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

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18 **Abstract**

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

38 Aside from the fundamental importance of identifying the carbon sources fuelling biomass  
39 production, stable isotopes have been integral in the tool box of palaeolimnologists seeking to  
40 identify how contributions from methane have waxed and waned over time. Here, we

41 synthesise the current state of knowledge in the use of stable isotopes to trace methane-  
42 derived carbon in primarily freshwater ecosystems.

43

44 Running head:

45 Identifying and tracking methane in food webs

46

47 Keywords:

48 Methanotrophy; chironomids; fatty acids; zooplankton; carbon; hydrogen; production; food  
49 webs; greenhouse gas; biogeochemical cycling; trophic transfer

50

51

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

54 The global carbon cycle was considered, until relatively recently, to be solely the flux and  
55 storage of carbon between the atmosphere, and terrestrial and oceanic pools. Within the total  
56 carbon budget, it has been noted that despite their relatively small area, inland freshwaters  
57 make a considerable contribution to the global methane (CH<sub>4</sub>) budget with emissions of CH<sub>4</sub>  
58 from freshwaters being at least comparable to the terrestrial CH<sub>4</sub> sink (Battin *et al.*, 2009).  
59 However, there is a considerable bias toward data from lakes and other wetlands, and the role  
60 of rivers remains poorly defined (Bastviken *et al.*, 2011). Emissions of CH<sub>4</sub> may be small in  
61 terms of carbon, but one must consider that CH<sub>4</sub> is a more potent greenhouse gas than CO<sub>2</sub>  
62 over century time scales; (Bastviken *et al.*, 2011) estimated that global CH<sub>4</sub> emissions  
63 expressed as CO<sub>2</sub> equivalents correspond to at least 25% of the estimated terrestrial  
64 greenhouse gas sink. Our understanding of the global carbon cycle will only be complete if  
65 we include the flux of carbon through inland freshwaters (Battin *et al.*, 2009); (Cole *et al.*,  
66 2007); Trimmer *et al.*, 2012); getting to grips with methane-fuelling of food webs is an  
67 interesting and important component of this. Indeed, (Cole, 2013) noted that “the role of  
68 methane in supporting food webs in lakes, and perhaps even beyond their shores, has come as  
69 a surprise” and that “the notion that lake methane partially supports higher organisms in  
70 surrounding terrestrial environments fundamentally changes our understanding of how  
71 aquatic food webs work”.

72 Methanogenesis is a universal terminal degradation process of organic matter in anoxic  
73 aquatic sediments when inorganic oxidants such as nitrate, ferric iron or sulphate are depleted  
74 (Conrad, 2005). Hence, in marine systems where there is typically a high concentration of  
75 sulphate, the sulphur cycle tends to dominate chemosynthesis, but in freshwaters where  
76 sulphate concentrations are typically lower (Hobbie, 1988) then methanogenesis dominates.  
77 Stable isotopes have been an incredibly useful tool in the identification and quantification of  
78 methanogenic and methanotrophic pathways (Conrad, 2005) and further identifying the  
79 constituents of the complex microbial community that is actively involved via stable isotope  
80 probing (SIP; e.g. (He *et al.*, 2012), but those aspects are not the focus of this review.  
81 Methane may be lost directly from the system via ebullition or the recently hypothesised  
82 micro-bubble pathway, stochastic processes notoriously difficult to quantify (Prairie and del  
83 Giorgio, 2013) or be effectively ‘piped’ to the surface via plants (Bergstrom *et al.*, 2007);  
84 (Sanders *et al.*, 2007). Alternatively, or in addition, it may subsequently serve as an energy  
85 and C source for methanotrophs (methane oxidising bacteria; MOB), typically at oxic-anoxic  
86 boundaries (if anaerobic CH<sub>4</sub> oxidation is excluded) in the sediment, or in the water column  
87 (Rudd and Taylor, 1980; (Kankaala *et al.*, 2007). It is essentially from this point in the cycle  
88 that stable isotopes have been key in tracing the use of methane-derived carbon (MDC) into,  
89 and through, food webs, particularly in freshwaters (Jones and Grey, 2011). A schematic of  
90 potential routes by which CH<sub>4</sub> produced in anoxic freshwater sediments may either by-pass  
91 or become incorporated into food webs is shown in Figure 1.

92

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

94 Anoxic water and sediments are typically rich in organic matter compared to the overlying  
95 oxic water, and anoxic metabolism may account for a substantial part (20–60%) of the carbon

96 metabolism and the heterotrophic microbial production within freshwater environments  
97 (Hessen and Nygaard, 1992). Methanogenesis in lakes has been reported corresponding to  
98 30-80% of the anaerobic mineralisation in waters and sediments (Bastviken, 2009). While  
99 seasonal variability in CH<sub>4</sub> oxidation is known to be considerable, especially in dimictic  
100 lakes, between 30-94% of the CH<sub>4</sub> reaching oxygenated layers is reputedly oxidised (Casper  
101 et al., 2000); (Morana *et al.*, 2015). In essence then, CH<sub>4</sub> is a major product of the C  
102 mineralisation in lakes, and a large proportion may be converted to microbial biomass  
103 equivalent in some instances to the total C fixation by heterotrophic bacteria and a significant  
104 proportion of primary production (Hessen and Nygaard, 1992; Bastviken *et al.*, 2003). Again,  
105 data from rivers are lacking, but across 15 rivers, in late summer, i.e. when one might expect  
106 the greatest contribution from photosynthesis, (Shelley *et al.*, 2014) conservatively calculated  
107 that net methanotrophy was equivalent to between 1% and 46% of benthic net photosynthetic  
108 production within the gravel beds of chalkstreams. Couple this to the apparently high (50%)  
109 carbon conversion efficiency of methanotrophs (relative to 10-30%, typical for bacteria in  
110 detrital-based food webs), regardless of marked spatial and temporal changes in ambient  
111 methane concentration, and it suggests that methanotrophs can sustain net production  
112 throughout the year (Trimmer *et al.*, 2015)).

