

1 **Geographically widespread ¹³C-depletion of grazing**
2 **caddis larvae: a ‘third way’ of fuelling stream food webs?**

3

4 **Running Title:** Widespread ¹³C-depletion in grazing caddis

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20 Summary

- 21 1) Stream ecosystems are supported by both ‘green’ (i.e, based on grazing) and ‘brown’
22 (i.e. detritus) food webs, whereas methane-derived carbon is not considered generally
23 to be important; here we add circumstantial evidence for this potential ‘third way’.
- 24 2) Grazing cased-caddis (Trichoptera) larvae in the family Glossosomatidae can be very
25 abundant in springs and headwaters and frequently have much lower stable carbon
26 isotope ratios (i.e. they are depleted in the heavier ^{13}C stable isotope) than the biofilm
27 (‘epilithon’) on the upper surfaces of the stones on which they live, and which is their
28 presumed diet. Evidence for similar isotopic depletion in other lotic invertebrates is
29 currently limited, however; even for glossosomatids it has been observed so far only
30 in some streams draining the southern English cretaceous chalk and in a few
31 headwaters in northern California. If this phenomenon proves to be more widespread,
32 among streams or taxa, it could imply a more general underpinning of stream food
33 webs by ‘isotopically light’ carbon derived from methane and accessed via consumers
34 feeding on methanotrophic bacteria.
- 35 3) Here we sampled 58 stream sites to examine whether caddis larvae are also ^{13}C -
36 depleted in streams draining other geologies. We focused mainly on carboniferous
37 limestone and sandstone, as well as on further chalk streams representative of most of
38 the British chalk aquifer: together, these new sites covered an area of almost 90,000
39 km^2 , around three times greater than that surveyed previously.
- 40 4) At all 58 sites methane gas was supersaturated relative to the atmospheric
41 equilibrium, and at 49 of them larvae were conspicuously ^{13}C -depleted (from -17.5 to
42 -3.6‰) relative to the bulk epilithon (components of which we know can oxidise
43 methane). Although still most pronounced on chalk, this phenomenon was
44 geographically and geologically much more widespread than shown previously and

45 suggests methane-derived carbon could indeed play a prominent role in stream food
46 webs (i.e. the ‘third way’).

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48 *Keywords:* methane, carbon, armoured grazers, geology, stable isotopes, streams

49

50 **INTRODUCTION**

51 Inland waters play a key role in the global carbon cycle, transporting, burying or respiring as
52 much as half of all terrestrial net ecosystem production (Cole *et al.*, 2007; Cole, 2013). Many
53 fresh waters are sources of carbon dioxide and methane to the atmosphere (Striegl *et al.* 2012;
54 Prairie & del Giorgio 2013), and Bastviken *et al.* (2011) suggested that, in terms of greenhouse
55 gas potential, their methane emissions may offset the entire terrestrial continental sink of
56 greenhouse gases. Running waters in particular are now recognised as major potential sources
57 of methane, while the review by Stanley *et al.* (2015) found that river water was almost
58 ubiquitously supersaturated with a mean concentration of 1.35 μM (about two orders of
59 magnitude greater than atmospheric equilibrium). The total annual global emissions of 26.8 Tg
60 of methane from running waters is similar to that released from wildfires, termites, methane
61 hydrates and permafrost combined, and represents about half the total freshwater contribution
62 and 20% of that from wetlands (Kirschke *et al.*, 2013). This lends a new weight to the
63 importance of the global carbon metabolism of rivers and streams.

64 In fresh waters, research has focussed on the generation of methane as a potent greenhouse gas
65 (Bastviken *et al.*, 2004; Walter *et al.*, 2006; Prairie & del Giorgio, 2013; Bridgham *et al.*, 2013).
66 However, not all the methane produced and transported by fresh waters is emitted to the
67 atmosphere. Some is oxidised by methanotrophic bacteria (MOB) and converted to microbial

68 biomass and carbon dioxide (CO₂) (Chen & Murrell, 2010; Trimmer *et al.*, 2012). Both these
69 products can potentially pass through food webs: as microbial biomass consumed directly as
70 food and in the form of CO₂ as an inorganic carbon source for photosynthetic organisms (Grey,
71 2016). However, the ecological significance of these pathways that divert dissolved methane
72 from the atmosphere remains largely unknown.

73 Secondary production and the trophic basis of food webs in rivers and streams have been
74 widely studied, and the conventional understanding has been that they are based on organic
75 carbon fixed by photosynthesis, either produced within the system (autochthonous) and/or
76 imported from the land (allochthonous) (Hynes, 1970; Vannote *et al.*, 1980; Thorp *et al.*, 1998).
77 The relative importance in various environmental settings of these respective “green” and
78 “brown” pathways is still widely debated (Hein *et al.*, 2003; Carpenter *et al.*, 2005; Doucett *et al.*,
79 2007; Risse-Buhl *et al.*, 2012; Benke & Wallace, 2014; Carroll, Thorp & Roach, 2016; Guo
80 *et al.*, 2016, 2018; Brett *et al.*, 2017; Neres-Lima *et al.*, 2017). The direct consumption of
81 methanotrophic bacteria, however, represents a potential ‘third way’, in which methane-
82 derived carbon could support river food webs (Trimmer *et al.*, 2012; Shelley, Grey & Trimmer,
83 2014), as it can in lakes (Taipale *et al.*, 2012). This pathway is of great interest, not only because
84 methane oxidation moderates atmospheric emissions of a potent greenhouse gas, but also
85 because methane-derived carbon is not widely acknowledged as being important in river food
86 webs.

87 The consumption of methanotrophic bacteria by animals can be detected by the marked
88 depletion of ¹³C in the tissues of consumers, and is widespread in lakes (Grey & Deines, 2005;
89 Deines *et al.*, 2007; Jones *et al.*, 2008; Taipale *et al.* 2012). Similarly, low δ¹³C values in lotic
90 animals could signal that the same phenomenon occurs in streams. Some of the first indications
91 of this came from Japan, where Kohzu *et al.* (2004) found ¹³C-depleted values in a variety of
92 macroinvertebrates in headwater streams, although this was mostly limited to animals found

93 near anoxic zones rich in detritus, or in those that lived mostly in the hyporheic zone. More
94 recently, Del Vecchia *et al.* (2016) also suggested that methane-derived carbon supported the
95 biomass of five different species of stonefly (Plecoptera) in the hyporheic zone of four
96 floodplain aquifers in the USA, again based on their low stable carbon isotope values.

97 The potential importance of methane-derived carbon as a basal resource for consumers in the
98 surface waters of a southern English chalk stream draining a largely agricultural catchment was
99 first suggested by Sanders *et al.* (2007), who showed that organic sediment patches under
100 aquatic macrophytes exhibited intense methanogenesis in summer. Dissolved methane reached
101 a mean summer peak of $1.5 \mu\text{mol L}^{-1}$, with pore-water concentrations two orders of magnitude
102 higher. Sanders *et al.* (2007) showed that much of this methane was apparently oxidised *in situ*,
103 and Shelley *et al.* (2015) found that this process is strongly substrate (i.e. methane) limited and
104 could match the rate of methanogenesis. Further, methanotrophs produce (particulate) carbon
105 equivalent to up to about 13% of that fixed by photosynthesis in some chalk streams (Shelley
106 *et al.*, 2017), with methanotrophic bacteria assimilating methane with an efficiency close to
107 50% (Trimmer *et al.*, 2015). A substantial amount of methane-derived microbial biomass is
108 therefore potentially available to primary consumers.

109 Evidence that this potential food source was being exploited by primary consumers was first
110 obtained by Trimmer *et al.* (2009), who found that the mean larval $\delta^{13}\text{C}$ values of two common
111 grazing cased-caddis (Trichoptera), *Agapetus fuscipes* (Glossosomatidae) and *Silo nigricornis*
112 (Goeridae), in one English chalk stream (the River Lambourn) were anomalously low. At -41.2
113 and -40.4 ‰, respectively, these values were up to 11.1 ‰ lower than any of the various
114 feasible autochthonous and allochthonous (all ultimately produced photosynthetically) food
115 resources sampled. Whilst not the first recorded instance of low glossosomatid larval $\delta^{13}\text{C}$
116 values (McNeely, Clinton & Erbe, 2006), methane had not been considered as a potential driver
117 of ^{13}C -depletion in caddis larvae.

