass equivalent in some instances to the total C fixation by heterotrophic bacteria and a significant proportion of primary production (Hessen and Nygaard, 1992; Bastviken *et al.*, 2003). Again, data from rivers are lacking, but across 15 rivers, in late summer, i.e. when one might expect the greatest contribution from photosynthesis, (Shelley *et al.*, 2014) conservatively calculated that net methanotrophy was equivalent to between 1% and 46% of benthic net photosynthetic production within the gravel beds of chalkstreams. Couple this to the apparently high (50%) carbon conversion efficiency of methanotrophs (relative to 10-30%, typical for bacteria in detrital-based food webs), regardless of marked spatial and temporal changes in ambient methane concentration, and it suggests that methanotrophs can sustain net production throughout the year (Trimmer *et al.*, 2015)).

The importance of a CH_4 pathway to food webs might yet increase further under climate change. Increases in temperature forecast for the coming decades may have profound implications for the cycling of carbon in aquatic ecosystems due to the differential temperature dependencies of carbon fixation by gross primary production (GPP) and carbon mineralisation by ecosystem respiration (ER). For example, (Yvon-Durocher et al., 2010) showed that warming of 4°C reduced the carbon sequestration capacity of freshwater mesocosms by 13%, shifting them towards net heterotrophy (i.e. net sources of CO_2 to the atmosphere) because ER responded more strongly to temperature than GPP. They also found that methanogenesis responded even more strongly than ER or GPP, with 20% more of the GPP being accounted for by CH_4 emissions with 4°C of warming (Yvon-Durocher *et al.*, 2011). Benthic community structure and how that contributes to a host of ecosystem processes, including microbial and macrofaunal decomposition rates, was also clearly affected by such warming (Dossena *et al.*, 2012). If it is assumed that delivery of organic matter does not change but temperature increases as predicted, then for example, the increased mineralisation will equate to a 4-27% (0.9-6.4 Tg C y^{-1}) decrease in organic carbon burial in boreal lakes (Gudasz *et al.*, 2010). However, very recent work in rivers suggests that methanotrophy has the potential to match methanogenesis enhanced by warming (Shelley *et al.*, 2015). How climate change might impact upon food web mediation of methane-derived carbon will be returned to later.

Why are stable carbon and hydrogen such useful tracers of methane?

Isotopic signatures of environmental CH_4, both $^{13}C/^{12}C$ and $^{2}H/^{1}H$, have been compiled by (Whiticar *et al.*, 1986) and (Bréas *et al.*, 2001) amongst others. An important characteristic of biogenic methane is that its carbon stable isotope composition is typically markedly ^{13}C-deplete compared to other putative basal resources in a food web. So, for freshwater lakes, CH_4 $\delta^{13}C$ may be as low as -110‰ to -50‰ dependent upon formation pathway; (Whiticar, 1999); (Deines and Grey, 2006); (Taipale *et al.*, 2007) relative to either allochthonous terrestrial plant detritus ($\delta^{13}C$ value from C3 plants typically -28‰ to -26‰ (Peterson and Fry, 1987); or autochthonous phytoplankton ($\delta^{13}C$ typically between -35 to -25‰; (Grey et al., 2000, Vuorio et al., 2006) but acknowledging that components of the phytoplankton such as their fatty acids may be ~10‰ further ^{13}C-depleted e.g. (Taipale et al., 2015)). However, CH_4 $\delta^{13}C$ values reported from sediments are not necessarily linked to the $\delta^{13}C$ values of sedimentary organic matter; instead they may be strongly influenced by the quality of the organic matter substrate and/or the predominant methanogenic pathway (Rinta et al., 2015), and of course to a certain extent as to whether some of the CH_4 has already been oxidised by MOB prior to analysis (Coleman et al., 1981). In marine hydrocarbon seep communities, $\delta^{13}C$ has been the primary isotope value examined, used to differentiate between animals with chemoautotrophic symbionts (–40‰ to –20‰) from those with methanotrophic symbionts (≤–40‰) (Brooks et al., 1987) and to identify the source CH_4 pool as either thermogenic ($\delta^{13}C$ = -45‰ to -40‰) or biogenic ($\delta^{13}C$ <-45‰) CH_4 (Sassen et al., 1999).

Isotopic fractionation during the use of CH_4 by MOB typically leads to further ^{13}C-depletion (by 0-30‰; (Summons et al., 1994, Templeton et al., 2006). For example, CH_4-consuming archaea isolated from anoxic marine sediments have been reported with $\delta^{13}C$ values as low as -96‰ (Orphan et al., 2001), while biomarkers (e.g., archaeol and hydroxyarchaeol) from such archaea within a CH_4-supported benthic microbial community in cold-seep sediments exhibited $\delta^{13}C$ values as low as -111‰ (Werne et al., 2002). Hence, the MOB biomass available to consumers has a strikingly low $\delta^{13}C$ and, because stable carbon isotope ratios differ little between consumers and their diets, assuming no selective assimilation or substantial biosynthesis (McCutchan Jr et al., 2003, Grey, 2006), this should allow its contribution to consumer biomass to be rather readily traced.