113 The importance of a CH<sub>4</sub> pathway to food webs might yet increase further under climate  
114 change. Increases in temperature forecast for the coming decades may have profound  
115 implications for the cycling of carbon in aquatic ecosystems due to the differential  
116 temperature dependencies of carbon fixation by gross primary production (GPP) and carbon  
117 mineralisation by ecosystem respiration (ER). For example, (Yvon-Durocher et al., 2010)  
118 showed that warming of 4°C reduced the carbon sequestration capacity of freshwater  
119 mesocosms by 13%, shifting them towards net heterotrophy (i.e. net sources of CO<sub>2</sub> to the  
120 atmosphere) because ER responded more strongly to temperature than GPP. They also found  
121 that methanogenesis responded even more strongly than ER or GPP, with 20% more of the  
122 GPP being accounted for by CH<sub>4</sub> emissions with 4°C of warming (Yvon-Durocher *et al.*,  
123 2011). Benthic community structure and how that contributes to a host of ecosystem  
124 processes, including microbial and macrofaunal decomposition rates, was also clearly  
125 affected by such warming (Dossena *et al.*, 2012). If it is assumed that delivery of organic  
126 matter does not change but temperature increases as predicted, then for example, the  
127 increased mineralisation will equate to a 4-27% (0.9-6.4 Tg C y<sup>-1</sup>) decrease in organic carbon  
128 burial in boreal lakes (Gudasz *et al.*, 2010). However, very recent work in rivers suggests that  
129 methanotrophy has the potential to match methanogenesis enhanced by warming (Shelley *et*  
130 *al.*, 2015). How climate change might impact upon food web mediation of methane-derived  
131 carbon will be returned to later.

132

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

134 Isotopic signatures of environmental CH<sub>4</sub>, both <sup>13</sup>C/<sup>12</sup>C and <sup>2</sup>H/<sup>1</sup>H, have been compiled by  
135 (Whiticar *et al.*, 1986) and (Bréas *et al.*, 2001) amongst others. An important characteristic of  
136 biogenic methane is that its carbon stable isotope composition is typically markedly <sup>13</sup>C-  
137 depleted compared to other putative basal resources in a food web. So, for freshwater lakes,  
138 CH<sub>4</sub> δ<sup>13</sup>C may be as low as -110‰ to -50‰ dependent upon formation pathway; (Whiticar,  
139 1999); (Deines and Grey, 2006); (Taipale *et al.*, 2007) relative to either allochthonous

140 terrestrial plant detritus ( $\delta^{13}\text{C}$  value from C3 plants typically -28‰ to -26‰ (Peterson and  
141 Fry, 1987); or autochthonous phytoplankton ( $\delta^{13}\text{C}$  typically between -35 to -25‰; (Grey et  
142 al., 2000, Vuorio et al., 2006) but acknowledging that components of the phytoplankton such  
143 as their fatty acids may be ~10‰ further  $^{13}\text{C}$ -depleted e.g. (Taipale et al., 2015)). However,  
144  $\text{CH}_4$   $\delta^{13}\text{C}$  values reported from sediments are not necessarily linked to the  $\delta^{13}\text{C}$  values of  
145 sedimentary organic matter; instead they may be strongly influenced by the quality of the  
146 organic matter substrate and/or the predominant methanogenic pathway (Rinta et al., 2015),  
147 and of course to a certain extent as to whether some of the  $\text{CH}_4$  has already been oxidised by  
148 MOB prior to analysis (Coleman et al., 1981). In marine hydrocarbon seep communities,  
149  $\delta^{13}\text{C}$  has been the primary isotope value examined, used to differentiate between animals with  
150 chemoautotrophic symbionts (-40‰ to -20‰) from those with methanotrophic symbionts  
151 ( $\leq -40$ ‰) (Brooks et al., 1987) and to identify the source  $\text{CH}_4$  pool as either thermogenic  
152 ( $\delta^{13}\text{C} = -45$ ‰ to -40‰) or biogenic ( $\delta^{13}\text{C} < -45$ ‰)  $\text{CH}_4$  (Sassen et al., 1999).

153 Isotopic fractionation during the use of  $\text{CH}_4$  by MOB typically leads to further  $^{13}\text{C}$ -depletion  
154 (by 0-30‰; (Summons et al., 1994, Templeton et al., 2006). For example,  $\text{CH}_4$ -consuming  
155 archaea isolated from anoxic marine sediments have been reported with  $\delta^{13}\text{C}$  values as low as  
156 -96‰ (Orphan et al., 2001), while biomarkers (e.g., archaeol and hydroxyarchaeol) from such  
157 archaea within a  $\text{CH}_4$ -supported benthic microbial community in cold-seep sediments  
158 exhibited  $\delta^{13}\text{C}$  values as low as -111‰ (Werne et al., 2002). Hence, the MOB biomass  
159 available to consumers has a strikingly low  $\delta^{13}\text{C}$  and, because stable carbon isotope ratios  
160 differ little between consumers and their diets, assuming no selective assimilation or  
161 substantial biosynthesis (McCutchan Jr et al., 2003, Grey, 2006), this should allow its  
162 contribution to consumer biomass to be rather readily traced.

163 Hydrogen isotope effects during methanogenesis of methylated substrates can lead to  
164 deuterium depletions as large as -531‰, whereas, bacterial D/H discrimination for the  $\text{CO}_2$ -  
165 reduction pathway is significantly less (-250‰ to -170‰; Whiticar, 1999). Very little is  
166 known regarding the  $\delta\text{D}$  values of MOB. However, when compared to typical values of  
167 autochthonous (-290‰ to -215‰) and allochthonous (-160‰ to -125‰) resources, there is  
168 still great scope for the use of  $\delta\text{D}$  to trace  $\text{CH}_4$ -derived production (Doucett et al., 2007, Estep  
169 and Dabrowski, 1980), especially when in combination with  $\delta^{13}\text{C}$  (e.g. (Deines et al., 2009)).  
170 The more distinct the sources, and indeed, the more tracers used, the more confidence can be  
171 assigned to estimates of contribution to diet derived from any of the recently published  
172 mixing models (e.g. (Parnell et al., 2013)). Problems arise using isotopic tracers when a  
173 relatively minor contribution from MDC results in  $\delta$  values that could be arrived at via  
174 alternative pathways (see 'The zone of contention' section below).

175

#### 176 *Methane use across a spectrum of sources*

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

182 signal, and so the importance of MDC might have been overlooked in many of these systems  
183 (Figure 2).

