Ancient and methane-derived carbon subsidizes contemporary food webs

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While most global productivity is driven by modern photosynthesis, river ecosystems are supplied by locally fixed and imported carbon that spans a range of ages. Alluvial aquifers of gravel-bedded river floodplains present a conundrum: despite no possibility for photosynthesis in groundwater and extreme paucity of labile organic carbon, they support diverse and abundant large-bodied consumers (stoneflies, Insecta: Plecoptera). Here we show that up to a majority of the biomass carbon composition of these top consumers in four floodplain aquifers of Montana and Washington is methane-derived. The methane carbon ranges in age from modern to up to >50,000 years old and is mostly derived from biogenic sources, although a thermogenic contribution could not be excluded. We document one of the most expansive ecosystems to contain site-wide macroinvertebrate biomass comprised of methane-derived carbon and thereby advance contemporary understanding of basal resources supporting riverine productivity.
wo landmark papers in 1974 and 1988 revolutionized our view of river systems. These works demonstrated that the shallow alluvial aquifers of river floodplains were abundantly populated by diverse large-bodied hyporheic stoneflies (Insecta: Plecoptera) that spent their nymphal stages entirely underground before emerging from the river channel as flying adults. The papers highlighted the broad extent of surface and groundwater interchange, and additionally underscored the importance of hydrologic and biogeochemical connectivity for maintaining biodiversity and productivity of river ecosystems. In the decades that followed, knowledge of the importance of riverine aquifers has expanded but the question has persisted: how do these abundant large-bodied consumers survive in the highly oligotrophic, dark and carbon-limited environment of the aquifer?

River floodplains worldwide are underlain by shallow alluvial aquifers where interstitial flow is driven by penetration of river water into the bed sediments. In gravel-bed systems these aquifers are extremely porous and generally well-oxygenated. The aquifers may contain diverse and abundant meiofauna as well as large-bodied stoneflies (Supplementary Fig. 1). The presence of speciose communities is a conundrum because productivity is generally limited by labile organic carbon.

Results

Methane sources in the Nyack aquifer. At Nyack, we collected samples from seven wells (Fig. 1) previously shown to contain the full suite of aquifer biota (Supplementary Fig. 1). One of the wells had a residence time of 45 days while all others ranged from 117 to 305 days (Supplementary Table 1). We sampled at two depths, 1 and 4 m below the base-flow water table, approximately every three weeks from August 2013 to August 2015. In addition, we collected samples near the bottom of well HA10 specifically to target potential shale off-gassing of methane because this well had high methane concentrations deeper in the well. Only three wells, HA10, HA12 and HA17, yielded methane concentrations high enough for us to measure stable isotope values. Only wells HA10 and HA12 occasionally had high enough concentrations to measure radiocarbon. In these two wells, maximum concentration reached 10% saturation (Supplementary Fig. 3B).

In wells HA10, HA12 and HA17, we compared our measured methane stable isotope ratios to known characterizations of methane sources based on the ratios of deuterium and carbon stable isotope ratios (δD and δ13C) generally clustered at values suggesting a mix of acetoclastic (reduction of organic carbon) and hydrogenotrophic (reduction of carbon dioxide) methanogenesis. HA10 samples deviated from the clustering, suggesting either high levels of microbial oxidation or a thermogenic methane contribution, likely from outgassing of the underlying Kishenehn shale formation. We therefore also measured concentrations of higher chain hydrocarbons—ethane and propane—in these three wells (Fig. 2b). The high ratios of methane concentrations to concentrations of these higher chain hydrocarbons suggested that the aquifer did not contain a thermogenic methane subsidy. However, none of the samples from which we were able to measure ethane and propane concentrations coincided with heavy carbon isotopic ratios. Therefore, the cause of the heavy isotopic ratios of methane was still unresolved and the possibility of a thermogenic methane subsidy remained valid.