Hydrogen isotope effects during methanogenesis of methylated substrates can lead to deuterium depletions as large as -531‰, whereas, bacterial D/H discrimination for the CO_2-reduction pathway is significantly less (-250‰ to -170‰; Whiticar, 1999). Very little is known regarding the δD values of MOB. However, when compared to typical values of autochthonous (-290‰ to -215‰) and allochthonous (-160‰ to -125‰) resources, there is still great scope for the use of δD to trace CH_4-derived production (Doucett et al., 2007, Estep and Dabrowski, 1980), especially when in combination with $\delta^{13}C$ (e.g. (Deines et al., 2009)). The more distinct the sources, and indeed, the more tracers used, the more confidence can be assigned to estimates of contribution to diet derived from any of the recently published mixing models (e.g. (Parnell et al., 2013)). Problems arise using isotopic tracers when a relatively minor contribution from MDC results in δ values that could be arrived at via alternative pathways (see ‘The zone of contention’ section below).

Methane use across a spectrum of sources

As appreciation of the possibility of MDC providing an alternative energy source to food webs has grown, so the emphasis on research has shifted from point sources to ever more diffuse sources, and less intuitively obvious locations where it might be relevant. The proportion of MDC contributing to food webs at more diffuse sources may well be smaller (but still of significance); as such, there is likely to be greater ambiguity in the stable isotope signal, and so the importance of MDC might have been overlooked in many of these systems (Figure 2).

It is perhaps unsurprising that the use of CH_4 (amongst other chemosynthetic production) is strongly evident at point sources such as deep-sea vents and seeps, whale, kelp and wood falls, and some sewage outflows, typically far beyond the direct reach of photosynthetically active radiation (although of course photosynthetic production can ‘fall-out’ of the water column to benthic communities). The potential for chemosynthesis to fuel entire animal communities in the ocean was first noted around 35 years ago (e.g. (Rau and Hedges, 1979)). Early attention focussed on megafaunal or epifaunal taxa such as molluscs or pogonophorans but there was a broad suite of smaller infaunal deposit feeding and omnivorous invertebrates whose mode of nutrition remained largely ignored until the application of stable isotope analyses by the likes of (Van Dover and Fry, 1994), (Colaço et al., 2002) and (Levin and Michener, 2002). For example, Levin and Michener (2002) looked at a variety of sites including CH_4 seeps in the Gulf of Alaska, on the Oregon margin, and on the northern California slope and found that seep macrofauna exhibited lighter $\delta^{13}C$ (and $\delta^{15}N$) values than those in non-seep sediments. Significant contributions were found from MDC to macrofaunal biomass from sediments of pogonophoran fields (32%–51%) and clam beds (12%–40%) in the Gulf of Alaska, and in microbial mat sediments on the Oregon margin (20%–44%). Some polychaetes exhibited extremely low values of $\delta^{13}C$ (-90.6‰) at these point sources (see Figure 2a).

Within the last 15 years, research on MDC and food webs primarily focussed on lakes, particularly stratifying lakes in temperate and boreal systems, and much of this work has been extensively reviewed by Jones & Grey (2011). Tube-dwelling chironomid larvae appear key in lake sediments. Field studies from lakes across Alaska, England, Finland and Germany (amongst others) have demonstrated that chironomids can assimilate MDC extensively (up to 70% of larval biomass; (Jones et al., 2008). The degree to which they do may vary within lakes on a temporal (Deines et al., 2007c, Grey et al., 2004c) or spatial (Deines and Grey, 2006; (Gentzel et al., 2012) scale, or by taxa (Jones and Grey, 2004, Kelly et al., 2004, Jones et al., 2008), and among lakes with ‘strength’ of stratification (Grey et al., 2004c, Deines et al., 2007b, Hershey et al., 2015). Chironomid larvae are bioengineers; they bioturbate the sediment while ‘digging’ and maintaining their burrows and draw down oxygenated water, bringing it into contact with anoxic sediment. The sediments on the burrow walls have been shown to exhibit higher methane oxidation rates and higher densities of MOB than the surrounding bulk or surficial sediments (Kajan and Frenzel, 1999); Gentzel et al., 2012). Larvae thereby appear to create the perfect micro-niche for the coupling of methanogenesis and methanotrophy (Deines et al., 2007a); (Kelly et al., 2004); see route 3 in Figure 1).

It was assumed from field studies that the low $\delta^{13}C$ values for taxa such as *Chironomus plumosus* (e.g. -70‰ to -50‰; (Jones et al., 2008)) reflected ingestion of the MOB on their burrow walls (Deines et al., 2007a) akin to ‘gardening’ by trichopteran caddis flies on the biofilms that develop on caddis cases (also studied by using stable isotopes; (Ings et al., 2012). By using ^{13}C-labelled CH_4 additions directly into sediments housing chironomid larvae under controlled experimental settings, Deines et al., (2007a) have elegantly demonstrated that larvae assimilate MDC via MOB; this was further supported by phospholipid fatty acids diagnostic for MOB and significantly enriched by the ^{13}C-labelled methane being detected in the larval tissues. In a series of parallel experiments, they showed that larvae could also obtain MDC via ^{13}C-labelled Type II MOB introduced into the water column above sediments. Type I and Type II MOB use different pathways for formaldehyde assimilation (ribulose monophosphate and serine, respectively) and typically favour different environmental conditions; Type I appear to be dominant in environments in which CH_4 is limiting and combined nitrogen and copper concentrations are relatively high, whereas Type II appear where there are high CH_4 concentrations, low dissolved oxygen, and limiting concentrations of combined nitrogen and/or copper (Hanson and Hanson, 1996). The ability to access MDC via two discrete routes might account for some of the incredible inter-individual variability that has been observed in chironomid stable isotope ratios (e.g. (Grey et al., 2004b); Figure 2).

