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A case study of the past CH$_4$ cycle in lakes by the combined use of dual isotopes (carbon and hydrogen) and ancient DNA of methane-oxidizing bacteria: rearing experiment and application to Lake Remoray (eastern France)

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Abstract This study aims at estimating the potential of the hydrogen stable isotope (δ^2H) analysis of chironomid remains (HC) to reconstruct past changes in the methane (CH$_4$) cycle in lakes. A rearing experiment was first designed to assess the contribution of hydrogen derived from diet to the chironomid biomass and the offset between larvae and their HC. Results from the rearing experiment show that hydrogen stable isotope applied to the chironomid HC seems to be a powerful geochemical tracer because a large proportion (about 85 %) of the total hydrogen in the chironomid biomass is derived from the diet and that the application of a corrected factor to the HC values allows estimating the δ^2H of the chironomid larvae. We have then tested the interest of the HC δ^2H analysis in addition to other approaches to reconstruct CH$_4$ production and consumption along a sediment core covering the last 1500 years and collected in the deepest part of Lake Remoray (Jura Mountains, France). δ^{13}C and δ^2H analyses were performed on both HC and sedimentary organic matter and were combined with an analysis of ancient DNA (aDNA) of methane-oxidizing bacteria (MOB). Results show that MOB is an important part of the total bacteria aDNA all along the studied period. Moreover, δ^{13}C data confirm that MOB may be an important source of carbon for the chironomid biomass (between 20 and 54 %). Finally, δ^2H values appear fastidious to obtain in the paleolimnological context; however, this tracer may provide evidence that CO$_2$ reduction is the main dominant process of methanogenesis in Lake Remoray. δ^2H analysis could be useful to distinguish the long-term evolution and the relative contribution of the two pathways of methanogenesis. However, δ^2H rearing experiments are needed to better estimate the offset and the contribution values.

Keywords Chironomidae · Hydrogen stable isotopes · Carbon stable isotopes · Methane-oxidizing bacteria · Methanogenesis · Ancient DNA

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Introduction

Freshwater lakes contribute up to 16 % of the global methane (CH$_4$) emissions (Bastviken et al. 2008) and have a non-negligible play in the current global warming (Kirschke et al. 2013). Methanogenesis is a pathway of organic matter degradation under anoxic conditions (Rudd and Hamilton 1978), which caused by

Archean bacteria. According to the very high trophic fractionation of methanogens, biogenic CH_4 is known to exhibit very low $\delta^{13}C$ values (currently from -70 to -50 ‰; Whiticar 1999). Methanogenesis involves two pathways: carbon dioxide reduction and acetate fermentation. According to their metabolic synthesis, biogenic CH_4 exhibits also very low δ^2H values (from -400 to -250 ‰ for the acetate fermentation and from -250 to -150 ‰ for the carbon dioxide reduction; Whiticar et al. 1986; Whiticar 1999). The environmental conditions allowing the control of the type of substrate used during the methanogenesis are very different. Indeed, the quality of organic matter and the temperature could influence the prevalence of these two processes (Glissman et al. 2004; Mandic-Mulec et al. 2012).

However, up to 75 % of this gas is used as a source of energy and carbon by methane-oxidizing bacteria (MOB; Schubert et al. 2010). MOB has also a high trophic fractionation, leading to very low $\delta^{13}C$ values of this organism (from -90 to -60 ‰; Summons et al. 1994; Eller et al. 2007). Very few studies have focused on the hydrogen isotopic values for MOB, but they have suggested that MOB has a much more depleted δ^2H signatures than CH_4 (e.g., Burke et al. 1988; Liptay et al. 1998). MOB can then be assimilated by metazoan organisms, as a complementary food, supporting then the higher trophic level of the lake food web (Ravinet et al. 2010). Lake sediments are known to be "hot spots" of CH_4 production (Bastviken et al. 2004). Some evidences of MOB contribution to the benthic metazoan have been highlighted and particularly for the chironomid larvae (Diptera). In fact, Jones et al. (2008) have shown that up to 80 % of their biomass can derive from MOB assimilation in case of hypoxic hypolimnia. The recognition of the trophic reliance on CH_4 has provided a new perspective on lacustrine trophic functioning (e.g., Jones et al. 2008), but it is still poorly studied. The understanding of such mechanism is hampered by the lack of monitoring data (Naeher et al. 2014). Therefore, long-term reconstructions of past CH_4 dynamics in aquatic food web are needed.

The use of lake sediments as ecological archives allows providing reliable information about the temporal evolution of CH_4 cycle contribution to the benthic food web (van Hardenbroek et al. 2009). Non-biting midges (Chironomidae, Diptera) are ubiquitous organisms whose larvae grow in various environments, including lake sediment. The most chitinous part of

their exoskeletons (head capsule, "HC") is preserved well in surface sediment. The HC chemical composition stays stable for several millennia (Verbruggen et al. 2010). $\delta^{13}C$ analysis of these remains is considered to be a reliable descriptor for tracking temporal changes in sources of carbon assimilated by chironomid larvae. Indeed, the $\delta^{13}C$ offset between larvae and head capsules is low (Heiri et al. 2012; Frossard et al. 2013), and the trophic fractionation (difference in $\delta^{13}C$ values between food and larvae) is assumed to be negligible or very low (Goedkoop et al. 2006; Frossard et al. 2013). Recently, it was demonstrated that $\delta^{13}C$ of chironomid remains could be used to highlight incorporation of CH_4-derived carbon (van Hardenbroek et al. 2009). In addition, the quantitative analysis of ancient DNA (hereafter "aDNA") of MOB has demonstrated a strong correlation between an increase in the percent of MOB in the total bacteria present in sediment and a decrease in $\delta^{13}C$ values of HC (Belle et al. 2014). This methodology allows collecting reliable information about past MOB dynamics and transfers into benthic food web (by reconstruction of chironomid paleodiet). However, the long-term temporal evolution of the biogenic CH_4 synthesis remains poorly understood and needs the calibration of other descriptors such as hydrogen stable isotopes.