Of the three wells with methane present, only HA10 and HA12 had high enough concentrations to determine radiocarbon ages. The methane in well HA10 was consistently older than that of
HA12, and all methane samples that we aged corresponded with methanogenic $^{13}\text{C}$ signatures and a lack of measurable higher level hydrocarbons. Methane in HA12 ranged from 335 ± 15 years BP to 1970 ± 20 years BP, and methane in HA10 ranged from 2350 ± 15 years BP to 6910 ± 140 years BP (Supplementary Table 2). Because radiocarbon ages of dissolved methane samples are the average ages of all sources of methane present, the highly aged methane from HA10 could have included a substantial proportion of ancient methane that is radiocarbon dead; radiocarbon-dead methane could have come from off-gassed methane from the Tertiary age shale underlying the floodplain. For example, the most aged HA10 sample of 6,900 years BP (11/24/2014) could have included up to 58% radiocarbon-dead methane with 42% modern methane: if we assumed that thermogenic methane had a $\delta^{13}\text{C}$ value of $-50\%$ (refs 13,14) and that microbial methane had a $\delta^{13}\text{C}$ value of $-100\%$ (see methods), then the same sample that had a measured $\delta^{13}\text{C}$ value of $-70.6\%$ could have included a maximum of 59% thermogenic methane. The closeness of these estimates suggested that this sample in particular could have had a substantial thermogenic contribution, though we did not have measured ethane and propane concentrations from the same day to verify or refute this possibility. We concluded that measurable dissolved methane in the aquifer was mainly produced via microbial methanogenesis of modern and ancient organic matter, but a subsidy from thermogenic methane was likely, at least in the HA10 well.

Methane-derived carbon in Nyack aquifer stonefly biomass. Five species of amphibitic stoneflies were very abundant in our

Figure 1 | Floodplain locations and characteristics. (a). The four floodplains studied are overlaid on Google Earth Imagery. The main research site was the Nyack Floodplain. (b). Aerial imagery of the Nyack Floodplain shows the locations of the 7 wells studied (see Supplementary Table 1). (c). A view of the Nyack floodplain, near well HA02, shows the pristine nature, landscape complexity, and spatial heterogeneity typical of Nyack. (d). A cross-section of the Nyack bed-sediments highlights the heterogeneity of the matrix: sorted cobbles allow extreme hydraulic conductivity, while the fine sediment presents the opportunity to retain organic matter and develop localized hypoxia or anoxia6.
samples from the Nyack wells: *Paraperla frontalis*, *Isocapnia grandis*, *I. crinita*, *I. integra* and *Kathroperla perdita*. These stoneflies were by far the largest-bodied organisms living in the aquifers. Mature nymphs of *P. frontalis* and *K. perdita* are 2.5–3 cm long, and have mouth parts typically associated with carnivory (elongate mandibles with sharp apical teeth). The *Isocapnia* spp are smaller, 1–2 cm long and have mouthparts associated with herbivory (short mandibles and stout apical teeth). However, guts of all species contained amorphous, particulate organic-matter (POM), especially in early instars. We concluded that in these dark, organic-carbon-limited aquifers, these large consumers eat whatever organic matter they encounter. Stonefly samples from each species had a wide range of variation in $\delta^{15}C$ (Table 1). This variation indicated that the stoneflies were consuming methane-derived carbon, rather than deriving low $\delta^{13}C$ values from a symbiosis with methanogenic microbes. Consumption of methane-derived carbon would occur by stoneflies directly or indirectly (via additional trophic linkages) consuming methane oxidizing bacteria (MOB), likely entrained in the amorphous POM. Stonefly biomass $\delta^{13}C$ values were significantly different between species and between dates of collection (ANOVA analysis $F$ test $P<0.05$) but we observed that the abundance of each species was not consistent across wells. To isolate differences between wells of collection, we pooled both species and dates of collection by well for all subsequent analyses.