When stratification of the water column becomes too pronounced, generally in duration, and the benthic sediments become inhospitable even for the hypoxic tolerant chironomid larvae, Jones & Grey (2011) hypothesised that MDC is more likely to be taken up in the water column at the oxic-anoxic boundary by zooplankton. Again, evidence for this is mostly derived from the field from small Finnish boreal lakes with marked oxyclines (e.g.(Jones et al., 1999); (Taipale et al., 2007, Taipale et al., 2008), but see (Bastviken et al., 2003); (Santer et al., 2006); (Schilder et al., 2015a). Pelagic zooplankton δ^{13}C values are typically not as low as those reported from similar lakes for benthic chironomids, perhaps again reflecting the more diffuse nature of the source CH_4, and / or the more mobile feeding capability of zooplankton in the water column relative to tube dwelling chironomids in the sediments. Some of the lowest values reported are for *Daphnia* spp. from small, strongly stratifying lakes with anoxic hypolimnia; for example, -47‰ in a kettle lake, Plußsee (Harrod and Grey, 2006), or -46‰ from Mekkojarvi (Taipale et al., 2008). Laboratory support for zooplankton uptake of MOB is sparse, but (Kankaala et al., 2006) measured growth rates of *Daphnia* in replicated cultures fed microbial suspensions with or without addition of CH_4 and found that their δ^{13}C values indicated consumption of ^{13}C-depleted MOB, as have (Deines and Fink, 2011) using ^{13}C-labelling of CH_4.

Evidence of MDC contributions to biomass in polymictic (permanently mixed) lakes is rarer. Such lakes are often shallow and contain considerable stands of macrophytes; while methanogenesis is certainly proceeding in the sediments, much of the CH_4 produced might be routed via the plant stems and via ebullition (routes 1&2 in Figure 1) and hence, side-step incorporation into the food web (although see reference to (Agasild et al., 2014), below). Since the whole water column is well oxygenated, there is no distinct boundary where MOB will accumulate and thus it is unlikely that zooplankton will feed heavily upon MOB (Jones and Grey, 2011). In the benthos, there is also typically a more consistent supply of ^{13}C-enriched phytoplankton production from above which will 'swamp' the lower δ^{13}C values from MOB. Examples of such lakes with permanently oxic sediment surface layers in which MDC has been shown to make only a limited (maximum ~20%) or negligible contribution to chironomid biomass include Großer Binnensee and Schöhsee in north Germany (Grey et al., 2004c, Deines et al., 2007c), Lough Neagh and Rostherne Mere in the UK (Kelly et al., 2004), Izunuma in Japan (Yasuno et al., 2012), and Võrtsjärv in Estonia (Agasild et al., 2014, Cremona et al., 2014). Interestingly, the latter lake was sampled at various sites and it was only at one particular site dominated by vegetation that low δ^{13}C values were recorded in both zooplankton and chironomids. Agasild et al. (2014) postulated that the stands of macrophytes prevented wind mixing from disturbing the sediments, and that dissolved oxygen in the water column was reduced by the restricted circulation of water and gas exchange between the water surface and the atmosphere and by increased oxygen demand from the decomposition of organic matter; all processes which would lead to greater MDC being available to the food web.

Within the last five years has come the first convincing evidence of MDC contributing to food webs in free-flowing, well oxygenated streams and rivers, where because of the turbulent nature, the source of CH_4 could be considered to be most diffuse. One of the first studies claiming a river food web to be fuelled by MDC was by (Kohzu et al., 2004) who reported *Helodes* sp. beetle larvae and adults with $\delta^{13}C$ values as low as -69.8‰ but these were from stagnant backwater pools akin to stratifying lakes, and while these may be important habitats on some lotic systems, they were not from the free flowing, main-stem river food web. Since then, considerable research on the chalk streams of the UK, highly productive, ground water fed systems has revealed that trichopteran larvae may play a similar role to chironomids in lakes, the main conduit for MDC to route into the wider food web (e.g. (Trimmer et al., 2009, Trimmer et al., 2010). In contrast, (Mbaka et al., 2014) studied small inline impoundments with extremely short residence times on a river system in Germany but could find negligible evidence of MDC contributing to chironomids from the sediments there. How MDC might contribute significantly to river food webs clearly requires more research.