118 All the evidence so far comes from a number of southern English chalk streams, which are
119 groundwater-fed, free-flowing systems with very high alkalinity and primary production
120 (Rovelli *et al.*, 2017). Chalk is a form of very soft limestone formed by the shells of
121 coccolithophores laid down in shallow Cretaceous seas and forming highly fractured and
122 extremely permeable thick beds. It is globally a rather unusual geological formation, largely
123 restricted to southern England, northern France and a few other areas of NW Europe (Darling
124 and Gooddy, 2006). However, methane in river waters is abundant almost everywhere (Stanley
125 *et al.* 2015) and, in UK aquifers, high concentrations in groundwater are by no means confined
126 to the southern chalk (Darling and Gooddy 2006, Bell *et al.* 2017). This implies that methane
127 could be far more widespread and important as a basal resource than suspected previously.
128 Moreover, grazing glossosomatid caddis species, and potentially other specialist grazers, are
129 often extremely abundant and widespread in suitable headwaters (mainly on various permeable
130 strata, but not only in chalk streams) (Douglas, 1958; Elliott, 1982; Arens, 1990; Becker, 2011).
131 Therefore, we aimed to measure the $\delta^{13}\text{C}$ of glossosomatid larvae across a much greater number
132 and variety of sites than we had done previously.

133 On this evidence, we expected (1) that methane would be ubiquitous (at supersaturated
134 concentrations) in streams on all geologies: in this study these were primarily chalk,
135 carboniferous limestone and sandstone but included opportunistically a few sites draining other
136 formations (permeable and non-permeable) where we knew the larvae occurred. If this proved
137 to be the case, we also expected (2) that low glossosomatid $\delta^{13}\text{C}$ values, relative to epilithon
138 and other putative food resources, would be widespread, suggesting a general role for methane
139 in stream food webs. Finally (3), because methane oxidation by methanotrophs is limited by
140 the supply of the dissolved gas, both epilithic and caddis larval $\delta^{13}\text{C}$ should correlate with
141 stream methane concentration rather than with the bedrock geology of the streams *per se*.

142 **METHODS**

143 **Site selection.**

144 Fifty-eight streams were chosen on the basis of geology and the occurrence of glossosomatid
145 caddis larvae (either *Agapetus fuscipes* or *Glossosoma conformis*). Fifty-five of the streams
146 were drawn from various permeable geological formations: chalk (n=31), limestone (9),
147 sandstone (14) and from one site on the south Wales coalfield (mixed sedimentary rock
148 formations of coal, mudstone, siltstone and sandstone) (Table 1; Fig.1). The three remaining
149 sites were on London clay (2) while the last was an isolated spring amongst the volcanic
150 geology of the NW Lake District (mafic lava). Of the 31 sites on chalk, 14 lay outside the area
151 of central southern England sampled in previous work (Trimmer *et al.*, 2009; Shelley *et al.*,
152 2014), and were either in the north of the Thames basin (sites 1, 8, 9, 41, 44, 52, 53 and 58 in
153 Fig. 1; Table S1), to the south and east of London (sites 2, 3, 42 and 55), or towards the northern
154 extent of the chalk in England (sites 37 and 38). Samples were collected in the spring or summer
155 of 2010, 2011 or 2014 (see Table 1).

156 **Ambient stream water methane concentration**

157 Samples (analytical replicates) of surface water (n=5) were taken from the mid-channel and
158 mid-depth at each site. Each sample was drawn up with a 60 mL gas-tight syringe (after Pretty,
159 Hildrew & Trimmer 2006) and discharged through a 150 mm length of polytetrafluoroethylene
160 tubing into a gas-tight vial (12.5 mL Exetainer, Labco, High Wycombe, UK), allowed to
161 overflow (~three times) to minimise outgassing to the atmosphere. Samples were immediately
162 fixed with 100 μ L ZnCl₂ (50% w/v) bactericide and capped. In the laboratory, a 2 mL
163 headspace of analytical grade helium (BOC) was introduced into each vial using a two-way
164 valve and 5 mL gastight syringe. Samples were placed on rollers (Denley Spiramix, Thermo
165 Denley, Colchester, UK) to allow the gas inside the vials to equilibrate between the water and
166 the headspace. After this, a sub-sample of the headspace (100 μ L) was injected into a gas

167 chromatograph fitted with a flame ionising detector (GC-FID; Agilent Technologies, UK Ltd.,
168 South Queensferry, UK (for details see Sanders *et al.* 2007). The methane concentration in the
169 headspace of the vial was calculated from the area of the peak, calibrated against the areas of
170 known standards. The total amount of methane gas in each vial (headspace + water) was
171 calculated using solubility coefficients (Yamamoto, Alcauskas & Crozier, 1976). The mean of
172 the methane concentration in these five samples yielded an overall average value for each site
173 which was then used in subsequent analyses.

174 **Collection of caddis larvae and putative food resources for stable isotope analysis.**

175 We sought to collect sufficient cased-caddis larvae for at least three measurements (analytical
176 replicates) of $\delta^{13}\text{C}$ (each ~ 15 individuals and totalling ~ 0.5 mg dry mass) to contribute to an
177 overall mean $\delta^{13}\text{C}$ value for caddis for each stream that could be used for further analysis (i.e.
178 treating each stream as a replicate). Caddis were picked directly from rocks and gravels, placed
179 in 50 mL tubes (Falcon, Fisher Scientific, Loughborough, UK) and frozen immediately,
180 therefore including in the sample some gut contents (a potential source of error). In streams
181 where larvae were scarce, fewer analytical replicates could be collected (Table 1).

182 Glossosomatidae are known to graze on epilithon (both algal and detrital components (Becker,
183 1990) but, in order to understand how larvae acquire low $\delta^{13}\text{C}$ values, we also collected other
184 feasible stream food sources (e.g. macrophytes, mosses, filamentous algae and allochthonous
185 leaf litter) for $\delta^{13}\text{C}$ analysis. Epilithon was removed with a toothbrush from the substratum and
186 leaf litter, macrophytes, mosses and filamentous algae were collected by hand. Three samples
187 (analytical replicates, each ~ 0.8 mg dry mass) of each food resource, where present, were
188 collected per site to gain mean $\delta^{13}\text{C}$ site values for each food resource. Fewer than three such
189 samples could be collected for some sites, where food resources were scarce. All samples were
190 then stored in 50 mL tubes (as above) and, along with the caddis larvae, frozen in the field in

191 a portable freezer (17 L, Engel, Australia). In the laboratory, caddis larvae were thawed out
192 and carefully removed from their cases using forceps under a dissecting microscope, cleaned
193 of any debris and rinsed. Epilithon, terrestrial detritus and macrophytes were acidified (4 %
194 HCl, 24h) to remove any inorganic carbon (Hedges & Stern, 1984). All samples of caddis and
195 putative food sources were then oven dried to constant mass at 60 °C and ground to a fine
196 powder using an agate pestle and mortar.

197 Samples were weighed into ultra-clean tin capsules, then combusted using an elemental
198 analyser coupled to a continuous flow isotope ratio mass spectrometer (CF/IRMS, Thermo-
199 Finnigan, Delta Matt Plus, Bremen, Germany). Isotope calibration was carried out using the
200 international standard for carbon (Ref. 8542, sucrose -10.47 ‰ $\delta^{13}\text{C}$ vs. Vienna-PeeDee
201 Belemnite [VPDB], National Institute for Standards and Technology) and values are expressed
202 using the standard delta (δ) notation with units of per mille (‰) (Equation 1).

$$203 \quad \delta = [(R_{\text{SAMPLE}} / R_{\text{STANDARD}} - 1)] * 1000 \quad \text{[Equation 1]}$$

204 Urea was used to determine carbon content and check linearity, and Cyclohexanone-2,4-
205 dinitrophenylhydrazone ($\text{C}_{12}\text{H}_{14}\text{N}_4\text{O}_4$), was used as an internal standard to check the precision
206 of the carbon stable isotope values (Trimmer *et al.* 2009).

207 **Data analysis.**

208 Statistical analyses were performed with the R statistical software v3.1.1. (R Core
209 Development Team, 2014). To test for differences in methane concentration in the water
210 between the three main geologies (chalk, limestone and sandstone), we performed a one-way
211 ANOVA with a *post hoc* Games-Howell test to assess differences between pairs. Methane
212 concentration was first \log_{10} transformed to normalise for positive skew.