184 It is perhaps unsurprising that the use of CH<sub>4</sub> (amongst other chemosynthetic production) is  
185 strongly evident at point sources such as deep-sea vents and seeps, whale, kelp and wood  
186 falls, and some sewage outflows, typically far beyond the direct reach of photosynthetically  
187 active radiation (although of course photosynthetic production can ‘fall-out’ of the water  
188 column to benthic communities). The potential for chemosynthesis to fuel entire animal  
189 communities in the ocean was first noted around 35 years ago (e.g. (Rau and Hedges, 1979)).  
190 Early attention focussed on megafaunal or epifaunal taxa such as molluscs or pogonophorans  
191 but there was a broad suite of smaller infaunal deposit feeding and omnivorous invertebrates  
192 whose mode of nutrition remained largely ignored until the application of stable isotope  
193 analyses by the likes of (Van Dover and Fry, 1994), (Colaço et al., 2002) and (Levin and  
194 Michener, 2002). For example, Levin and Michener (2002) looked at a variety of sites  
195 including CH<sub>4</sub> seeps in the Gulf of Alaska, on the Oregon margin, and on the northern  
196 California slope and found that seep macrofauna exhibited lighter δ<sup>13</sup>C (and δ<sup>15</sup>N) values  
197 than those in non-seep sediments. Significant contributions were found from MDC to  
198 macrofaunal biomass from sediments of pogonophoran fields (32%–51%) and clam beds  
199 (12%–40%) in the Gulf of Alaska, and in microbial mat sediments on the Oregon  
200 margin (20%–44%). Some polychaetes exhibited extremely low values of δ<sup>13</sup>C (-90.6‰) at  
201 these point sources (see Figure 2a).

202 Within the last 15 years, research on MDC and food webs primarily focussed on lakes,  
203 particularly stratifying lakes in temperate and boreal systems, and much of this work has been  
204 extensively reviewed by Jones & Grey (2011). Tube-dwelling chironomid larvae appear key  
205 in lake sediments. Field studies from lakes across Alaska, England, Finland and Germany  
206 (amongst others) have demonstrated that chironomids can assimilate MDC extensively (up to  
207 70% of larval biomass; (Jones et al., 2008). The degree to which they do may vary within  
208 lakes on a temporal (Deines et al., 2007c, Grey et al., 2004c) or spatial (Deines and Grey,  
209 2006; (Gentzel et al., 2012) scale, or by taxa (Jones and Grey, 2004, Kelly et al., 2004, Jones  
210 et al., 2008), and among lakes with ‘strength’ of stratification (Grey et al., 2004c, Deines et  
211 al., 2007b, Hershey et al., 2015). Chironomid larvae are bioengineers; they bioturbate the  
212 sediment while ‘digging’ and maintaining their burrows and draw down oxygenated water,  
213 bringing it into contact with anoxic sediment. The sediments on the burrow walls have been  
214 shown to exhibit higher methane oxidation rates and higher densities of MOB than the  
215 surrounding bulk or surficial sediments (Kajan and Frenzel, 1999); (Gentzel et al., 2012).  
216 Larvae thereby appear to create the perfect micro-niche for the coupling of methanogenesis  
217 and methanotrophy (Deines et al., 2007a); (Kelly et al., 2004); see route 3 in Figure 1).

218 It was assumed from field studies that the low δ<sup>13</sup>C values for taxa such as *Chironomus*  
219 *plumosus* (e.g. -70‰ to -50‰; (Jones et al., 2008)) reflected ingestion of the MOB on their  
220 burrow walls (Deines et al., 2007a) akin to ‘gardening’ by trichopteran caddis flies on the  
221 biofilms that develop on caddis cases (also studied by using stable isotopes; (Ings et al.,  
222 2012). By using <sup>13</sup>C-labelled CH<sub>4</sub> additions directly into sediments housing chironomid  
223 larvae under controlled experimental settings, Deines et al., (2007a) have elegantly  
224 demonstrated that larvae assimilate MDC via MOB; this was further supported by  
225 phospholipid fatty acids diagnostic for MOB and significantly enriched by the <sup>13</sup>C-labelled  
226 methane being detected in the larval tissues. In a series of parallel experiments, they showed

227 that larvae could also obtain MDC via  $^{13}\text{C}$ -labelled Type II MOB introduced into the water  
228 column above sediments. Type I and Type II MOB use different pathways for formaldehyde  
229 assimilation (ribulose monophosphate and serine, respectively) and typically favour different  
230 environmental conditions; Type I appear to be dominant in environments in which  $\text{CH}_4$  is  
231 limiting and combined nitrogen and copper concentrations are relatively high, whereas Type  
232 II appear where there are high  $\text{CH}_4$  concentrations, low dissolved oxygen, and limiting  
233 concentrations of combined nitrogen and/or copper (Hanson and Hanson, 1996). The ability  
234 to access MDC via two discrete routes might account for some of the incredible inter-  
235 individual variability that has been observed in chironomid stable isotope ratios (e.g. (Grey et  
236 al., 2004b); Figure 2).

237 When stratification of the water column becomes too pronounced, generally in duration, and  
238 the benthic sediments become inhospitable even for the hypoxic tolerant chironomid larvae,  
239 Jones & Grey (2011) hypothesised that MDC is more likely to be taken up in the water  
240 column at the oxic-anoxic boundary by zooplankton. Again, evidence for this is mostly  
241 derived from the field from small Finnish boreal lakes with marked oxyclines (e.g.(Jones et  
242 al., 1999); (Taipale et al., 2007, Taipale et al., 2008), but see (Bastviken et al., 2003); (Santer  
243 et al., 2006); (Schilder et al., 2015a). Pelagic zooplankton  $\delta^{13}\text{C}$  values are typically not as low  
244 as those reported from similar lakes for benthic chironomids, perhaps again reflecting the  
245 more diffuse nature of the source  $\text{CH}_4$ , and / or the more mobile feeding capability of  
246 zooplankton in the water column relative to tube dwelling chironomids in the sediments.  
247 Some of the lowest values reported are for *Daphnia* spp. from small, strongly stratifying  
248 lakes with anoxic hypolimnia; for example, -47‰ in a kettle lake, Plußsee (Harrod and Grey,  
249 2006), or -46‰ from Mekkojarvi (Taipale et al., 2008). Laboratory support for zooplankton  
250 uptake of MOB is sparse, but (Kankaala et al., 2006) measured growth rates of *Daphnia* in  
251 replicated cultures fed microbial suspensions with or without addition of  $\text{CH}_4$  and found that  
252 their  $\delta^{13}\text{C}$  values indicated consumption of  $^{13}\text{C}$ -depleted MOB, as have (Deines and Fink,  
253 2011) using  $^{13}\text{C}$ -labelling of  $\text{CH}_4$ .