Unless there is almost 100% trophic transfer of MDC higher into the food web, then obviously mixing with non-MDC food sources results in a dilution of the indicator isotope in question, and the ability to trace MDC further using stable isotopes alone is weakened (see below). An apparent gradient is thus evident from point to diffuse source of methane. For example, on a species-specific basis, some mobile benthic predators (eels, sea stars, and predatory snails) have been shown on the basis of their low $\delta^{13}C$ (and $\delta^{15}N$ & $\delta^{34}S$) values to obtain close to 100% of their nutrition from CH_4 seep production in the Gulf of Mexico (MacAvoy et al., 2002). From stratifying lakes, (Harrod and Grey, 2006) and (Ravinet et al., 2010) have found isotopic evidence of MDC contributing (up to ~12%) to bream (*Abramis brama*) and to ruffe (*Gymnocephalus cernuus*), respectively, while in a shallow, well-mixed Pantanal (tropical) wetland lake (Sanseverino et al., 2012) could trace MDC into various fish species. Even from the very shallow lake Võrtsjärv, Agasild *et al.* (2014) reported that at sites amongst the macrophytes where zooplankton and chironomid larvae were most ^{13}C-deplete, there was a corresponding decrease in $\delta^{13}C$ for roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and the apex predator, pike (*Esox lucius*), indicative of trophic transfer of MDC to the very top of the food web. To date, evidence from rivers has not been reported, but given the extremely abundant nature of the primary consumers (particularly cased caddis flies) that appear key to linking MOB into the food web in such systems, the pathway is certainly in place (Trimmer et al., 2012). Evidence of the transfer of MDC across ecosystem boundaries is still limited. Aquatic invertebrates such as *Helodes* sp., Chloroperlidae spp., Leuctridae spp. and *Sialis* sp. have all been recorded from Malaise traps on stream banks, i.e. post emergence, with $\delta^{13}C$ values from -69.8 to -51.8‰ (Kohzu et al., 2004) but there has still been only one study quantifying transfer of MDC and that was into riparian spiders (up to 18% of their biomass) mediated by emerging chironomid imagos from stratifying lakes (Jones and Grey 2011). The potential is clear to see for vertebrate predators as well, such as barn swallows (*Hirundo rustica*) which, using stable isotopes, have been identified as prioritising such abundant prey at specific times of the year (Parnell et al., 2013). Of course, we should also consider how alteration of a food web, for example by introduction of a top predator for recreation or as a function of range expansion might cause cascading effects down to biogeochemical cycling near the base of a food web. By experimentally manipulating fish density in a previously fish-less lake, (Devlin et al., 2015) showed that a trophic cascade from fish to microbes affected methane efflux to the atmosphere and reduced the amount of MDC assimilated into the biomass of zooplankton that remained (assessed from *Daphnia* $\delta^{13}C$ values). It may well be that such improved quantitative understanding of the influence of higher trophic consumers on carbon budgets creates future opportunity for management and policy to identify and implement new options for mitigating greenhouse gas release at regional scales (Schmitz et al., 2014).

The zone of contention

Various authors (e.g. (Deines et al., 2009)) have acknowledged that confidence in the use of isotopic tracers of MDC from field studies must be tempered where/when alternative explanations for such isotope values can arise. The 'zone of contention' for $\delta^{13}C$ from consumers in freshwater lakes for example typically occurs between -40‰ and -30‰. Chironomid larvae could exhibit such a value if they assimilated: a) a small percentage from very low $\delta^{13}C$ MOB and a greater percentage from relatively high $\delta^{13}C$ phytoplankton (e.g. (Grey et al., 2004b)); b) alternative chemosynthetic sources of carbon such as sulphur bacteria (e.g. (Deines et al., 2009); (Roach et al., 2011)); or c) phytoplankton with very low $\delta^{13}C$. It should be remembered that these scenarios are not mutually exclusive. Scenario c may arise because a substantial part of the dissolved CO_2 pool may originate from respiration of autochthonous and allochthonous organic matter and have low $\delta^{13}C$ (from -20‰ to -15‰: (Lennon et al., 2006); (Kankaala et al., 2010)). The degree of fractionation of that CO_2 by phytoplankton is uncertain and extremely variable, but in lakes might range from 0‰ to 15‰ (with values near the upper end of the range probably most widespread; (Bade et al., 2006)). Therefore, it is not uncommon to find $\delta^{13}C$ values for lake phytoplankton of < -30‰ (e.g. (Grey et al., 2000); (Vuorio et al., 2006)), and anything feeding selectively on ^{13}C-depleted phytoplankton (or assimilating selectively from components thereof such as fatty acids) will show correspondingly low $\delta^{13}C$ values (Pel et al., 2003). The same has been shown for rivers (Finlay et al., 1999). The situation is even more complex when a proportion of the low $\delta^{13}C$ values for CO_2 could have originated from the oxidation of CH_4, and hence in effect, be an indirect contribution from MDC (Route 5 in Figure 1). Further dilution of the MDC signal with trophic transfer up the food web has already been mentioned.