213 Caddis larvae and each putative food resource $\delta^{13}\text{C}$ values were pooled for each stream to
214 provide one mean $\delta^{13}\text{C}$ value for caddis and one for each food source per site for analysis (i.e.
215 the stream was the replicate). Equation 2 was used to calculate the isotope difference between
216 glossosomatid larval $\delta^{13}\text{C}$ and bulk epilithon (after Fry 2006), where δ_1 is the $\delta^{13}\text{C}$ (‰) of the
217 larval sample and δ_2 is that of the epilithon:

$$218 \quad \delta_{1,2} (\text{‰}) = [(\delta_1 - \delta_2) / (\delta_2 + 1000)] * 1000 \quad \text{[Equation 2]}$$

219 Potential variation in the trophic fractionation of $\delta^{13}\text{C}$ from source to consumer was
220 incorporated assuming a discrimination factor of $0.4 \pm 1.20 \text{‰}$ (mean \pm 1SD), after Moore &
221 Semmens (2008). Thus, 95% of larvae were expected to fall within -1.95 to + 2.75 ‰ of the
222 epilithic baseline at each site.

223 To test whether caddis and epilithic signatures were related to bedrock type, two separate one-
224 way ANOVAs with *post hoc* Games-Howell tests were performed, with caddis and epilithon
225 $\delta^{13}\text{C}$ s (mean values per site) as the dependent variables and bedrock as the independent
226 variable. Linear and polynomial regressions were used to assess whether there was a linear or
227 curvilinear correlation between methane concentration (log transformed) and the $\delta^{13}\text{C}$ of the
228 caddis larvae or of the epilithon of all of the stream sites.

229

230 **RESULTS**

231 **Ambient methane concentration in stream water**

232 At every site, methane was supersaturated in stream water relative to the atmosphere
233 (equilibril value = $0.002 \mu\text{mol L}^{-1}$ at 10°C), arithmetic mean ranging from $0.007 - 0.58 \mu\text{mol}$
234 L^{-1} with a mean of $0.11 \mu\text{mol L}^{-1}$ across the 58 streams, thus sustaining hypothesis (1). After
235 logarithmic transformation of the data, chalk streams had significantly higher concentrations

236 (back-transformed mean $0.08 \mu\text{mol L}^{-1}$) and, on average, around twice that of the
237 untransformed values for those streams draining sandstone ($0.04 \mu\text{mol L}^{-1}$) (Fig. 2);
238 concentrations in limestone streams were lower again, and half that of sandstone ($0.02 \mu\text{mol}$
239 L^{-1}) (Fig. 2).

240 **^{13}C -depletion in caddis larvae relative to putative food resources**

241 Two species of glossosomatid caddis (*Agapetus fuscipes* and *Glossosoma conformis*) were
242 found in the 58 streams surveyed, although they did not coexist at any site: *Agapetus fuscipes*
243 was present in 56 streams and *Glossosoma conformis* in the two (sandstone) streams in South
244 Wales (sites 13 and 20: Table 1). Caddis larval $\delta^{13}\text{C}$ ranged from -44.3 to -27.0 ‰, and epilithon
245 $\delta^{13}\text{C}$ from -39.5 to -22.8 ‰ (Table 1). Larvae were depleted in ^{13}C relative to the epilithon at
246 the same site by at least -1.95 ‰ (i.e. values were below the lower 95 % CL) at 49 of the sites,
247 and in some cases by > -10 ‰ (Fig. 3) (sustaining hypothesis 2). This depletion varied among
248 geologies but was not restricted to chalk. Larval $\delta^{13}\text{C}$ values fell within the expected limits of
249 potential fractionation relative to the epilithon at a further eight out of 58 sites: five draining
250 sandstone, two on chalk and one on the coal measures (Fig. 3). One limestone stream (site 18)
251 contained caddis larvae that were enriched (by 5.6 ‰) relative to the epilithon.

252 Among the other feasible (even if unlikely) food resources available at each site, particulate
253 detritus of terrestrial origin and various macrophytes were the most common (see Supporting
254 Information Table S2). The $\delta^{13}\text{C}$ of terrestrial detritus ranged from -31.0 to -27.2 ‰, and that
255 of mosses and macrophytes from -44.5 to -24.2 ‰. In most cases, larvae had very depleted
256 carbon isotope signatures relative to these alternatives (Fig. 4) and there was no consistent
257 relationship between larval signature and any of the resources considered. Overall, no feasible
258 mixture of these alternative food sources could explain the isotopic signature of the larvae at
259 most of the sites.

260 **The effect of geology**

261 Caddis larvae exhibited statistically different $\delta^{13}\text{C}$ values between the three main geological
262 types of chalk, limestone and sandstone ($F_{2,51}=13.79$, $P<0.001$), with significantly more
263 depletion on chalk than sandstone ($P=0.003$) but no other pairwise geological differences.
264 Thus, ^{13}C -depletion of caddis larvae was especially pronounced on chalk but also widespread
265 elsewhere. There was no statistically significant difference in the $\delta^{13}\text{C}$ of bulk epilithon among
266 the three main geologies. This partially supports hypothesis 3.

267 **Caddis $\delta^{13}\text{C}$ and methane concentration**

268 There was no overall relationship between methane concentration and caddis $\delta^{13}\text{C}$ for the 58
269 streams (Fig. 5), nor between methane concentration and epilithon $\delta^{13}\text{C}$, thus the further
270 stipulation of our third hypothesis, that bulk methane concentration would correlate with the
271 stable carbon isotopic ratios of larvae and their putative food source (epilithon), was not
272 supported.

273

274 **DISCUSSION**

275 **Methane is ubiquitous**

276 Methane exceeded atmospheric equilibril concentrations at all 58 sites, supporting
277 suggestions that supersaturated methane may be common in running waters worldwide (De
278 Angelis & Lilley, 1987; Jones & Mulholland, 1998; Stanley *et al.*, 2015). Methane is therefore,
279 a pervasive potential basal resource in lotic food webs, particularly since it is so readily and
280 efficiently transformed into microbial biomass (Trimmer *et al.*, 2015; Shelley *et al.*, 2015). The
281 methane in these streams is likely to have come from a variety of sources, including
282 groundwater aquifers linked to underlying ancient oil and gas reserves (Cramer *et al.*, 1999;

283 Bell *et al.*, 2017) as well as more recent ‘autochthonous’ methane produced by *in-situ* anaerobic
284 methanogenesis under macrophyte beds (Sanders *et al.*, 2007; Wilcock & Sorrell, 2008; Old *et*
285 *al.*, 2014). This suggests there may be considerable amounts of previously ignored forms of
286 both new and old carbon cycling through riverine food webs, with potentially important
287 implications for river ecosystems.

288

289 **Widespread ¹³C-depletion in cased caddis** Irrespective of its ultimate provenance,
290 remarkably low $\delta^{13}\text{C}$ values in these grazing cased-caddis suggests that the incorporation of
291 methane-derived carbon into stream food webs may be far more geographically and
292 geologically widespread than lowland southern English chalk streams (Trimmer *et al.* 2009).
293 If these low $\delta^{13}\text{C}$ values are indeed caused by the assimilation of methane-derived carbon
294 (Trimmer *et al.*, 2009; Grey 2016), this has important implications for our wider understanding
295 of the extent to which resources based on methane-derived carbon contribute to stream food
296 webs in general.

297 The most straightforward explanation for ¹³C-depleted caddis is that they are feeding on
298 methanotrophic bacteria that have assimilated isotopically light carbon from methane, a
299 pathway postulated by Trimmer *et al.* (2009). This suggests that many stream communities
300 could be partially powered by chemosynthesis – the ‘third way’ of fuelling stream food webs.
301 Recently, Shelley *et al.* (2017) demonstrated that the production of methanotrophs can be
302 equivalent to up to about 13% of the total of net primary production in chalk streams (reaching
303 a maximum in shaded patches and where methane is abundant). This represents a considerable
304 potential basal resource for primary consumers and is in addition to the well-known “brown”
305 (based on terrestrial detritus) and “green” (based on *in situ* photosynthesis) pathways in stream
306 food webs (e.g. Trimmer *et al.* 2012).

307 Nevertheless, glossosomatid $\delta^{13}\text{C}$ varied markedly across our 58 sites, suggesting the relative
308 contribution of methanotrophic bacteria to the diet may be influenced by local factors such as
309 larval density, micro-distribution and the availability of other food. The $\delta^{13}\text{C}$ of other feasible,
310 although highly unlikely, food sources were also explored as potential alternatives to epilithic
311 biofilms, but in only a few cases (resources and sites) did they give a better match to the isotopic
312 ratio of the larvae. At a few sites, moss (six sites), macrophyte tissues (two), or even leaf litter
313 (three) could have contributed to the larval carbon isotopic ratio, but this was patchy and
314 inconsistent (Fig. 5; Table S2): there is no compelling case to suggest the larvae are feeding
315 widely on resources other than epilithon, which reflects what is known of their feeding ecology
316 (Douglas, 1958; Arens, 1990; Becker, 1990, 2011; Nijboer, 2004).