254 Evidence of MDC contributions to biomass in polymictic (permanently mixed) lakes is rarer.  
255 Such lakes are often shallow and contain considerable stands of macrophytes; while  
256 methanogenesis is certainly proceeding in the sediments, much of the  $\text{CH}_4$  produced might be  
257 routed via the plant stems and via ebullition (routes 1&2 in Figure 1) and hence, side-step  
258 incorporation into the food web (although see reference to (Agasild et al., 2014), below).  
259 Since the whole water column is well oxygenated, there is no distinct boundary where MOB  
260 will accumulate and thus it is unlikely that zooplankton will feed heavily upon MOB (Jones  
261 and Grey, 2011). In the benthos, there is also typically a more consistent supply of  $^{13}\text{C}$ -  
262 enriched phytoplankton production from above which will 'swamp' the lower  $\delta^{13}\text{C}$  values  
263 from MOB. Examples of such lakes with permanently oxic sediment surface layers in which  
264 MDC has been shown to make only a limited (maximum ~20%) or negligible contribution to  
265 chironomid biomass include Großer Binnensee and Schöhsee in north Germany (Grey et al.,  
266 2004c, Deines et al., 2007c), Lough Neagh and Rostherne Mere in the UK (Kelly et al.,  
267 2004), Izunuma in Japan (Yasuno et al., 2012), and Võrtsjärv in Estonia (Agasild et al., 2014,  
268 Cremona et al., 2014). Interestingly, the latter lake was sampled at various sites and it was  
269 only at one particular site dominated by vegetation that low  $\delta^{13}\text{C}$  values were recorded in  
270 both zooplankton and chironomids. Agasild et al. (2014) postulated that the stands of  
271 macrophytes prevented wind mixing from disturbing the sediments, and that dissolved

272 oxygen in the water column was reduced by the restricted circulation of water and gas  
273 exchange between the water surface and the atmosphere and by increased oxygen demand  
274 from the decomposition of organic matter; all processes which would lead to greater MDC  
275 being available to the food web.

276 Within the last five years has come the first convincing evidence of MDC contributing to  
277 food webs in free-flowing, well oxygenated streams and rivers, where because of the  
278 turbulent nature, the source of CH<sub>4</sub> could be considered to be most diffuse. One of the first  
279 studies claiming a river food web to be fuelled by MDC was by (Kohzu et al., 2004) who  
280 reported *Helodes* sp. beetle larvae and adults with  $\delta^{13}\text{C}$  values as low as -69.8‰ but these  
281 were from stagnant backwater pools akin to stratifying lakes, and while these may be  
282 important habitats on some lotic systems, they were not from the free flowing, main-stem  
283 river food web. Since then, considerable research on the chalk streams of the UK, highly  
284 productive, ground water fed systems has revealed that trichopteran larvae may play a similar  
285 role to chironomids in lakes, the main conduit for MDC to route into the wider food web (e.g.  
286 (Trimmer et al., 2009, Trimmer et al., 2010). In contrast, (Mbaka et al., 2014) studied small  
287 inline impoundments with extremely short residence times on a river system in Germany but  
288 could find negligible evidence of MDC contributing to chironomids from the sediments there.  
289 How MDC might contribute significantly to river food webs clearly requires more research.

290 Unless there is almost 100% trophic transfer of MDC higher into the food web, then  
291 obviously mixing with non-MDC food sources results in a dilution of the indicator isotope in  
292 question, and the ability to trace MDC further using stable isotopes alone is weakened (see  
293 below). An apparent gradient is thus evident from point to diffuse source of methane. For  
294 example, on a species-specific basis, some mobile benthic predators (eels, sea stars, and  
295 predatory snails) have been shown on the basis of their low  $\delta^{13}\text{C}$  (and  $\delta^{15}\text{N}$  &  $\delta^{34}\text{S}$ ) values to  
296 obtain close to 100% of their nutrition from CH<sub>4</sub> seep production in the Gulf of Mexico  
297 (MacAvoy et al., 2002). From stratifying lakes, (Harrod and Grey, 2006) and (Ravinet et al.,  
298 2010) have found isotopic evidence of MDC contributing (up to ~12%) to bream (*Abramis*  
299 *brama*) and to ruffe (*Gymnocephalus cernuus*), respectively, while in a shallow, well-mixed  
300 Pantanal (tropical) wetland lake (Sanseverino et al., 2012) could trace MDC into various fish  
301 species. Even from the very shallow lake Vörtsjärv, Agasild *et al.* (2014) reported that at sites  
302 amongst the macrophytes where zooplankton and chironomid larvae were most <sup>13</sup>C-deplete,  
303 there was a corresponding decrease in  $\delta^{13}\text{C}$  for roach (*Rutilus rutilus*), perch (*Perca*  
304 *fluviatilis*) and the apex predator, pike (*Esox lucius*), indicative of trophic transfer of MDC to  
305 the very top of the food web. To date, evidence from rivers has not been reported, but given  
306 the extremely abundant nature of the primary consumers (particularly cased caddis flies) that  
307 appear key to linking MOB into the food web in such systems, the pathway is certainly in  
308 place (Trimmer et al., 2012). Evidence of the transfer of MDC across ecosystem boundaries  
309 is still limited. Aquatic invertebrates such as *Helodes* sp., Chloroperlidae spp., Leuctridae  
310 spp. and *Sialis* sp. have all been recorded from Malaise traps on stream banks, i.e. post  
311 emergence, with  $\delta^{13}\text{C}$  values from -69.8 to -51.8‰ (Kohzu et al., 2004) but there has still  
312 been only one study quantifying transfer of MDC and that was into riparian spiders (up to  
313 18% of their biomass) mediated by emerging chironomid imagos from stratifying lakes  
314 (Jones and Grey 2011). The potential is clear to see for vertebrate predators as well, such as  
315 barn swallows (*Hirundo rustica*) which, using stable isotopes, have been identified as  
316 prioritising such abundant prey at specific times of the year (Parnell et al., 2013). Of course,  
317 we should also consider how alteration of a food web, for example by introduction of a top  
318 predator for recreation or as a function of range expansion might cause cascading effects

319 down to biogeochemical cycling near the base of a food web. By experimentally  
320 manipulating fish density in a previously fish-less lake, (Devlin et al., 2015) showed that a  
321 trophic cascade from fish to microbes affected methane efflux to the atmosphere and reduced  
322 the amount of MDC assimilated into the biomass of zooplankton that remained (assessed  
323 from *Daphnia*  $\delta^{13}\text{C}$  values). It may well be that such improved quantitative understanding of  
324 the influence of higher trophic consumers on carbon budgets creates future opportunity for  
325 management and policy to identify and implement new options for mitigating greenhouse gas  
326 release at regional scales (Schmitz et al., 2014).