In such scenarios, only with the addition of alternative but complementary tracers can the assimilation of MDC be assigned with confidence. Hence, the addition of further stable isotopes such as δD (e.g. (Belle et al., 2015, Deines et al., 2009, van Hardenbroek et al., 2015), $\delta^{34}S$ (Grey and Deines, 2005), and to a certain extent $\delta^{15}N$ (Grey et al., 2004b, Stephen et al., 2002); see later discussion), have proved useful in ascertaining the use of MDC. Radio isotopes might offer some support under certain situations; for example. (Opsahl and Chanton, 2006) studied the food webs of troglobitic organisms in the Upper Floridian aquifer and found that crayfish trapped from remote sinkhole conduits were not only on average ~10‰ ^{13}C-depleted relative to their counterparts at accessible springs at the surface but that there was a strong correlation with radiocarbon ($\Delta^{14}C$) depletion relative to modern values, indicative of a chemosynthetic food source. Concurrent analysis of phospholipid fatty acids (PLFAs) which are diagnostic for MOB, as well as compound-specific analysis of the isotope ratios of those PLFAs has also been invaluable. For example, (Taipale et al., 2009) demonstrated a strong relationship between the $\delta^{13}C$ values of *Daphnia* and the proportion of MOB-specific PLFAs in *Daphnia*. These methods have also highlighted the indirect route via methane-oxidation and uptake of the resulting ^{13}C-depleted CO_2 by autotrophs (Route 5 in Figure 1). For bog-pool food webs in Estonia, (Duinen et al., 2013) suggested that the most parsimonious explanation for relatively low $\delta^{13}C$ values of algae-derived polyunsaturated fatty acids found in insects was that MOB were creating CO_2 from the oxidation of CH_4 which was assimilated by their direct algal 'neighbours' within the biofilm community. (Sanseverino et al., 2012) used the presence of MOB-diagnostic PLFAs in various benthivorous and detritivorous fishes to support claims of MDC assimilation in Brazilian wetlands as the fish $\delta^{13}C$ values were <-36‰; low relative to the other food web components in question but clearly not the marked ^{13}C-depletion classically associated with CH_4. Further correlative evidence may be provided by concurrent assessment of the methanogen / methanotroph community by molecular methods. (Eller et al., 2005) reported zones of aerobic and anaerobic CH_4 oxidation in the water column of a strongly stratifying lake, where high cell numbers of MOB were also detected by fluorescence *in situ* hybridisation techniques. It was around this depth in the same lake that (Santer et al., 2006) found that one of the cyclopoid copepod species, *Diacyclops bicuspidatus*, consistently maintained highest density and exhibited $\delta^{13}C$ values ~10‰ lower than epilimnetic species and the photosynthetic particulate organic matter sampled during the same time interval, and proposed the role of MDC in its diet.

Looking back: hindcasting

A particular area of research related to CH_4-fuelling of food webs that has emerged most recently aims to identify or determine past 'methane environments', predominantly in lakes. Insight into past variations of CH_4 availability in lakes would further our understanding of the timing and magnitude of the response of lake CH_4 production and emissions to changing environmental conditions. Palaeolimnologists have long recognised that recalcitrant remains of organisms such as the strongly sclerotized head capsules of chironomids or the ephippia of daphniids, can be found in abundance and well preserved in most lake sediment records. Chironomid remains, especially the larval head capsules, can be found abundantly in lake sediments. Indeed, exoskeleton fragments originating from moulting and deceased larvae, or zooplankton resting eggs, are preserved for tens to hundreds of thousands of years at a quality which allows microscopic identification usually to genus, or species morphotype, but sometimes also to species level (van Hardenbroek et al., 2011). Since lake sediments can be dated using radiometric and / or other dating methods, these remains can be used to reconstruct historical community composition and by inference the water quality, ambient temperature, or a particular habitat structure (Eggermont and Heiri, 2012). Head capsules and exoskeletons comprise mainly chitin and proteins and, on the basis that their chemical composition does not seem to be strongly affected by decomposition processes, they can be used to develop palaeo-environmental reconstructions based upon stable isotope composition (Perga, 2010, Perga, 2011); (Heiri et al., 2012).

(Heiri et al., 2012) recently reviewed the available stable isotope studies based on fossil chironomids (which had mainly examined the elements C, N, H and O), and identified four key areas: (1) developing the methodology for preparing samples for isotopic analysis, (2) studies rearing chironomid larvae under controlled laboratory conditions to determine those factors affecting stable isotope composition, (3) ecosystem-scale studies relating stable isotope measurements of fossil chironomid assemblages to environmental conditions, and (4) developing the first down-core records describing past changes in the stable isotope composition of chironomid assemblages. In a relatively short period of time since that review, a number of publications have arisen expanding upon those areas, further demonstrating the usefulness of the technique, and including other complementary tracer evidence to support suppositions when the stable isotopes alone might prove ambiguous.