317 Are there other possible explanations for the depleted stable carbon isotopic ratios of these
318 epilithic grazers? Chemolithoautotrophy driven by the oxidation of ammonium and reduced
319 sulphur could both contribute ^{13}C -deplete carbon to the biofilm (Ruby, Jannasch & Deuser,
320 1987; Sakata *et al.*, 2008). While we recognise the significant potential for ammonium
321 oxidation in both chalk-gravels and sand sediments (Lansdown *et al.*, 2016), its likely depletion
322 in ^{13}C carbon could not account for that measured here in the caddis and we have no evidence
323 to suggest significant sulphur cycling either (see Supporting Information; Fig. S1, Table S3
324 and supplementary Discussion).

325 Further, in streams where dissolved inorganic carbon is very abundant, algae can discriminate
326 against ^{13}C and take up the lighter ^{12}C isotope (Finlay, Power & Cabana, 1999; Finlay, 2001).
327 As a consequence, algal cells favoured by grazing caddis may be more ^{13}C -depleted than the
328 bulk biofilm within which they are embedded (and whose $\delta^{13}\text{C}$ we measured). In addition,
329 methanotrophs produce CO_2 as a product of methane oxidation. This CO_2 is likely to be ^{13}C -
330 depleted (although very scarce compared to the bulk DIC) and could 'label' constituent
331 organisms, such as algae, disproportionately to other non-photosynthetic components. Where

332 methanotrophic bacteria and algal cells are abundant, and in intimate proximity within the
333 biofilm, carbon exchange may be more readily facilitated, so these small-scale transfers could
334 be key to shaping the entry of methane-derived carbon into the wider food web.

335 This last explanation for low $\delta^{13}\text{C}$ values in caddis larvae, which can contribute a large fraction
336 of overall secondary production in streams, demands that some algae or cell components are
337 very highly ^{13}C -depleted relative to the bulk biofilm and are themselves grazed or assimilated
338 highly selectively by the caddis. There is some support for selective feeding within the genus
339 *Glossosoma*: McNeely *et al.*, (2006) attributed the low glossosomatid $\delta^{13}\text{C}$ values in small
340 unproductive streams in Northern California to selective uptake of algae, although the influence
341 of methane was not assessed in their study. Similarly, *Glossosoma intermedium* caddis larvae
342 are thought to employ nutrient-specific foraging, grazing on phosphorus-rich periphyton on
343 conspecific cases instead of phosphorus-limited periphyton on stream bed cobbles (Mooney,
344 Strauss & Haro, 2014, 2016). Overall, therefore, the uptake of methane derived carbon in our
345 study could be by direct ingestion of methanotrophs, which we think the most likely. Methane
346 could also be accessed indirectly by grazing epilithic algae that have assimilated the very C^{13} -
347 depleted inorganic carbon respired by methanotrophs. In either case (i.e. direct or indirect
348 uptake), we would expect the amount of dissolved methane in stream water to correlate with
349 the $\delta^{13}\text{C}$ of the caddis larvae (since methane oxidation rates in rivers rise linearly with methane
350 concentration; Shelley *et al.*, 2014, 2015). This was not found, although methane oxidation
351 rates can vary between different stream gravel biofilm communities (Shelley *et al.*, 2014), and
352 the exact circumstances in which methane-derived carbon is ingested and subsequently
353 assimilated by caddis larvae remain uncertain.

354 Caddis larval $\delta^{13}\text{C}$ could be independent of stream methane concentration if MOB are
355 consumed only sporadically, perhaps as a “supplementary” resource. Methane-oxidising
356 bacteria could thus constitute a kind of ‘ship’s biscuit’ for these grazers – basic rations when

357 higher quality food is unavailable. Glossosomatid larvae characteristically form very high-
358 density aggregations and have been shown to graze on the surfaces of neighbouring
359 conspecifics' cases (Cox & Wagner, 1989; Cavanaugh, Haro & Jones, 2004; Mooney *et al.*,
360 2014, 2016). Note also that biofilms on caddis cases were shown to oxidise methane (Trimmer
361 *et al.*, 2009) and thus must contain MOB. Little is known about the dietary quality of MOB as
362 a food resource for invertebrate grazers. However, in feeding trials carried out by Taipale *et al.*
363 (2012), the zooplankter *Daphnia magna* could not grow and reproduce on MOB alone, but did
364 so on various mixtures of bacteria with phytoplankton. On the assumption that depleted stable
365 carbon isotope values in *Agapetus* were attributable solely to the direct assimilation of
366 methanotrophs, Trimmer *et al.* (2009) used a simple mixing model to calculate that up to 30%
367 of the biomass production of the caddis in a single stream could be accounted for by methane-
368 derived carbon. Similar assumptions here suggest this value is not exceptional and indeed, at
369 the sites with the greatest disparity between caddis and bulk epilithon, estimates would
370 evidently be even higher. Such a conclusion is still premature, however, since our evidence is
371 circumstantial.

372 Overall, our study shows quite clearly that conspicuous ¹³C-depletion of glossosomatid caddis
373 larvae and high concentrations of dissolved methane are common. Widespread food-web
374 linkages involving methane-derived carbon, probably via direct grazing of MOB or perhaps
375 via photosynthetic uptake of depleted carbon respired by MOB, could be routine rather than
376 exceptional. Distinguishing unequivocally between these two would require further evidence,
377 such as the presence of assimilated biomarkers of MOB in the tissues of the caddis.