327

### 328 *The zone of contention*

329 Various authors (e.g. (Deines et al., 2009)) have acknowledged that confidence in the use of  
330 isotopic tracers of MDC from field studies must be tempered where/when alternative  
331 explanations for such isotope values can arise. The 'zone of contention' for  $\delta^{13}\text{C}$  from  
332 consumers in freshwater lakes for example typically occurs between -40‰ and -30‰.  
333 Chironomid larvae could exhibit such a value if they assimilated: a) a small percentage from  
334 very low  $\delta^{13}\text{C}$  MOB and a greater percentage from relatively high  $\delta^{13}\text{C}$  phytoplankton (e.g.  
335 (Grey et al., 2004b)); b) alternative chemosynthetic sources of carbon such as sulphur  
336 bacteria (e.g. (Deines et al., 2009); (Roach et al., 2011)); or c) phytoplankton with very low  
337  $\delta^{13}\text{C}$ . It should be remembered that these scenarios are not mutually exclusive. Scenario c  
338 may arise because a substantial part of the dissolved  $\text{CO}_2$  pool may originate from respiration  
339 of autochthonous and allochthonous organic matter and have low  $\delta^{13}\text{C}$  (from -20‰ to -15‰:  
340 (Lennon et al., 2006); (Kankaala et al., 2010)). The degree of fractionation of that  $\text{CO}_2$  by  
341 phytoplankton is uncertain and extremely variable, but in lakes might range from 0‰ to 15‰  
342 (with values near the upper end of the range probably most widespread; (Bade et al., 2006)).  
343 Therefore, it is not uncommon to find  $\delta^{13}\text{C}$  values for lake phytoplankton of < -30‰ (e.g.  
344 (Grey et al., 2000); (Vuorio et al., 2006)), and anything feeding selectively on  $^{13}\text{C}$ -depleted  
345 phytoplankton (or assimilating selectively from components thereof such as fatty acids) will  
346 show correspondingly low  $\delta^{13}\text{C}$  values (Pel et al., 2003). The same has been shown for rivers  
347 (Finlay et al., 1999). The situation is even more complex when a proportion of the low  $\delta^{13}\text{C}$   
348 values for  $\text{CO}_2$  could have originated from the oxidation of  $\text{CH}_4$ , and hence in effect, be an  
349 indirect contribution from MDC (Route 5 in Figure 1). Further dilution of the MDC signal  
350 with trophic transfer up the food web has already been mentioned.

351 In such scenarios, only with the addition of alternative but complementary tracers can the  
352 assimilation of MDC be assigned with confidence. Hence, the addition of further stable  
353 isotopes such as  $\delta\text{D}$  (e.g. (Belle et al., 2015, Deines et al., 2009, van Hardenbroek et al.,  
354 2015),  $\delta^{34}\text{S}$  (Grey and Deines, 2005), and to a certain extent  $\delta^{15}\text{N}$  (Grey et al., 2004b,  
355 Stephen et al., 2002); see later discussion), have proved useful in ascertaining the use of  
356 MDC. Radio isotopes might offer some support under certain situations; for example.  
357 (Opsahl and Chanton, 2006) studied the food webs of troglobitic organisms in the Upper  
358 Floridian aquifer and found that crayfish trapped from remote sinkhole conduits were not  
359 only on average  $\sim 10\%$   $^{13}\text{C}$ -depleted relative to their counterparts at accessible springs at the  
360 surface but that there was a strong correlation with radiocarbon ( $\Delta^{14}\text{C}$ ) depletion relative to  
361 modern values, indicative of a chemosynthetic food source. Concurrent analysis of  
362 phospholipid fatty acids (PLFAs) which are diagnostic for MOB, as well as compound-  
363 specific analysis of the isotope ratios of those PLFAs has also been invaluable. For example,  
364 (Taipale et al., 2009) demonstrated a strong relationship between the  $\delta^{13}\text{C}$  values of *Daphnia*

365 and the proportion of MOB-specific PLFAs in *Daphnia*. These methods have also  
366 highlighted the indirect route via methane-oxidation and uptake of the resulting  $^{13}\text{C}$ -depleted  
367  $\text{CO}_2$  by autotrophs (Route 5 in Figure 1). For bog-pool food webs in Estonia, (Duinen et al.,  
368 2013) suggested that the most parsimonious explanation for relatively low  $\delta^{13}\text{C}$  values of  
369 algae-derived polyunsaturated fatty acids found in insects was that MOB were creating  $\text{CO}_2$   
370 from the oxidation of  $\text{CH}_4$  which was assimilated by their direct algal 'neighbours' within the  
371 biofilm community. (Sanseverino et al., 2012) used the presence of MOB-diagnostic PLFAs  
372 in various benthivorous and detritivorous fishes to support claims of MDC assimilation in  
373 Brazilian wetlands as the fish  $\delta^{13}\text{C}$  values were  $<-36\%$ ; low relative to the other food web  
374 components in question but clearly not the marked  $^{13}\text{C}$ -depletion classically associated with  
375  $\text{CH}_4$ . Further correlative evidence may be provided by concurrent assessment of the  
376 methanogen / methanotroph community by molecular methods. (Eller et al., 2005) reported  
377 zones of aerobic and anaerobic  $\text{CH}_4$  oxidation in the water column of a strongly stratifying  
378 lake, where high cell numbers of MOB were also detected by fluorescence *in situ*  
379 hybridisation techniques. It was around this depth in the same lake that (Santer et al., 2006)  
380 found that one of the cyclopoid copepod species, *Diacyclops bicuspidatus*, consistently  
381 maintained highest density and exhibited  $\delta^{13}\text{C}$  values  $\sim 10\%$  lower than epilimnetic species  
382 and the photosynthetic particulate organic matter sampled during the same time interval, and  
383 proposed the role of MDC in its diet.  
384