Firstly, it is important to determine whether there is any isotopic offset between the recalcitrant parts of organisms recovered from palaeolimnological samples and the whole body that is typically analysed for the study of contemporary relationships in food webs. It is also important to determine whether the 'clean up' protocols that palaeo-samples typically require have any significant effect upon isotopic integrity. To answer both of these questions for chironomid head capsules, (van Hardenbroek *et al*., 2010) trialled various commonly used chemical methods for sediment processing and found that treatment with 10% KOH, 10% HCl, or 40% HF showed no detectable effect on $\delta^{13}C$, whereas, perhaps unsurprisingly, a combination of boiling, accelerated solvent extraction and heavy chemical oxidation resulted in a small but statistically significant decrease in $\delta^{13}C$ values (0.2‰). Then, using a modification of the culturing experimental protocol by (Deines et al., 2007a), they demonstrated with MOB grown on ^{13}C-labelled methane, that methanogenic carbon is transferred into chironomid head capsules (van Hardenbroek *et al*., 2010). (Frossard et al., 2013) have also looked at head capsule to whole organism isotopic offsets for chironomid larvae and reported from experimental rearing on three different diets that the head capsules were ^{13}C-depleted by 0.9‰ relative to whole biomass. For zooplankton, Perga (2010) has shown that the C and N stable isotope compositions of the daphniid exoskeleton and those of the whole body are strongly correlated. Exoskeleton $\delta^{13}C$ values were similar to those of the whole body but were strongly depleted in ^{15}N (-7.9‰), reflecting its derivation from excretory ammonia of dietary origin, known to be ^{15}N-depleted compared with dietary organic nitrogen (Schimmelmann, 2011). Further elegant experiments have shown that the stable isotopic composition of *Daphnia* ephippia provides information on that of the parent *Daphnia*, and of the food and water they were exposed to during formation. (Schilder et al., 2015b) demonstrated that there were only small offsets between *Daphnia* and ephippia relative to the range of variation in *Daphnia* stable isotopic composition reported from down-core studies. Interestingly however, their work also indicated that temperature may have a minor influence on the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values of *Daphnia* body tissue and ephippia which has implications for water temperature reconstruction work using oxygen isotopes, as well as highlighting the care with which controlled feeding experiments need to be conducted (*sensu* (Perga and Grey, 2010). The suite of organism remains has been further extended recently, as it now appears bryozoan statoblasts and zooids have the potential to act as indicators of MDC (van Hardenbroek *et al*., 2015).

Prior to the interest in palaeo-reconstruction, site-specific, and hence, differing CH_4 production potential and oxidation had only been linked to living chironomid larvae (e.g. (Deines and Grey, 2006). More confidence in the potential of recalcitrant remains to provide information about past changes in CH_4 availability in lakes using sediment records has arisen since studies have been conducted across lake types and actually using remains from surficial sediments i.e. reflecting the most recent CH_4 history that can be measured concurrently. In a study of seven Swedish lakes, (van Hardenbroek *et al*., 2012) observed significant negative correlations between the $\delta^{13}C$ of Chironomini and both CH_4 fluxes at the lake surface, and CH_4 releases from the sediment. That dataset was built upon by incorporating samples from 10 Siberian lakes and expanding the suite of remains to include those of *Daphnia* and

Tanytarsini; the δ^{13}C of all three groups were correlated significantly with diffusive CH_4 flux in the combined Siberian and Swedish dataset suggesting that δ^{13}C in the biomass of these invertebrates was affected by CH_4 availability (van Hardenbroek *et al.*, 2013). (Schilder et al., 2015a) measured *Daphnia* ephippial δ^{13}C values from the surface sediments of 15 small European lakes, and found a strong correlation to the late summer aqueous CH_4 concentration in both the surface water and above the sediment.

Down-core work is providing some tantalising evidence of past CH_4 variability over time. Adding to their proof-of-concept work on which invertebrate remains are useful tracers of methane-derived carbon (van Hardenbroek et al., 2013) went on to measure the δ^{13}C of invertebrate remains from a sediment record (covering the past ~1000 years) of a shallow thermokarst lake in northeast Siberia. Those taxa most sensitive to CH_4 availability (Chironomini, Tanytarsini, and *Daphnia*) exhibited the lowest δ^{13}C values in sediments deposited from ca AD 1250 to ca AD 1500, and after AD 1970, which coincided with periods of warmer climate (indicated by an independent local temperature record). As a consequence, the discrepancy in δ^{13}C between CH_4-sensitive taxa and bulk organic matter was higher in these sections than in other parts of the core, whereas the δ^{13}C of other invertebrate taxa did not show the same trend. They concluded that there was higher CH_4 availability in the study lake during warmer periods and that the energy sources of some key benthic invertebrates changed accordingly. (Wooller et al., 2012) managed to reconstruct the CH_4 history of Qalluuraq Lake, a shallow Alaskan tundra lake, over a period ~12,000y in this manner, and similar work has been conducted on large, deep sub-alpine lakes, particularly in France. A change from oligotrophic status associated with anthropogenic nutrient enrichment over the last 150 years was examined for associated shifts in the basal resources available to the benthic food web (Frossard et al., 2015). Chironomid head capsule δ^{13}C values started to decrease with the onset of eutrophication in both Lake Annecy and Lake Bourget; the estimates of the MDC contribution to chironomid biomass ranged from <5% prior to the 1930s to nearly 30% in recent years.