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383

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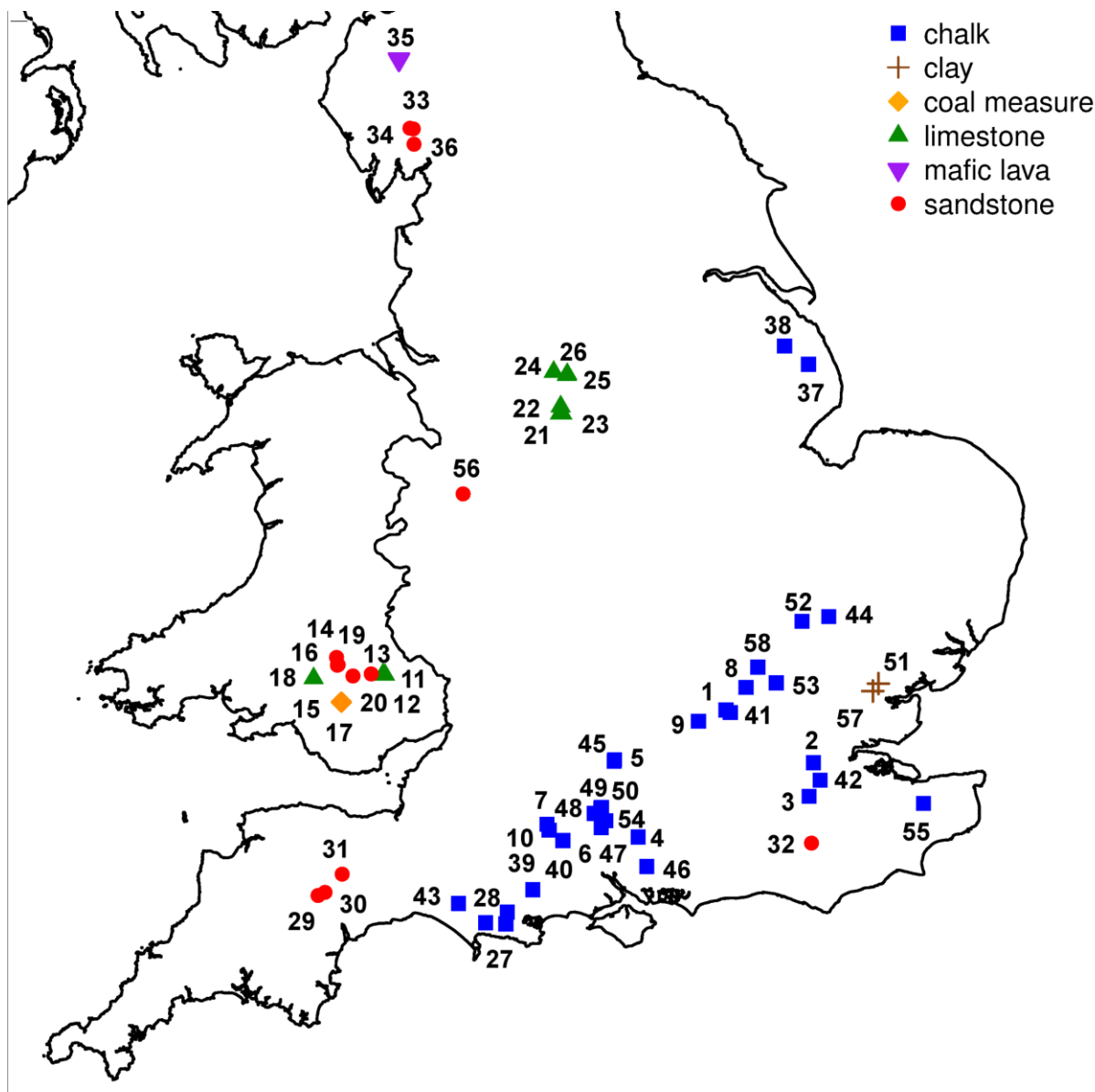
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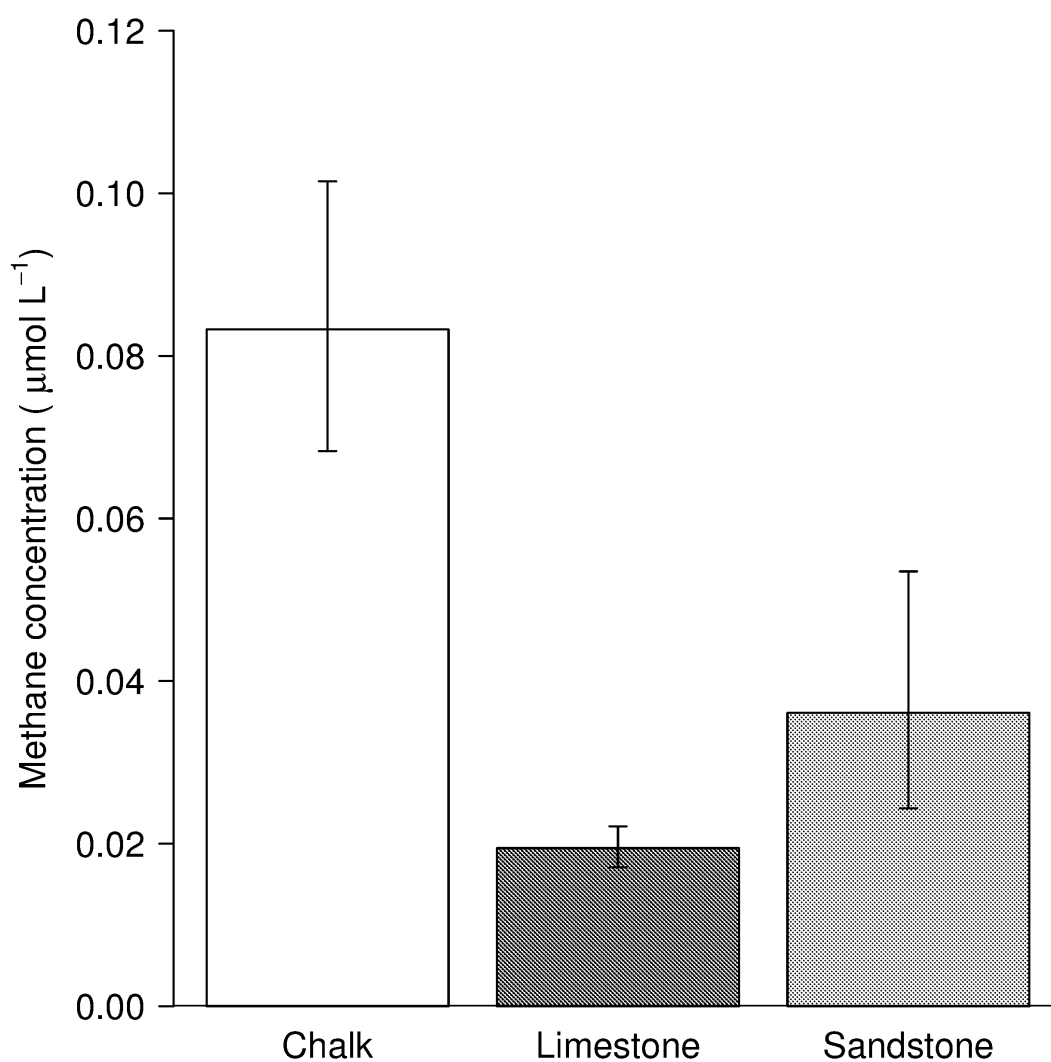
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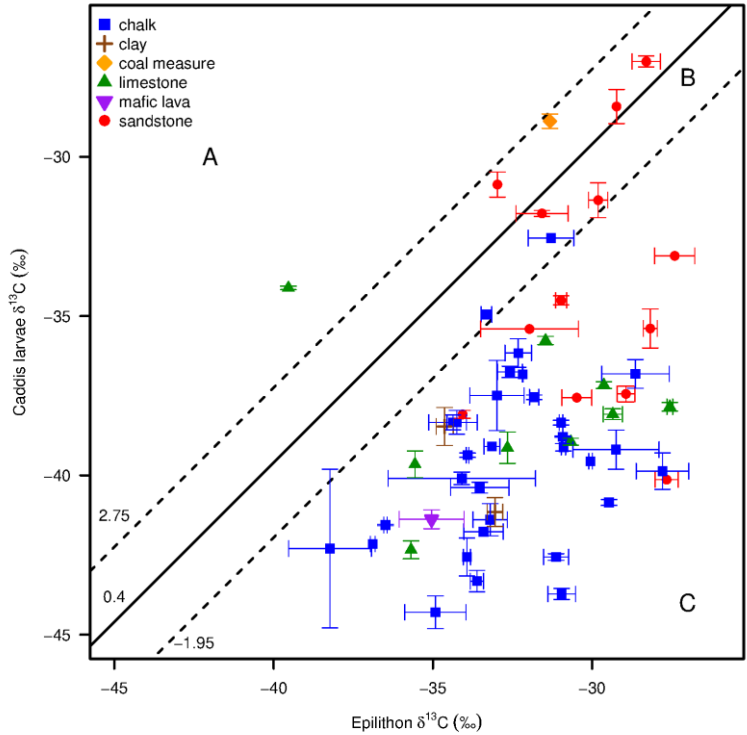
570 **Figure 1.** Map of England and Wales showing the 58 sites across six geologies 1. Chess
 571 (upper), 2. Cray, 3. Darent (upper), 4. Itchen, 5. Lambourn at Bagnor, 6. Test, 7. Till, 8. Ver, 9.
 572 Wye at High Wycombe, 10. Wylve, 11. Nant Onneu, 12. Nant Wenllan, 13. Nant Cleisfer, 14.
 573 Craig Cerrig Gleisiad (lower), 15. Craig Cerrig Gleisiad (middle), 16. Craig Cerrig Gleisiad
 574 (upper), 17. Dare, 18. Upper Tawe, 19. Traeth Mawr, 20. Owls Grove, 21. Manifold, 22.
 575 Milldale, 23. Dove, 24. Wye (upper), 25. Wye (middle) 26. Wye (lower), 27. Tadnoll, 28. Bere,
 576 29. Creedy, 30. Stockleigh, 31. Lowman, 32. Crowborough Warren, 33. Hodgehow Wood, 34.

577 Belle Grange Beck, 35. Carrock Beck, 36. Ghyll Head, 37. Belleau, 38. Welton le Wold, 39.
578 Allen, 40. Bourne, 41. Chess (lower), 42. Darenth, 43. Frome, 44. Granta, 45. Lambourn at
579 Boxford, 46. Meon, 47. Test (upper), 48. Pillhill, 49. Andover, 50. Anton, 51. Brain, 52. Shep,
580 53. Mimram (lower), 54. Sheringham, 55. Great Stour, 56. Telford, 57. Ter, 58. Mimram
581 (upper).