We used standard linear two-source mixing models to determine the methane contribution to stonefly biomass in the aquifers. We accounted for methane carbon isotope fractionation by MOB by implementing the most conservative possible estimate of the MOB $\delta^{13}C$ signature as our lower boundary, and the average of our methane $\delta^{13}C$ signatures as an upper boundary, terming these our 'conservative' and 'average' estimates of methane-derived carbon in biomass, respectively (see Methods). We found that stonefly biomass from all wells, including those wells with no measurable methane, included methane-derived carbon (Supplementary Fig. 4). Using a stratified average of both the conservative and average estimates of methane-derived carbon in biomass at each well on the floodplain, we determined that 37.3–66.5% of Nyack aquifer stonefly biomass carbon was methane-derived. Our results therefore showed that the amphibitic stoneflies, the top consumers in the specious aquifer food web at Nyack, were substantially dependent on methane-derived carbon.

**Biomass subsidized by ancient carbon.** Wells HA10 and HA12 were the only wells from which we were able to date the dissolved methane because methane concentrations were low to undetectable in the other wells. Thus only in these two wells were we able to compare methane and stonefly biomass ages. To measure a biomass age most representative of river-supplied carbon, we additionally dated stonefly biomass from the well with the shortest flow path and the lowest overall stonefly methane-derived carbon in biomass: well HA02 (Supplementary Table 1, Supplementary Fig. 5). Biomass radiocarbon ages of individual stoneflies from these three wells were strongly correlated with calculated levels of methane-derived carbon in biomass $\log (\text{Age} + 1,000)$ regressed against the average estimate of methane-derived carbon in biomass per individual; $R^2 = 0.56$, $F$ test $P = 2.328 \times 10^{-10}$, $n = 52$ (Fig. 3). This indicated that: (a) a broad range of methane ages was present in the aquifer, (b) the carbon derived from sources other than methane was modern and (c) stoneflies assimilated methane carbon at least 6,900 years BP old.

We used the measured radiocarbon and $\delta^{13}C$ values of stonefly biomass, methane, and organic matter to parameterize a Bayesian mixing model to estimate the contribution of aged or ancient methane to stonefly biomass in all wells. We estimated the distribution of radiocarbon values for organic matter (or all non-methane carbon sources) by weighting stonefly biomass ages by the per cent non-methane contributions calculated from a two-source mixing model of $^{13}C$ signatures. We then created four scenarios considering two possibilities that represented opposite ends of ranges for each methane $\delta^{13}C$ values and the oldest possible methanogenic methane contribution (Supplementary Table 3). Regardless of scenario, the $\delta^{13}C$ values and radiocarbon ages of the stoneflies were significantly different among the three wells (Fig. 4), suggesting that stoneflies were in fact dependent on local food resources that varied spatially. Where river-supplied...
The data showed that the Nyack flood food web was a widespread phenomenon. *P. frontalis*, *K. perdita* and *I. grandis* were present at all floodplains. We used the same standard two-source mixing model on δ13C values used on Nyack to estimate ranges of methane-derived carbon in stonfly biomass. We parameterized the model using the source estimates for organic matter and methane calculated at Nyack. We found higher estimates of methane-derived carbon in biomass at Nyack than at any other floodplain, but overall methane contributions were high across the other floodplains as well, ranging from 8.5 to 36.5% (Fig. 5, Table 1). This was surprising given that, of fifteen wells analysed across all other floodplains, only three (two at Methow, one on the Jocko) had measurable dissolved methane concentrations (Supplementary Fig. 5). This was similar to the case we found at Nyack, where methane-derived carbon in biomass existed at all wells regardless of methane concentrations.