To date, values for chironomid head capsules have not been reported as ^{13}C-depleted as for live organisms. This is in part a frustrating function of the requirement for multiple head capsules to be pooled to provide sufficient material for elemental and isotopic analysis. It is also likely associated with the fact that the sampling of the remains of organisms at a specific location (depth) might not truly reflect the location where the animal assimilated its diet, due perhaps to resuspension of sediments and/or focussing of material (Battarbee, 1999). Hence, the ‘strength’ of a MDC signal that one can find in a contemporary sample derived from fresh larvae with values for individuals <-70‰, will always be dampened (i.e. less ^{13}C-depleted) by pooling and/or dilution effects in palaeolimnological samples. As a consequence, the usefulness of δ^{13}C alone as a tracer deteriorates (see *the zone of contention* section above). One very promising approach is the analysis of ancient DNA (aDNA) from the methanotroph community. (Belle et al., 2014) has elegantly demonstrated how aDNA can be used to complement stable isotopes in a study of a sediment core from the deepest zone of Lake Narlay, representing the last 1500 years of sediment accumulation. A significant change was noted since ca AD1600, with an increase in the proportion of MOB in the total bacteria community, and a corresponding decrease in chironomid head capsule δ^{13}C. These trends suggest that assimilation of MOB may account for up to 36% of chironomid biomass, with evidence for preferential assimilation of methanotroph type I and the NC10 phylum. Parallel strands of evidence are clearly required whenever there is ambiguity in stable isotope data, and the development of aDNA will surely grow in this particular field.

Looking forward: knowledge gaps

To date, the majority of studies on CH_4 in food webs have solely concentrated on the stable carbon isotopes as a tracer. However, equally evident to the very low and varying $\delta^{13}C$ values in consumers part-fuelled by biogenic CH_4 have been low and highly variable $\delta^{15}N$ values; indeed, one of the most striking patterns to emerge from studies involving chironomids and CH_4 is the strong, positive relationship between $\delta^{13}C$ and $\delta^{15}N$ (Grey et al., 2004a) which appears to have some species-specific basis (Kelly et al., 2004). These relationships appear consistent and widespread (Figure 3) and while most likely linked to assimilation of MOB, a test of the potential mechanisms underpinning such low $\delta^{15}N$ values in consumer tissues is currently lacking.

In Grey *et al*. (2004a), it was postulated that nitrogen within chironomid tubes may be continuously cycled between the larva and microbial consortia; for example, chironomids excrete nitrogen in the form of ammonium directly into their tubes and the overlying water (Devine and Vanni, 2002), and via essential fractionation of ammonia, any microbial community taking up that nitrogen source would be ^{15}N-depleted (Macko et al., 1987, Ings et al., 2012). More specifically, both Type I and II MOB can fix atmospheric N_2 into ammonium and share similar pathways to oxidize ammonia/ammonium as autotrophic ammonium oxidizing bacteria (Lee and Childress, 1994) and thus, are likely to exhibit correspondingly low $\delta^{15}N$ values. However, ammonium oxidation rates are typically low and high ammonium concentrations may inhibit CH_4 oxidation. In addition, some MOB can convert nitrate back to N_2 and such denitrifying methanotrophs may outcompete other MOB in nitrogen-rich, low oxygen environments (Stein and Klotz, 2011), which are characteristic of many of the lakes where low $\delta^{13}C$ and $\delta^{15}N$ values in chironomids have been found (Jones et al., 2008). To examine the underlying causal mechanisms for the strong, consistent, and widespread relationship between chironomid $\delta^{13}C$ and $\delta^{15}N$ values, more research is required to characterise the stable isotope values of potential nitrogen sources, to measure potential N-fractionation by MOB, and to use complementary methods such as molecular biomarker profiling (PLFAs and 16S rRNA genes) of chironomid gut contents.

While $\delta^{13}C$ values for dissolved CH_4 are relatively easily measured in the lab as well as in the field nowadays, and hence are available from a wide range of aquatic environments, more robust end-member values for MOB are required if we are to improve estimates for the quantitative contribution of CH_4-carbon to total carbon budgets and production figures for different ecosystems. To date, such estimates have relied on some of the earliest simple two-source mixing models (i.e. only using one stable isotope: carbon) by applying a range of trophic fractionation factors for MOB (reported from a very small number of laboratory experiments) to values of CH_4 gas to derive one end-member. Direct measures of MOB $\delta^{13}C$ from aquatic environments are badly needed. Currently, it is possible to measure the $\delta^{13}C$ of MOB-specific PLFAs extracted from aquatic sediments, but how these relate to the values from whole MOB cells still needs to be established. More laboratory studies of how carbon isotope fractionation between CH_4 and MOB may vary with different environmental and cell growth conditions would be extremely useful, acknowledging that ‘controlling’ every parameter even in the lab can be extremely difficult (e.g. Perga & Grey, 2010).