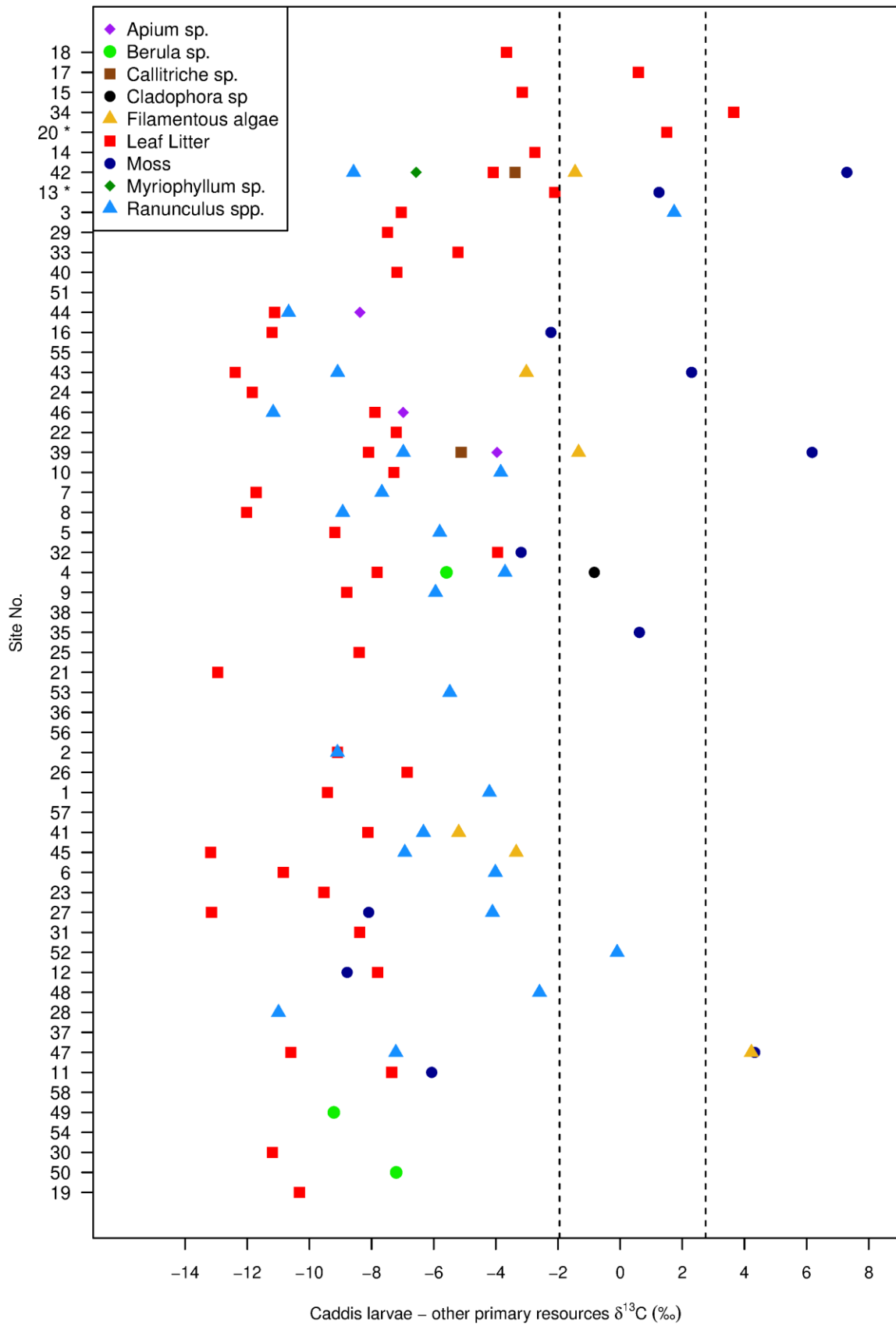
582
583 **Figure 2.** Mean (± 1 SE) methane concentration ($\mu\text{mol L}^{-1}$, after back transformation) for the
584 three main geological types; chalk ($n = 31$), sandstone ($n = 14$), limestone ($n = 9$).

585

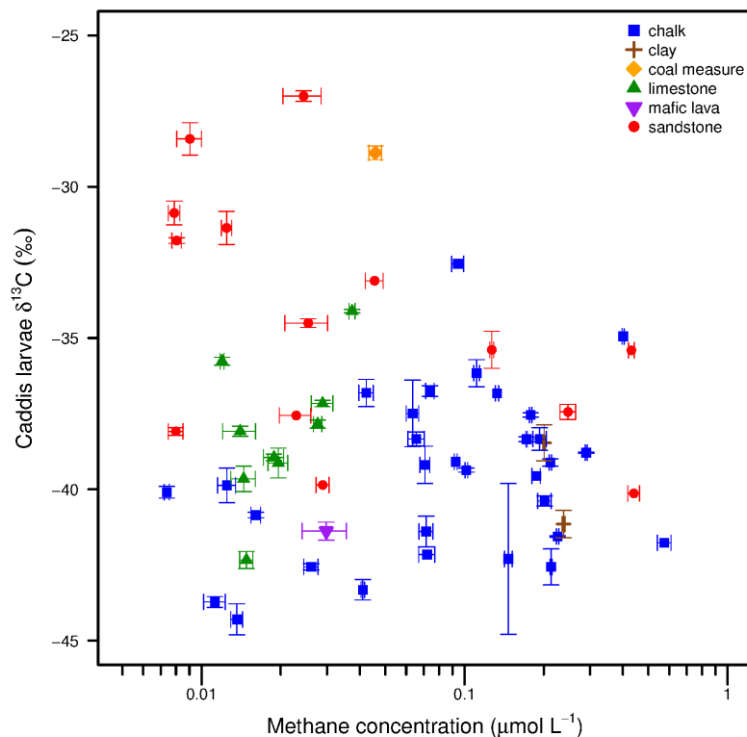


586

587 **Figure 3.** Caddis $\delta^{13}\text{C}$ relative to epilithon $\delta^{13}\text{C}$ (points are site means, limits around site
 588 points are calculated from analytical replicates). Black and broken lines are the mean
 589 expected larval signatures and upper and lower 95% CL, calculated assuming a fractionation
 590 of $0.4 \pm 1.2\text{‰}$ (mean ± 1 SD; Moore & Semmens 2008) and that epilithon is the sole food
 591 source. Sites above the upper limit (area A on the plot) indicate larval enrichment in ^{13}C ,
 592 while those below indicate depletion (area C; sites within the expected limits are in area B).
 593 Colours indicate the different geologies.



595 **Figure 4.** The $\delta^{13}\text{C}$ of glossosomatid larvae, relative to alternative primary food resources.
 596 For each site, each symbol (specifying a putative resource) denotes the isotopic ratio of the
 597 caddis relative to that particular resource. Sites have been ordered by the largest negative
 598 difference between larvae and epilithon (from least difference at the top to greatest at the
 599 bottom). The area between the broken lines (95% CL) indicates the expected larval $\delta^{13}\text{C}$
 600 assuming a mean fractionation value of $+0.4 \pm 1.2\text{‰}$ (mean ± 1 SD; Moore & Semmens
 601 2008).
 602



603
 604 **Figure 5.** Stream ambient methane concentration ± 1 SE (logarithmic scale) and caddis
 605 larval $\delta^{13}\text{C} \pm 1$ SE, suggesting no overall difference in methane concentration among
 606 sandstone, limestone and chalk, but greater overall depletion of caddis on chalk than the
 607 others (see text for details).

608 **Table 1.** Mean $\delta^{13}\text{C} \pm 1\text{SE}$ for epilithon and caddis larvae. Sites are grouped in to geologies
609 then numerically by site number (facilitating comparison with Fig. 1). † indicates the two
610 sites where *Glossosoma conformis* was found rather than *Agapetus fuscipes*. Where only two
611 samples contribute to a mean, both values are given as a range.