#### 385 *Looking back: hindcasting*

386 A particular area of research related to  $\text{CH}_4$ -fuelling of food webs that has emerged most  
387 recently aims to identify or determine past 'methane environments', predominantly in lakes.  
388 Insight into past variations of  $\text{CH}_4$  availability in lakes would further our understanding of the  
389 timing and magnitude of the response of lake  $\text{CH}_4$  production and emissions to changing  
390 environmental conditions. Palaeolimnologists have long recognised that recalcitrant remains  
391 of organisms such as the strongly sclerotized head capsules of chironomids or the ephippia of  
392 daphniids, can be found in abundance and well preserved in most lake sediment records.  
393 Chironomid remains, especially the larval head capsules, can be found abundantly in lake  
394 sediments. Indeed, exoskeleton fragments originating from moulting and deceased larvae, or  
395 zooplankton resting eggs, are preserved for tens to hundreds of thousands of years at a quality  
396 which allows microscopic identification usually to genus, or species morphotype, but  
397 sometimes also to species level (van Hardenbroek et al., 2011). Since lake sediments can be  
398 dated using radiometric and / or other dating methods, these remains can be used to  
399 reconstruct historical community composition and by inference the water quality, ambient  
400 temperature, or a particular habitat structure (Eggermont and Heiri, 2012). Head capsules and  
401 exoskeletons comprise mainly chitin and proteins and, on the basis that their chemical  
402 composition does not seem to be strongly affected by decomposition processes, they can be  
403 used to develop palaeo-environmental reconstructions based upon stable isotope composition  
404 (Perga, 2010, Perga, 2011); (Heiri et al., 2012).

405 (Heiri et al., 2012) recently reviewed the available stable isotope studies based on fossil  
406 chironomids (which had mainly examined the elements C, N, H and O), and identified four  
407 key areas: (1) developing the methodology for preparing samples for isotopic analysis, (2)  
408 studies rearing chironomid larvae under controlled laboratory conditions to determine those  
409 factors affecting stable isotope composition, (3) ecosystem-scale studies relating stable  
410 isotope measurements of fossil chironomid assemblages to environmental conditions, and (4)

411 developing the first down-core records describing past changes in the stable isotope  
412 composition of chironomid assemblages. In a relatively short period of time since that  
413 review, a number of publications have arisen expanding upon those areas, further  
414 demonstrating the usefulness of the technique, and including other complementary tracer  
415 evidence to support suppositions when the stable isotopes alone might prove ambiguous.

416 Firstly, it is important to determine whether there is any isotopic offset between the  
417 recalcitrant parts of organisms recovered from palaeolimnological samples and the whole  
418 body that is typically analysed for the study of contemporary relationships in food webs. It is  
419 also important to determine whether the ‘clean up’ protocols that palaeo-samples typically  
420 require have any significant effect upon isotopic integrity. To answer both of these questions  
421 for chironomid head capsules, (van Hardenbroek *et al.*, 2010) trialled various commonly used  
422 chemical methods for sediment processing and found that treatment with 10% KOH, 10%  
423 HCl, or 40% HF showed no detectable effect on  $\delta^{13}\text{C}$ , whereas, perhaps unsurprisingly, a  
424 combination of boiling, accelerated solvent extraction and heavy chemical oxidation resulted  
425 in a small but statistically significant decrease in  $\delta^{13}\text{C}$  values (0.2‰). Then, using a  
426 modification of the culturing experimental protocol by (Deines *et al.*, 2007a), they  
427 demonstrated with MOB grown on  $^{13}\text{C}$ -labelled methane, that methanogenic carbon is  
428 transferred into chironomid head capsules (van Hardenbroek *et al.*, 2010). (Frossard *et al.*,  
429 2013) have also looked at head capsule to whole organism isotopic offsets for chironomid  
430 larvae and reported from experimental rearing on three different diets that the head capsules  
431 were  $^{13}\text{C}$ -depleted by 0.9‰ relative to whole biomass. For zooplankton, Perga (2010) has  
432 shown that the C and N stable isotope compositions of the daphniid exoskeleton and those of  
433 the whole body are strongly correlated. Exoskeleton  $\delta^{13}\text{C}$  values were similar to those of the  
434 whole body but were strongly depleted in  $^{15}\text{N}$  (-7.9‰), reflecting its derivation from  
435 excretory ammonia of dietary origin, known to be  $^{15}\text{N}$ -depleted compared with dietary  
436 organic nitrogen (Schimmelmann, 2011). Further elegant experiments have shown that the  
437 stable isotopic composition of *Daphnia* ephippia provides information on that of the parent  
438 *Daphnia*, and of the food and water they were exposed to during formation. (Schilder *et al.*,  
439 2015b) demonstrated that there were only small offsets between *Daphnia* and ephippia  
440 relative to the range of variation in *Daphnia* stable isotopic composition reported from down-  
441 core studies. Interestingly however, their work also indicated that temperature may have a  
442 minor influence on the  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  values of *Daphnia* body tissue and ephippia  
443 which has implications for water temperature reconstruction work using oxygen isotopes, as  
444 well as highlighting the care with which controlled feeding experiments need to be conducted  
445 (*sensu* Perga and Grey, 2010). The suite of organism remains has been further extended  
446 recently, as it now appears bryozoan statoblasts and zooids have the potential to act as  
447 indicators of MDC (van Hardenbroek *et al.*, 2015).

448  
449 Prior to the interest in palaeo-reconstruction, site-specific, and hence, differing  $\text{CH}_4$   
450 production potential and oxidation had only been linked to living chironomid larvae (e.g.  
451 (Deines and Grey, 2006). More confidence in the potential of recalcitrant remains to provide  
452 information about past changes in  $\text{CH}_4$  availability in lakes using sediment records has arisen  
453 since studies have been conducted across lake types and actually using remains from surficial  
454 sediments i.e. reflecting the most recent  $\text{CH}_4$  history that can be measured concurrently. In a  
455 study of seven Swedish lakes, (van Hardenbroek *et al.*, 2012) observed significant negative  
456 correlations between the  $\delta^{13}\text{C}$  of Chironomini and both  $\text{CH}_4$  fluxes at the lake surface, and  
457  $\text{CH}_4$  releases from the sediment. That dataset was built upon by incorporating samples from  
458 10 Siberian lakes and expanding the suite of remains to include those of *Daphnia* and

459 Tanytarsini; the  $\delta^{13}\text{C}$  of all three groups were correlated significantly with diffusive  $\text{CH}_4$  flux  
460 in the combined Siberian and Swedish dataset suggesting that  $\delta^{13}\text{C}$  in the biomass of these  
461 invertebrates was affected by  $\text{CH}_4$  availability (van Hardenbroek *et al.*, 2013). (Schilder *et al.*,  
462 2015a) measured *Daphnia* ephippial  $\delta^{13}\text{C}$  values from the surface sediments of 15 small  
463 European lakes, and found a strong correlation to the late summer aqueous  $\text{CH}_4$  concentration  
464 in both the surface water and above the sediment.