**Discussion.** The data showed that the Nyack flood food web was heavily subsidized by methane with various carbon ages (from modern to millennial aged or fossil), most of which was methanogenically produced. Although we could not verify a thermogenic methane contribution through presence of ethane and propane concentrations, the documented existence of carboniferous shale at Nyack⁸, presence of highly aged carbon, and presence of heavy methane δ13C added credence to the possibility of a thermogenic contribution to the aquifer food web. Because methanogenesis occurs mainly in anoxic environments and MOB are likely that stoneflies were directly or indirectly consuming resources produced at these interfaces. In fact, the heaviness of biofilm and organic matter δ13C signatures relative to stonfly biomass signatures suggested that stoneflies preferentially consume methane-derived carbon. This could explain their abundance in such a carbon-limited system. Furthermore, because the amphibitic stoneflies emerge from the river as flying or crawling adults, they are exporters of labile organic carbon from the aquifer to the floodplain surface, as well as top consumers in a food web that clearly sequesters methane, a powerful greenhouse gas.

All of the floodplains which we studied had substantial site-wide methane subsidies to top consumers, and Nyack additionally had a millennial-aged to fossil methane subsidy. There are multiple possibilities for the origin of the millennial-aged to ancient methane at Nyack: if methanogenic, it could have been produced from buried organic matter.
deposited since the last glaciation \(^25\); it also could have come from outgassing of thermogenic methane.

This is the first report of a methane-derived carbon contribution to top consumer species across multiple river ecosystems, and the first report of an ancient methane-derived carbon contribution to any freshwater consumers. While methane cycling and aged carbon have each been studied in rivers \(^{26-32}\), the few published studies that document a river food web methane subsidy are site-specific and do not report that the methane-derived carbon was millennial-aged or ancient. For example, Caraco et al. documented an ancient carbon subsidy to zooplankton in the Hudson River Estuary \(^{28}\), Kohzu et al. showed that some macroinvertebrate production was fuelled by biogenic methane produced from detritus in backwater pools \(^{29}\), and Trimmer et al. showed that caddis fly species derived up to 30% of their biomass from methanogenic methane in the River Lambourn \(^{31}\). Additionally, the aquifers that we studied are dark. Therefore, the \(\delta^{13}C\) depletion in stonfly biomass could not have occurred from fractionation of isotopically light CO\(_2\) during photosynthesis, as has been found in previous studies \(^{33}\).

River floodplains are among the most threatened ecosystems in the world owing to dams, revetments and gravel mining, among many other perturbations \(^{34}\). The surprising details of groundwater ecology described herein provide a broader basis for river protection and conservation. The amphibian stonflies are prolific denizens in coupled river-aquifer ecosystems characterized by unperturbed spatial and biogeochemical complexity that produces exploitable food web carbon while at the same time providing natural filtration processes that maintain water quality for downstream reaches.

**Methods**

**Sample collection and processing.** The Nyack floodplain was equipped with seven 3-inch PVC wells with 2 mm slot openings down the length of the pipe. The wells were drilled 8–10 m using a hollow auger drilling rig (Supplementary Table 1) for aquifer characteristics measured at each well. Wells HA02, HA07, HA08, HA10, HA12 and HA15 (Fig. 1) were equipped with sensors and data loggers that recorded dissolved oxygen, temperature, depth and specific conductance on an hourly basis. We used a peristaltic pump equipped with PTFE (Teflon) tubing to draw water from two to three depths at each well—1 and 4 m below the baseflow water table at all wells, with an additional depth of 0.5 m above bedrock (well base) at well HA10. The Kalispell floodplain had seven existing wells drilled similarly (slotted but not screened, and drilled to maximum possible depth). The Jocko had three wells, and the Methow four. We sampled these wells also at 1 and 4 m below the base flow water table.

We sampled methane concentrations approximately every three weeks at all wells on Nyack and in Kalispell for two years, from August 2013 to August 2015.

**Figure 4 | Bayesian modelling outcomes showing carbon contributions of methane and organic matter to stonfly biomass.** Per cent contributions from each modern methane, ancient methane, and organic matter were plotted for each well (colours) for each of the four mixing-model scenarios explained in text and Supplementary Table 4 (symbols). The shaded areas represent the full range of possibilities for source contributions considering the four scenarios. Error bars represent s.d.’s of each estimate. The shaded lines on the methane axis (left) represent the potential mixtures of modern and ancient methane in each well from which we could measure methane ages (HA10 and HA12). Wells HA10 and HA12 were the only two wells on the floodplain with high methane concentrations, and well HA02 was closest to the river with the shortest flow path and lowest levels of methane-derived carbon contribution to stonfly biomass across all samples.