Bedrock geology	Site no. (year sampled)	Epilithon $\delta^{13}\text{C}$	n =	<i>Agapetus fuscipes</i> $\delta^{13}\text{C}$	n =
Chalk	1 (2010)	-30.93 to -30.91	2	-38.79 \pm 0.02	3
Chalk	2 (2010)	-31.03 to -30.92	2	-38.35 \pm 0.08	3
Chalk	3 (2010)	-33.32 \pm 0.17	3	-34.95	1
Chalk	4 (2010)	-31.82 \pm 0.14	3	-37.54 \pm 0.07	3
Chalk	5 (2010)	-33.91 \pm 0.04	3	-39.37 \pm 0.07	3
Chalk	6 (2010)	-30.90 \pm 0.04	3	-39.12 \pm 0.12	3
Chalk	7 (2010)	-36.49 \pm 0.05	3	-41.56 \pm 0.02	3
Chalk	8 (2010)	-36.89 \pm 0.08	3	-42.15	1
Chalk	9 (2010)	-33.15 \pm 0.25	3	-39.09	1
Chalk	10 (2010)	-32.18 \pm 0.01	3	-36.83	1
Chalk	27 (2011)	-33.41 \pm 0.62	3	-41.77	1
Chalk	28 (2011)	-30.11 to -29.99	2	-39.56	1
Chalk	37 (2011)	-33.61 \pm 0.21	3	-43.32 \pm 0.34	3
Chalk	38 (2011)	-34.09 \pm 2.31	3	-40.09 \pm 0.20	3
Chalk	39 (2011)	-32.99 \pm 0.85	4	-38.59 to -36.39	2
Chalk	40 (2011)	-32.32 \pm 0.41	6	-36.16 \pm 0.45	5
Chalk	41 (2011)	-28.64 \pm 1.06	6	-36.81 \pm 0.45	5
Chalk	42 (2011)	-31.29 \pm 0.72	6	-32.55	1
Chalk	43 (2011)	-38.23 \pm 1.30	6	-42.30 \pm 2.49	3
Chalk	44 (2011)	-34.37 \pm 0.76	5	-38.34 \pm 0.23	3
Chalk	45 (2011)	-33.20 \pm 0.54	5	-41.39 \pm 0.51	6
Chalk	46 (2011)	-32.58 \pm 0.39	6	-36.75 \pm 0.17	3
Chalk	47 (2011)	-29.25 \pm 1.35	6	-39.19 \pm 0.62	6
Chalk	48 (2014)	-34.92 \pm 0.96	3	-44.30 \pm 0.51	3
Chalk	49 (2014)	-31.13 \pm 0.39	3	-42.56 \pm 0.10	3
Chalk	50 (2014)	-31.39 to -30.52	2	-43.72 \pm 0.18	3
Chalk	52 (2014)	-33.93 \pm 0.11	3	-43.16 to -41.97	2
Chalk	53 (2014)	-33.53 \pm 0.92	3	-40.38 \pm 0.17	3
Chalk	54 (2014)	-27.79 \pm 0.82	3	-39.87 \pm 0.57	3
Chalk	55 (2014)	-34.25 \pm 0.30	3	-38.33 \pm 0.37	3
Chalk	58 (2014)	-29.47	1	-40.85 \pm 0.09	3
Clay	51 (2014)	-34.90 to -34.36	2	-38.46 \pm 0.60	3
Clay	57 (2014)	-33.05 \pm 0.24	3	-41.6 to -40.7	2
Coal	17 (2011)	-31.32	1	-28.88 \pm 0.23	4
Limestone	11 (2011)	-27.56 \pm 0.09	3	-37.84 \pm 0.14	3
Limestone	12 (2011)	-29.35 \pm 0.30	3	-38.08 \pm 0.17	3
Limestone	18 (2011)	-39.53	1	-34.11 \pm 0.05	3
Limestone	21 (2011)	-35.68	1	-42.34 \pm 0.28	3
Limestone	22 (2011)	-31.46	1	-35.77 \pm 0.13	3
Limestone	23 (2011)	-30.67	1	-38.94 \pm 0.11	3
Limestone	24 (2011)	-35.56	1	-39.65 \pm 0.42	3
Limestone	25 (2011)	-32.66	1	-39.13 \pm 0.49	3
Limestone	26 (2011)	-29.63	1	-37.16 \pm 0.11	3
Mafic Lava	35 (2011)	-35.04 \pm 1.02	3	-41.38 \pm 0.30	4
Sandstone	13 (2011)	-29.81 \pm 0.30	3	-31.36 \pm 0.55†	3
Sandstone	14 (2011)	-31.57 \pm 0.82	3	-31.78 \pm 0.09	3
Sandstone	15 (2011)	-33.81 \pm 0.11	1	-30.87 \pm 0.39	3
Sandstone	16 (2011)	-34.07	1	-38.09 \pm 0.13	3

Sandstone	19 (2011)	-22.78 ± 0.34	3	$-39.86 - -39.85$	2
Sandstone	20 (2011)	-29.23	1	$-28.42 \pm 0.54†$	3
Sandstone	29 (2011)	-31.97 ± 1.54	3	-35.4	1
Sandstone	30 (2011)	-27.66 ± 0.36	3	-40.13	1
Sandstone	31 (2011)	$-29.22 - -28.66$	2	-37.44 ± 0.25	3
Sandstone	32 (2011)	-27.41 ± 1.09	3	-33.11	1
Sandstone	33 (2011)	-30.98 ± 0.17	3	-34.51 ± 0.14	3
Sandstone	34 (2011)	-28.30 ± 0.44	3	-27.01 ± 0.17	3
Sandstone	36 (2011)	-30.48 ± 0.47	3	-37.56	1
Sandstone	56 (2014)	-28.18 ± 0.22	3	-35.39 ± 0.61	3

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614 **Supporting Information**

615

616 **Table S1.** Stream number, site names, latitude and longitude.

No	Stream site	Latitude	Longitude	No	Stream site	Latitude	Longitude
1	Chess (upper)	51.678619	-0.531497	30	Stockleigh	50.828669	-3.596573
2	Cray	51.432946	0.137360	31	Lowman	50.913828	-3.465383
3	Darent (upper)	51.276710	0.104090	32	Crowborough	51.058296	0.122215
4	Itchen	51.086330	-1.203529	33	Warren	54.385572	-2.921985
5	Lambourn at Bagnor	51.439692	-1.386321	34	Hodgehow Wood	54.388649	-2.947094
6	Test	51.130964	-1.485299	35	Belle Grange Beck	54.709711	-3.031610
7	Till	51.146125	-1.899308	36	Carrock Beck	54.314272	-2.916455
8	Ver	51.783835	-0.378468	37	Ghyll Head	53.289143	0.099984
9	Wye at High			38	Belleau		
9	Wycombe	51.626327	-0.741859	38	Welton le Wold	53.374073	-0.083635
10	Wylfe	51.118594	-1.883152	39	Allen	50.840853	-2.007536
11	Nant Onneu	51.844590	-3.153215	40	Bourne	51.070008	-1.778369
12	Nant Wenllan	51.841701	-3.137769	40	Chess at		
13	Nant Cleisfer	51.846579	-3.243810	41	Chorleywood	51.666700	-0.498589
14	Craig Cerrig Gleisiad			42	Darent (lower)	51.350933	0.188828
14	Lower	51.889191	-3.496174	43	Frome	50.777333	-2.576144
15	Craig Cerrig Gleisiad			44	Granta	52.113786	0.254714
15	Middle	51.887046	-3.502176	44	Lambourn at		
16	Craig Cerrig Gleisiad			45	Boxford	51.446572	-1.383592
16	Upper	51.887423	-3.500837	46	Meon	50.949369	-1.136356
17	Dare	51.714891	-3.472319	47	Test at Wherwell	51.162231	-1.450025
18	Upper Tawe	51.826556	-3.683616	48	Pillhill	51.196944	-1.538667
19	Traeth Mawr	51.923453	-3.508511	49	Andover	51.219711	-1.487791
20	Owl's Grove	51.836834	-3.382922	50	Anton	51.224461	-1.484427
21	Manifold	53.058900	-1.800589	51	Brain	51.801374	0.634004
22	Milldale	53.092537	-1.795177	52	Shep	52.091401	0.049623
23	Dove	53.059931	-1.783559	53	Mimram (lower)	51.804682	-0.146922
24	Wye Upper	53.253473	-1.848458	54	Sheringham	51.224461	-1.484427
25	Wye Middle	53.239425	-1.748029	55	Great Stour	51.243697	0.977561
26	Wye Lower	53.243428	-1.745233	56	Telford	52.685119	-2.541128
27	Tadnoll	50.687380	-2.368156	57	Ter	51.766550	0.591073
28	Bere	50.736946	-2.203922	58	Mimram (upper)	51.877870	-0.288111
29	Creedy	50.814149	-3.649864				

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Table S2. Mean $\delta^{13}\text{C} \pm 1\text{SE}$ for other primary resources sampled from stream sites. The number of samples contributing to each mean value is indicated in brackets.