465 Down-core work is providing some tantalising evidence of past  $\text{CH}_4$  variability over time.  
466 Adding to their proof-of-concept work on which invertebrate remains are useful tracers of  
467 methane-derived carbon (van Hardenbroek *et al.*, 2013) went on to measure the  $\delta^{13}\text{C}$  of  
468 invertebrate remains from a sediment record (covering the past ~1000 years) of a shallow  
469 thermokarst lake in northeast Siberia. Those taxa most sensitive to  $\text{CH}_4$  availability  
470 (Chironomini, Tanytarsini, and *Daphnia*) exhibited the lowest  $\delta^{13}\text{C}$  values in sediments  
471 deposited from ca AD 1250 to ca AD 1500, and after AD 1970, which coincided with periods  
472 of warmer climate (indicated by an independent local temperature record). As a consequence,  
473 the discrepancy in  $\delta^{13}\text{C}$  between  $\text{CH}_4$ -sensitive taxa and bulk organic matter was higher in  
474 these sections than in other parts of the core, whereas the  $\delta^{13}\text{C}$  of other invertebrate taxa did  
475 not show the same trend. They concluded that there was higher  $\text{CH}_4$  availability in the study  
476 lake during warmer periods and that the energy sources of some key benthic invertebrates  
477 changed accordingly. (Wooller *et al.*, 2012) managed to reconstruct the  $\text{CH}_4$  history of  
478 Qalluuraq Lake, a shallow Alaskan tundra lake, over a period ~12,000y in this manner, and  
479 similar work has been conducted on large, deep sub-alpine lakes, particularly in France. A  
480 change from oligotrophic status associated with anthropogenic nutrient enrichment over the  
481 last 150 years was examined for associated shifts in the basal resources available to the  
482 benthic food web (Frossard *et al.*, 2015). Chironomid head capsule  $\delta^{13}\text{C}$  values started to  
483 decrease with the onset of eutrophication in both Lake Annecy and Lake Bourget; the  
484 estimates of the MDC contribution to chironomid biomass ranged from <5% prior to the  
485 1930s to nearly 30% in recent years.

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

504 strands of evidence are clearly required whenever there is ambiguity in stable isotope data,  
505 and the development of aDNA will surely grow in this particular field.

506

507 *Looking forward: knowledge gaps*

508 To date, the majority of studies on CH<sub>4</sub> in food webs have solely concentrated on the stable  
509 carbon isotopes as a tracer. However, equally evident to the very low and varying  $\delta^{13}\text{C}$  values  
510 in consumers part-fuelled by biogenic CH<sub>4</sub> have been low and highly variable  $\delta^{15}\text{N}$  values;  
511 indeed, one of the most striking patterns to emerge from studies involving chironomids and  
512 CH<sub>4</sub> is the strong, positive relationship between  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (Grey et al., 2004a) which  
513 appears to have some species-specific basis (Kelly et al., 2004). These relationships appear  
514 consistent and widespread (Figure 3) and while most likely linked to assimilation of MOB, a  
515 test of the potential mechanisms underpinning such low  $\delta^{15}\text{N}$  values in consumer tissues is  
516 currently lacking.

517 In Grey *et al.* (2004a), it was postulated that nitrogen within chironomid tubes may be  
518 continuously cycled between the larva and microbial consortia; for example, chironomids  
519 excrete nitrogen in the form of ammonium directly into their tubes and the overlying water  
520 (Devine and Vanni, 2002), and via essential fractionation of ammonia, any microbial  
521 community taking up that nitrogen source would be <sup>15</sup>N-depleted (Macko et al., 1987, Ings et  
522 al., 2012). More specifically, both Type I and II MOB can fix atmospheric N<sub>2</sub> into  
523 ammonium and share similar pathways to oxidize ammonia/ammonium as autotrophic  
524 ammonium oxidizing bacteria (Lee and Childress, 1994) and thus, are likely to exhibit  
525 correspondingly low  $\delta^{15}\text{N}$  values. However, ammonium oxidation rates are typically low and  
526 high ammonium concentrations may inhibit CH<sub>4</sub> oxidation. In addition, some MOB can  
527 convert nitrate back to N<sub>2</sub> and such denitrifying methanotrophs may outcompete other MOB  
528 in nitrogen-rich, low oxygen environments (Stein and Klotz, 2011), which are characteristic  
529 of many of the lakes where low  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in chironomids have been found (Jones  
530 et al., 2008). To examine the underlying causal mechanisms for the strong, consistent, and  
531 widespread relationship between chironomid  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, more research is required  
532 to characterise the stable isotope values of potential nitrogen sources, to measure potential N-  
533 fractionation by MOB, and to use complementary methods such as molecular biomarker  
534 profiling (PLFAs and 16S rRNA genes) of chironomid gut contents.

535 While  $\delta^{13}\text{C}$  values for dissolved CH<sub>4</sub> are relatively easily measured in the lab as well as in the  
536 field nowadays, and hence are available from a wide range of aquatic environments, more  
537 robust end-member values for MOB are required if we are to improve estimates for the  
538 quantitative contribution of CH<sub>4</sub>-carbon to total carbon budgets and production figures for  
539 different ecosystems. To date, such estimates have relied on some of the earliest simple two-  
540 source mixing models (i.e. only using one stable isotope: carbon) by applying a range of  
541 trophic fractionation factors for MOB (reported from a very small number of laboratory  
542 experiments) to values of CH<sub>4</sub> gas to derive one end-member. Direct measures of MOB  $\delta^{13}\text{C}$   
543 from aquatic environments are badly needed. Currently, it is possible to measure the  $\delta^{13}\text{C}$  of  
544 MOB-specific PLFAs extracted from aquatic sediments, but how these relate to the values  
545 from whole MOB cells still needs to be established. More laboratory studies of how carbon  
546 isotope fractionation between CH<sub>4</sub> and MOB may vary with different environmental and cell  
547 growth conditions would be extremely useful, acknowledging that ‘controlling’ every  
548 parameter even in the lab can be extremely difficult (e.g. Perga & Grey, 2010).