**Figure 5 | Methane-derived carbon contributions to stonfly biomass across floodplains.** Boxplots of methane-derived carbon contribution to stonfly biomass for each of the floodplains studied using both the average and conservative estimation techniques (see Supplementary Table 1 for the two estimates). These estimates each assumed an extreme end of a range of potential source \(\delta^{13}C\) values for methane (see text). The values displayed above each bar are the average methane-derived carbon in biomass values (stratified by well) for each floodplain. Boxes represent the interquartile range with median and whiskers extend to 1.5 times the inter-quartile range for each set of floodplain estimates.
We sampled four times during 2014–2015, once in July 2014 and four times from March to September 2015 on the Jocko and on the Methow. We used a modified active-sampling method: we pumped sample water into a BOD bottle, allowing it to overflow for one to two minutes before withdrawing 1–7 ml using a 22-gauge needle attached to a two-way stopper and 10 ml syringe. In the lab, we had capped 9.83 ml glass scintillation vials with PTFE-lined grey butyl stoppers and combusted them for 24 h at 800 °C until preparation for stable isotope analysis. We froze samples and stored them at −80 °C until preparation for stable isotope analysis. We took the same collection approach for collecting organic matter and biolimn samples for stable isotope analysis, but samples were collected in June to August 2014 during the high water period. We used a modified reduction method39 then measured for radiocarbon (14C) on a compact accelerator mass spectrometer (AMS) facility.

After arriving at the UC-I Keck lab, we carried the samples to the University of California at Davis Stable Isotope Facility for 2H and 13C analyses, where they were analysed on a Thermol Scientific Delta V Plus isotope ratio mass spectrometer (IRMS, Thermo Scientific, Bremen, DE) according to the method of Dijkstra et al.12 Long-term s.e. was 0.2% for δ2H and 2% for δ13C.

To calculate potential methane source contributions, we considered three sources: modern methanogenic methane, ancient methanogenic methane, and thermogenic methane. All methane can be consumed by MOB, which fractionate the dissolved methane by methanogenesis, the methane produced is drastically depleted in 13C (−50 to −80%) due to methanogens preferentially assimilating lighter carbon isotope in their metabolism (δ13C (ref. 14)). Methane in freshwater systems can also be released from thermogenic sources such as shale or coal, though this has not been documented as an ecological subsidy. In this case, hydrocarbons are produced as a result of abiotic pressure and temperature conditions. Thermogenic methane carbon and hydrogen are both isotopically heavier than the methanogenic methane, and the thermogenic methane is usually accompanied by higher level hydrocarbons such as ethane and propane.15 It is also radiocarbon-dead, in excess of 50,000 years, and all thermogenic methane is thermogenically decomposed to produce highly aged methane. All methane can then be consumed by MOB, which fractionate the dissolved methane by preferentially assimilating the lighter carbon isotope, leaving the residual methane enriched in the heavier isotope. In the exponential phase of MOB growth, fractionation in MOB biomass is 30.3% (ref. 19). During normal growth phases, fractionation is 16% (refs 17, 19). A graphical summary of source determination using carbon and hydrogen isotopes is overlaid on Supplementary Fig. 3A. We used isotopic signatures in combination with radiocarbon dating, and measurement of ethane and propane concentrations to determine the methane source. These results are displayed in Supplementary Fig. 3A.

Our results suggested that the majority of dissolved methane was derived from a mixture of acetoclastic and hydrogenotrophic methanogenesis. The samples that deviated from this general classification were both taken from well HA10 at the deep sampling depth. Methane oxidation involves wide variation in deuterium fractionation depending on temperature, and all the samples collected at HA10 have heavier deuterium signatures.15–17 Therefore, these samples could have resulted from high levels of oxidation or a contribution from a thermogenic methane source. This range of possibilities was reinforced by radiocarbon dating, which showed that dissolved methane collected from HA10 was consistently older than dissolved methane from HA1.