Site No.	Terrestrial detritus	Moss spp.	<i>Ranunculus</i> spp.	<i>Berula</i> sp.	<i>Cladophora</i> sp.	<i>Apium</i> sp.	<i>Callitriche</i> sp.	<i>Myriophyllum</i> sp.	Filamentous algae
1	-29.65 ± 1.09 (3)	-	-34.73 ± 0.06 (3)	-	-	-	-	-	-
2	-29.52 ± 0.38 (3)	-	-29.52 ± 0.25 (3)	-	-	-	-	-	-
3	-28.10 ± 0.22 (3)	-	-36.62 ± 0.08 (3)	-	-	-	-	-	-
4	-29.96 ± 0.06 (3)	-	-33.97 ± 0.20 (3)	-32.14 ± 0.03 (3)	-36.74 ± 0.09 (3)	-	-	-	-
5	-30.46 ± 0.73 (3)	-	-33.75 ± 0.18 (3)	-	-	-	-	-	-
6	-28.59 ± 0.13 (3)	-	-35.24 ± 0.20 (3)	-	-	-	-	-	-
7	-30.20 ± 0.26 (3)	-	-34.15 ± 0.15 (3)	-	-	-	-	-	-
8	-30.49 ± 0.42 (3)	-	-33.52 ± 0.18 (3)	-	-	-	-	-	-
9	-30.56 ± 0.22 (3)	-	-33.35 ± 0.37 (3)	-	-	-	-	-	-
10	-29.76 ± 0.95 (3)	-	-33.11 ± 0.01 (3)	-	-	-	-	-	-
11	-30.72 ± 0.07 (3)	-31.97 ± 0.81 (3)	-	-	-	-	-	-	-
12	-30.51 ± 0.03 (3)	-29.55 ± 0.69 (3)	-	-	-	-	-	-	-
13	-29.32 ± 0.02 (3)	-32.57 ± 1.42 (3)	-	-	-	-	-	-	-
14	-29.11 ± 0.06 (3)	-	-	-	-	-	-	-	-
15	-27.80 ± 0.13 (3)	-	-	-	-	-	-	-	-
16	-27.19 (1)	-35.94 (1)	-	-	-	-	-	-	-
17	-29.45 ± 0.19 (6)	-	-	-	-	-	-	-	-
18	-30.57 ± 0.20 (3)	-	-	-	-	-	-	-	-
19	-29.84 ± 0.26 (3)	-	-	-	-	-	-	-	-
20	-29.88 ± 0.48 (3)	-	-	-	-	-	-	-	-
21	-29.77 ± 1.14 (3)	-	-	-	-	-	-	-	-
22	-28.77 ± 0.99 (3)	-	-	-	-	-	-	-	-
23	-29.70 ± 0.52 (3)	-	-	-	-	-	-	-	-
24	-28.14 ± 0.46 (3)	-	-	-	-	-	-	-	-
25	-30.99 ± 0.15 (3)	-	-	-	-	-	-	-	-
26	-30.52 ± 0.38	-	-	-	-	-	-	-	-
27	-29.00 (1)	-33.95 (1)	-37.81 ± 0.43 (3)	-	-	-	-	-	-
28	-	-	-28.88 ± 0.79 (3)	-	-	-	-	-	-
29	-28.16 - - 28.09 (2)	-	-	-	-	-	-	-	-
30	-29.67 - - 28.87 (2)	-	-	-	-	-	-	-	-

Site No.	Terrestrial detritus	Moss spp.	<i>Ranunculus</i> spp.	<i>Berula</i> sp.	<i>Cladophora</i> sp.	<i>Apium</i> sp.	<i>Callitriche</i> sp.	<i>Myriophyllum</i> sp.	Filamentous algae
31	-29.31 (1)	-	-	-	-	-	-	-	-
32	-29.28 ± 0.71 (3)	-30.12 -- 29.92 (2)	-	-	-	-	-	-	-
33	-29.44 (1)	-	-	-	-	-	-	-	-
34	--31.29 -- 29.81 (2)	-	-	-	-	-	-	-	-
35	-	-41.97 (1)	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-
39	-29.67 -- 29.59 (2)	-43.40 ± 0.80 (3)	-30.73 ± 0.75 (6)	-	-	-33.66 ± 0.97 (4)	-32.74 -- 32.34 (2)	-	-36.20 (1)
40	-29.19 ± 0.32 (4)	-	-	-	-	-	-	-	-
41	-28.93 ± 0.25 (6)	-	-30.68 ± 0.30 (5)	-	-	-	-	-	-31.78 ± 0.61 (3)
42	-28.58 ± 0.19 (6)	-39.56 ± 1.62 (3)	-24.18 ± 0.44 (6)	-	-	-	-29.27 ± 0.53 (6)	-26.16 ± 0.51 (3)	-31.14 ± 1.36 (6)
43	-30.40 -- 30.17 (2)	-44.50 ± 0.80 (3)	-33.51 ± 2.90 (3)	-	-	-	-	-	-40.10 -- 38.70 (2)
44	-27.52 ± 0.28 (6)	-	-27.97 ± 0.55 (3)	-	-	-30.21 (1)	-	-	-
45	-28.59 ± 0.29 (6)	-	-34.70 ± 1.29 (6)	-	-	-	-	-	-38.17 ± 0.46 (4)
46	-29.74 -- 28.44 (2)	-	-25.87 ± 0.35 (3)	-	-	-29.98 ± 0.78 (3)	-	-	-
47	-28.90 ± 0.29 (4)	-43.33 ± 0.76 (3)	-32.20 ± 0.72 (6)	-	-	-	-	-	-43.23 (1)
48	-	-	-41.81 ± 0.33 (3)	-	-	-	-	-	-
49	-	-	-	-33.66 ± 0.35 (3)	-	-	-	-	-
50	-	-	-	-36.78 ± 2.76 (3)	-	-	-	-	-
51	-	-	-	-	-	-	-	-	-
52	-	-	-42.47 (1)	-	-	-	-	-	-
53	-	-	-35.10 ± 0.20 (3)	-	-	-	-	-	-
54	-	-	-	-	-	-	-	-	-
55	-	-	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-	-	-
57	-	-	-	-	-	-	-	-	-
58	-	-	-	-	-	-	-	-	-

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627 **Figure S1 and Table S3.** Stable isotope carbon values for dissolved inorganic carbon ($\delta^{13}\text{C}$ -
628 DIC vs VPDB ‰) in river water, epilithon, *Agapetus* (overwhelmingly *Agapetus fuscipes* but
629 including two samples of the confamilial *Glossosoma conformis*) and ‘hypothetical’
630 biofilms based on average carbon fractionation, relative to the DIC, during autotrophy
631 coupled to ammonia oxidation [20‰ (Sakata *et al.*, 2008)) or sulphur oxidation [25‰, on
632 average (Ruby, Jannasch & Deuser, 1987). Data for $\delta^{13}\text{C}$ -DIC were collected from only 28 of
633 the 58 sites used in the main study and were analysed as described in Trimmer *et al.*(2015).

634

635 **Table S3.** All Pairwise Multiple Comparison Procedures (Tukey Test) for the $\delta^{13}\text{C}$ values in
636 above:

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638 Comparison	Diff of Ranks	q	P	P<0.050
639 DIC vs Sulphur at -25	2628.000	12.245	<0.001	Yes
640 DIC vs <i>Agapetus</i>	2426.000	11.304	<0.001	Yes
641 DIC vs Ammonia at -20	1498.000	6.980	<0.001	Yes
642 DIC vs Epilithon	1288.000	6.001	<0.001	Yes
643 Epilithon vs Sulphur at -25	1340.000	6.244	<0.001	Yes
644 Epilithon vs <i>Agapetus</i>	1138.000	5.302	0.002	Yes
645 Epilithon vs Ammonia at -20	210.000	0.978	0.958	No
646 Ammonia at -20 vs Sulphur at -25	1130.000	5.265	0.002	Yes
647 Ammonia at -20 vs <i>Agapetus</i>	928.000	4.324	0.019	Yes
648 <i>Agapetus</i> vs Sulphur at -25	202.000	0.941	0.964	No

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651 **Supplementary discussion in relation to the data presented in Figure S1 and Table S3.**

652 We know that there is a high ammonia oxidation potential in both chalk-gravel and sand
653 riverbed sediments (Lansdown *et al.*, 2016) that will be adding chemolithoautotrophic carbon
654 to the biofilm and that could have a $\delta^{13}\text{C}$ value (Sakata *et al.*, 2008) practically the same as
655 our bulk-epilithon (*see* rank Anova in Table S3). The glossomatids are still depleted relative
656 to both ($P=0.002$ and $P=0.019$, respectively), however. If the biofilm was predominantly
657 composed of carbon fixed by sulphur oxidising bacteria, fractionating on average by -25‰,
658 relative to the DIC (Ruby, Jannasch & Deuser, 1987), then that could account for the typical

659 $\delta^{13}\text{C}$ values in our samples of caddis. If this was the case, then we would expect the products
660 of sulphur cycling to be a significant component of the biofilm and to see evidence, for
661 example, of sulphate reduction as a source of reduced sulphur that could then be reoxidised
662 through chemolithoautotrophy to fix ^{13}C deplete carbon. However, our previous work in the
663 chalk shows sulphate to be conservative in the riverbed (Pretty, Hildrew & Trimmer, 2006).
664 As sulphur is one of the recognised macro-elements (H, O, C, N, P, S – by average rank
665 abundance) in living biomass (Sterner & Elser, 2003), we would expect some reduced
666 sulphur to result during decomposition but, in these predominantly oxic riverbeds, we would
667 not expect it to accumulate sufficiently for it to dominate the biofilm as, say, in a mat of
668 *Beggiatoa* spp. or *Thioploca* spp. where such chemolithoautotrophy is significant (Thamdrup
669 & Canfield, 1996; Hogslund *et al.*, 2009).

670

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