549 The geographic range of studies of MDC in food webs is still rather limited. Within  
550 freshwaters, Jones et al. (2008) is the only paper to synthesise data from across a wide  
551 latitudinal gradient and a distinct knowledge gap exists for the lower latitudes. Tropical  
552 regions are responsible for approximately half of the estimated CH<sub>4</sub> emissions from  
553 freshwater ecosystems to the atmosphere, although they have been consistently under-  
554 sampled (Bastviken *et al.*, 2011). Indeed, the permanently stratified (meromictic) Lake  
555 Kivu, within the western branch of the East African Rift, is one of the largest freshwater  
556 reservoirs of dissolved methane (CH<sub>4</sub>) on Earth. Given the relatively high magnitude of  
557 MOB production integrated over the entire water column reported by (Morana et al.,  
558 2015) (equivalent to 16–60% of the average photosynthetic primary production), and the  
559 substantial contribution of MDC to the overall biomass in the oxycline, suggest that MOB  
560 could potentially sustain a significant fraction of the pelagic food web in this lake. With  
561 few exceptions (like Lake Kivu), it should also be noted that the majority of studies have  
562 focussed upon relatively small stratifying stillwaters with strong oxygen gradients. The use of  
563 MDC in river food webs – substantial quantities of CH<sub>4</sub> are oxidised in large riverine  
564 systems, including the Amazon and the Hudson River (de Angelis and Scranton, 1993,  
565 Melack et al., 2004) – may prove to be a more widespread and significant ecosystem process  
566 than given credit at present (Trimmer et al., 2012). Whilst acknowledging that other  
567 chemosynthetic processes tend to dominate in marine systems, the use of MDC at pelagic  
568 boundaries, such as above the oxygen minimum zones of the various oceans, might well be  
569 locally important (but over vast areas) to zooplankton as it is in stratifying lakes subject to  
570 similar chemical gradients. There is very recent evidence for substantial oxidation of CH<sub>4</sub>  
571 within the water column above seeps off Svalbard, and carbon isotopic evidence that  
572 atmospheric methane above those seeps is not influenced by contributions from the seafloor  
573 source (Graves et al., 2015). Clearly then there must be MOB biomass accruing between the  
574 sediment and the surface that could be incorporated into food webs, a pathway that is only  
575 likely to increase in importance if gas hydrate destabilisation is promoted by warming of  
576 bottom waters.

577

578 Analyses of long-term data series from lakes demonstrate that many are subject to increasing  
579 average water temperature (Hampton et al., 2008, Schindler et al., 1990). While temperature  
580 exerts a strong control on CH<sub>4</sub> efflux via the physiological stimulation of microbial  
581 metabolism (Gedney et al., 2004, Yvon-Durocher et al., 2011), increasingly warm summer  
582 surface water temperatures may also increase the duration of stratification, Schmidt stability  
583 and hypolimnetic oxygen depletion (e.g. (Jankowski et al., 2006)), all of which will have  
584 ramifications for CH<sub>4</sub> dynamics and the routing of MDC into biomass (Jones and Grey,  
585 2011). Some limited yet tantalising empirical evidence for this arose from the physical  
586 manipulation of the depth of the thermocline in a lake (compared to a nearby reference lake)  
587 by installation of an impellor system (Forsius et al., 2010). As a consequence of deepening  
588 the thermocline, the dominant fish species, perch (*Perca fluviatilis*) were observed to become  
589 more <sup>13</sup>C-depleted; a function of increased surface area of sediment adjacent to oxygenated  
590 water ideal for chironomid uptake of MOB (route 3 in Figure 1), and the oxygenated water  
591 allowing perch to forage on the benthos (Rask et al., 2010). Further manifestations of climate  
592 change, such as an increase in both the frequency and severity of storms, could affect both the  
593 strength and duration of stratification in lakes, and increase the flux of carbon from the  
594 catchment. Not only might erosion from the terrestrial ecosystem provide the substrate for  
595 methanogenesis in aquatic ecosystems (e.g. Sanders et al. 2007), but increased concentration  
596 and use of dissolved organic and inorganic carbon in lakes and rivers (Schindler et al., 1997,  
597 Evans et al., 2005, Jones et al., 2001, Worrall et al., 2004) will shift the balance toward

598 heterotrophic rather than autotrophic functioning. Stable isotope tracers will remain key to  
599 unravelling the extent of MDC use in such food webs in future research.

600

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606

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967 Figure legends

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

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979 Figure 2. Stable isotope bi-plots of food webs across a spectrum of point to diffuse sources of  
980 methane with corresponding decrease in strength of  $\delta^{13}\text{C}$  value as a tracer of methane-derived  
981 carbon (MDC); blue boxes indicate components of the food web with small / negligible  
982 influence of MDC. (a) Gulf of Alaska (redrawn with permission from Levin and Michener,  
983 2002): solid symbols – pogonophoran field infauna; open symbols – clam field infauna. (b)  
984 Plußsee (strongly stratifying small lake, data from Harrod and Grey, 2006): open circles –  
985 fish; solid circles – macroinvertebrates; open squares – chironomid larvae. (c) Loch Ness  
986 (weakly stratifying large lake, data from Jones and Grey 2011): open circles – fish; solid  
987 circles – invertebrate & basal resources; open squares – chironomid larvae.

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990 Figure 3. Stable carbon and nitrogen isotope ratios of benthic chironomid larvae collected  
991 from stratifying lakes in Germany, England and Finland (data derived from Grey *et al.*  
992 (2004a&b), Deines *et al.* (2007a), Ravinet *et al.* (2010). Individuals were collected from a  
993 specific depth in each lake and on one date (except for Holzsee where the data are compiled  
994 from 12 sampling events in one year). Species are *Chironomus plumosus* (filled black  
995 markers, solid line), *Chironomus anthracinus* (filled grey markers, dashed line),  
996 *Prosilocerus jacuticus* (Jyväsjärvi only; open triangle, dashed line) and *Chironomus*  
997 *teniustylus* (Halsjärvi only; open marker, dashed line). Lines are least squares regressions for  
998 illustrative purposes only.

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