We therefore began to collect samples for the measurement of methane and propane concentrations in May 2015. These samples corresponded with stable isotope signatures and insect and radiocarbon ages. None of the samples from which we measured methane and propane concentrations were found to be isotopically heavy despite finding insect biomass carbon concentrations high enough to suggest a thermogenic methane contribution. In general, if the ratio of methane concentration to the summed concentrations of ethane and propane is <100, then the source is thermogenic. If it is >1,000, then the source is methanogenic. In between these two values, the ratios vary, indicating a mix of both sources. No samples had ratios significantly <1,000 (Supplementary Fig. 3B). We therefore concluded that the samples which we measured had no thermogenic methane source.
contribution, though we HA10 deep might still have a thermogenic methane source. We calculated methane-derived carbon contribution using either the average (Avg) or Conservative (Cons) approaches, we had two estimates for OM

Causes of stonefly biomass δ13C depletion. We also measured the age of dissolved CO2, which ranged from 1310 ± 15 to 1970 ± 20 years BP, suggesting that older methane carbon contributions were from organic material rather than DIC, which would be similar to the dissolved CO2 (Supplementary Table 2). The δ13C values ranged from -19.2 ± 0.7 to -14.8% (n = 6), which further indicates this carbon pool is not likely the main contributor for the stonefly biomass. Although the carbon isotope fractionation indicated by the low δ13C values in these estimates (Table 1) can occur via other pathways such as ammonium oxidation and sulphur oxidation, the resulting δ13C values would be far heavier than those we observed. Ammonium oxidation produces bulk biomass depleted in δ13C by 20% relative to CO2, which we measured as −16.6 ± 0.7% (ref. 45), and sulphur oxidation produces bulk biomass depleted in δ13C by 24.6 to 25.1% relative to CO2 (ref. 46).

Methane contribution to biomass: δ13C models. Regardless of methane source, it was necessary to account for the variation in isotopic signatures of methane across the floodplain as we proceeded to calculate methane-derived carbon contributions to stonefly biomass. We assumed that stoneflies consumed MOB as is suggested by the large variation in stonefly biomass δ13C values even within species (Table 1). We used a two-source mixing model22 on stonefly biomass signatures to calculate relative contributions of MOB and organic matter using δ13C values:

\[
\% \text{methane-derived carbon in biomass} = \frac{\text{Stonefly}^{13}\text{C} - \text{OM}^{13}\text{C}}{\text{Methane}^{13}\text{C} - \text{OM}^{13}\text{C}} \times 100
\]

To represent any possible contribution of organic matter to stonefly diet, we used ‘organic matter’ as a surrogate for any component of the stonefly biomass that was not methane-derived carbon. Means and s.e. of δ13C values for each organic matter class are displayed in Supplementary Table 4. Coarse particulate organic matter (CPOM) showed depletion relative to other organic matter pools because stonefly detritus was inevitably and visibly incorporated into the CPOM pools we collected via pumping. We used a stratified average of all OM pools, −27.83 ± 2.49%, which is approximately the literature estimate of photosynthetically fixed terrestrial carbon: −28% (ref. 22).

To calculate the δ13C value of MOB, we bracketed using our measured values of methane itself and maximum levels of fractionation by exponential growth of MOB (z = 30.3%)19. We preferred to use a Keeling plot47 to estimate methane signatures at the time of production, but our data showed extensive variation in isotopic signatures even in samples collected at times with high methane concentrations, making such an estimation technique unreliable (Supplementary Fig. 6). We therefore averaged all samples (n = 32) collected at times when methane concentration was > 1 μmol, yielding −68.79 ± 8.52%. This was termed our ‘Average’ estimate of source methane δ13C and therefore a suitable estimate for the heaviest possible isotopic ratio representative of MOB biomass. We then applied the fractionation factor to this estimate, yielding a most conservative estimate (lightest possible isotopic ratio) of −100.86% using the equation16:

\[
\% \text{source} = \frac{1000 + \delta^{13}\text{C}_{\text{source}}}{1000 + \delta^{13}\text{C}_{\text{product}}}
\]