The geographic range of studies of MDC in food webs is still rather limited. Within freshwaters, Jones et al. (2008) is the only paper to synthesise data from across a wide latitudinal gradient and a distinct knowledge gap exists for the lower latitudes. Tropical regions are responsible for approximately half of the estimated CH_4 emissions from freshwater ecosystems to the atmosphere, although they have been consistently under-sampled (Bastviken *et al*., 2011). Indeed, the permanently stratified (meromictic) Lake Kivu, within the western branch of the East African Rift, is one of the largest freshwater reservoirs of dissolved methane (CH_4) on Earth. Given the relatively high magnitude of MOB production integrated over the entire water column reported by (Morana et al., 2015) (equivalent to 16–60% of the average photosynthetic primary production), and the substantial contribution of MDC to the overall biomass in the oxycline, suggest that MOB could potentially sustain a significant fraction of the pelagic food web in this lake. With few exceptions (like Lake Kivu), it should also be noted that the majority of studies have focussed upon relatively small stratifying stillwaters with strong oxygen gradients. The use of MDC in river food webs – substantial quantities of CH_4 are oxidised in large riverine systems, including the Amazon and the Hudson River (de Angelis and Scranton, 1993, Melack et al., 2004) – may prove to be a more widespread and significant ecosystem process than given credit at present (Trimmer et al., 2012). Whilst acknowledging that other chemosynthetic processes tend to dominate in marine systems, the use of MDC at pelagic boundaries, such as above the oxygen minimum zones of the various oceans, might well be locally important (but over vast areas) to zooplankton as it is in stratifying lakes subject to similar chemical gradients. There is very recent evidence for substantial oxidation of CH_4 within the water column above seeps off Svalbard, and carbon isotopic evidence that atmospheric methane above those seeps is not influenced by contributions from the seafloor source (Graves et al., 2015). Clearly then there must be MOB biomass accruing between the sediment and the surface that could be incorporated into food webs, a pathway that is only likely to increase in importance if gas hydrate destabilisation is promoted by warming of bottom waters.

Analyses of long-term data series from lakes demonstrate that many are subject to increasing average water temperature (Hampton et al., 2008, Schindler et al., 1990). While temperature exerts a strong control on CH_4 efflux via the physiological stimulation of microbial metabolism (Gedney et al., 2004, Yvon-Durocher et al., 2011), increasingly warm summer surface water temperatures may also increase the duration of stratification, Schmidt stability and hypolimnetic oxygen depletion (e.g. (Jankowski et al., 2006)), all of which will have ramifications for CH_4 dynamics and the routing of MDC into biomass (Jones and Grey, 2011). Some limited yet tantalising empirical evidence for this arose from the physical manipulation of the depth of the thermocline in a lake (compared to a nearby reference lake) by installation of an impellor system (Forsius et al., 2010). As a consequence of deepening the thermocline, the dominant fish species, perch (*Perca fluviatilis*) were observed to become more ^{13}C-depleted; a function of increased surface area of sediment adjacent to oxygenated water ideal for chironomid uptake of MOB (route 3 in Figure 1), and the oxygenated water allowing perch to forage on the benthos (Rask et al., 2010). Further manifestations of climate change, such as an increase in both the frequency and severity of storms, could affect both the strength and duration of stratification in lakes, and increase the flux of carbon from the catchment. Not only might erosion from the terrestrial ecosystem provide the substrate for methanogenesis in aquatic ecosystems (e.g. Sanders et al. 2007), but increased concentration and use of dissolved organic and inorganic carbon in lakes and rivers (Schindler et al., 1997, Evans et al., 2005, Jones et al., 2001, Worrall et al., 2004) will shift the balance toward heterotrophic rather than autotrophic functioning. Stable isotope tracers will remain key to unravelling the extent of MDC use in such food webs in future research.

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Figure legends

Figure 1. Methane produced in anoxic sediments may be routed through plants (1) or lost from the sediments to the atmosphere via ebullition or micro-bubbles (2). If it reaches a boundary with oxygen at the sediment-water interface (under mixed or weakly stratified conditions), MOB oxidise it and create biomass which routes via benthic macroinvertebrates into benthic, pelagic, and terrestrial predators (3). Under strongly or permanently stratified conditions, methane will diffuse upwards through the water-column, and oxygen (and MOB) might first be encountered at the metalimnion, where zooplankton link MDC into higher predators (4). An indirect route for MDC could be via CO_2 derived from the oxidation of CH_4 might then be cycled through phytoplankton, and hence on to zooplankton (5), or indeed via sedimentation back down to benthic macroinvertebrates.

Figure 2. Stable isotope bi-plots of food webs across a spectrum of point to diffuse sources of methane with corresponding decrease in strength of $\delta^{13}C$ value as a tracer of methane-derived carbon (MDC); blue boxes indicate components of the food web with small / negligible influence of MDC. (a) Gulf of Alaska (redrawn with permission from Levin and Michener, 2002): solid symbols – pogonophoran field infauna; open symbols – clam field infauna. (b) Plußsee (strongly stratifying small lake, data from Harrod and Grey, 2006): open circles – fish; solid circles – macroinvertebrates; open squares – chironomid larvae. (c) Loch Ness (weakly stratifying large lake, data from Jones and Grey 2011): open circles – fish; solid circles – invertebrate & basal resources; open squares – chironomid larvae.

Figure 3. Stable carbon and nitrogen isotope ratios of benthic chironomid larvae collected from stratifying lakes in Germany, England and Finland (data derived from Grey *et al*. (2004a&b), Deines *et al*. (2007a), Ravinet *et al*. (2010). Individuals were collected from a specific depth in each lake and on one date (except for Holzsee where the data are compiled from 12 sampling events in one year). Species are *Chironomus plumosus* (filled black markers, solid line), *Chironomus anthracinus* (filled grey markers, dashed line), *Propsilocerus jacuticus* (Jyväsjärvi only; open triangle, dashed line) and *Chironomus teniustylus* (Halsjärvi only; open marker, dashed line). Lines are least squares regressions for illustrative purposes only.

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