We termed values of methane-derived carbon contributions using this estimate as our ‘Conservative’ estimate. We presented both sets of data in the results. Estimates were normally distributed. We found a significant effect of species and date of collection on methane-derived carbon in biomass using simple linear regression models and ANOVA analysis (main text). However, both of these variables were strongly confounded with well of collection, as stonefly life history and well conditions inevitably determined the environment which they inhabited at the time of sample collection and thereby influenced the quantities of each measured. In regards to date, we collected from time points over four seasons and during 1–2 years at all sites to avoid bias from sampling time. In order to compare overall levels of methane-derived carbon contributions to stonefly biomass across and within floodplains, therefore, we only considered weeks as strata and pooled species at all times of collection. Please see raw data files for dates of sample collection at each well.

Methane contribution to biomass: δ13C and Δ14C models. We submitted 52 stoneflies from wells HA02, HA10 and HA12 for combined stable isotope analysis and radiocarbon dating at the WM Keck facility at UC Irvine (see Methods above).

We then used to use both δ13C and Δ14C values for implementing a Bayesian framework stable isotope mixing model to infer contributions of various potential methane pools to stonefly biomass. This model considered aged methane, ancient methane, modern methane, and modern organic matter as potential sources, using scenarios of both average and conservative MOB δ13C values.

We inferred the source values for organic matter by taking a weighted average of δ14C values across the 52 stoneflies. Our weights (OM dependence) were calculated as 1− (methane-derived carbon contribution obtained via equation 1). Because we could calculate methane-derived carbon contribution using either the Average (Avg) or Conservative (Cons) approaches, we had two estimates for OM Δ14C: Avg: −13.7 ± 32.2% and Cons: −65.6 ± 75.9%. We used these in the Avg and Cons scenario types (Supplementary Table 3).

For each of the Avg and Cons scenarios, we also had two estimates for maximum methane age measured using Δ14C. The radiocarbon ages that we measured in methane, ranging from 335 to 6900 years BP, were by definition an average of the various carbon ages present in that methane sample. Each sample was a mixture of methane ages. We therefore treated the methane source to represent modern methane, taken as the Avg radiocarbon age of OM, and a second methane source as either aged or ancient methane. Aged methane was given the Δ14C value of the oldest measured methane (−580 ± 7.7%) and ancient methane was considered to be radiocarbon dead, or > 50,000 years in age (−100%). This contributed another dimension to the scenarios needed: Aged and Anc (ancient) methane. Again, the pathway for incorporation of either methane type to stonefly biomass would be via MOB and we therefore needed to consider the possibilities of minimum and maximum fractionation (Avg and Cons). The four scenarios and their associated source values and standard deviations are displayed in Supplementary Table 3.

We implemented the mixing model in the R platform48 using the SIAR package49. The SIAR package allows for the input of source mean stable isotope signatures and their standard deviations. It also requires the input of trophic enrichment factors and their standard deviation, which we took as widely used literature averages42. Individual stoneflies were grouped by well. The SIAR package uses a Monte Carlo Markov Chain simulation to calculate a distribution of possible contributions of each source to each group. We ran the model for 10,000 iterations with a burn-in of 1,000 runs for each scenario. We then compiled the run results and calculate mean and standard deviations of each source contribution to biomass in each well analysed (HA10, HA12 and HA02). Results for the four scenarios are all displayed in Fig. 4 (main paper).

Data availability. Data referenced in this study are tabulated in Supplementary Tables, and deposited at figshare, DOI 10.6084/m9.ﬁgsquare.3519782, or available on request from the corresponding author (AGD).

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