Deines et al. (2009) have shown that it could be possible to track the type of substrate used during the methanogenesis by δ^2H analysis of consumers. The use of hydrogen stable isotopes as a secondary trophic tracer in paleolimnological study needs the calibration of two assumptions. In fact, hydrogen from a biomass of consumer derives both from diet and water, and it is necessary to decipher the relative contribution of each sources. In addition, in paleolimnological studies, we have to check that the δ^2H analysis of the chironomid remains is a relevant proxy for the δ^2H value of larvae. Several studies have focused on the quantification of the relative contribution of hydrogen derived from the diet to the chironomid biomass. By δ^2H analyses of both water and chironomid larvae in an experiment, previous studies have shown that between 53 and 70 % of hydrogen in chironomid larvae are derived from their diet (Wang et al. 2009; Soto et al. 2013), making hydrogen isotopes a potential excellent trophic tracer. However, these studies used a constant food source and water with very different δ^2H signature (labeled water). In this context, it was not possible to assess the relationship between the diet contribution

values and the food sources. Complementary rearing experiments are needed to better estimate this contribution. In addition, no study exists concerning the second assumption and this is the first time that the relationship between δ^2H of larvae and their chitinous remains was studied.

An experiment was designed (1) to study the offset between the δ^2H of larvae and their HC and (2) to assess the contribution of hydrogen derived from different diet sources to the organic matter composition of living chironomid larvae. Based on these results, δ^2H and δ^{13}C analyses of subfossil chironomid remains were performed in a sediment core collected in the deepest part of Lake Remoray (Jura Mountains, eastern France) and were combined with MOB aDNA analysis to understand the evolution of CH_4 cycle in this lake over the last 1500 years.

Materials and methods

Dietary origin of chironomid δ^2H

Rearing experiment conditions

Chironomus riparius egg masses were supplied by an ecotoxicology laboratory (IRSTEA, Lyon) and cultured in a 50 L aquarium with approximately 5 cm of fine silica sand. Larvae were grown at approximately 20 °C with a photoperiod of 16-h light/8-h dark and fed with Tetramin (commercial fish food from Tetra).

Ten egg masses were isolated in a 100-mL beaker until hatching. Then, larvae ($n = 100$) belonging to the pelagic first instar were introduced into a 250-mL beaker, with 2 cm of fine silica sand and 200 mL of water. Larvae were then fed using three different types of food that were reduced to fine powder, specifically Tetramin (flakes of leftover fish), Oat (terrestrial plant) and *Groenlandia densa* (freshwater plant) at a rate of 0.4 mg of carbon per larva per day (Doi et al. 2007). Additionally, 150 mL of tap water (with a conductivity of approximately 350 µS cm^{-1}) was replenished daily to prevent the evaporation and increase in ammonium concentration. The study was maintained in the dark to prevent any algae development. Three replicates per treatment were performed. To carry out a potential gradient in the experimental chamber, the position of the nine culture media was randomly

distributed. Culture media were maintained at 20 °C using a thermostatic cabinet (Aqua® Lytic).

Sample collection

Three δ^2H measurements were performed per each food sources. δ^2H analysis was also performed on the larvae, HC and water in each growing medium. Before pupation, four larvae belonging to the fourth instar were collected and introduced into a beaker with distilled water over 24 h to allow gut clearance (Feuchtmayr and Grey 2003). Water samples were also taken at the end of the experiment (between 18 and 23 days) and centrifuged (5 min at 145,000 rotations per minute) to remove any particulate organic matter. Then, water samples were conditioned into a 2-mL collection tube and stored at 4 °C. The substrate of each beaker was sieved through a 200 µm mesh; HC belonging to the fourth instar were picked from the residue. The number of HC collected varied between 40 and 55 to reach the minimal mass of 0.3 mg. Solid samples (larvae, HC and food sources) were then dried at 60 °C for 72 h prior to the isotopic analysis (see "Stable isotope analysis" section below).

Paleolimnological application to Lake Remoray

Study site

Lake Remoray (46°46′12″ N; 6°15′49″ E) is located in the Jura Mountains in eastern France. The water depth of this moderate size lake (95 ha) reaches 27 m (Fig. 1a). The bedrock of its catchment is composed by carbonate substratum. This lake is dimictic, and its tropholytic zone has been hypoxic at the end of summer and during winter stratification (personal unpublished data), since ca. AD 1970. Borderelle et al. (2008) have shown that *Chironomus* sp. larvae from a 20 m water depth exhibited low δ^{13}C values (around −47.9 ‰), suggesting a potentially high transfer of CH_4-derived carbon through a benthic food web.

A 186-cm-long sediment core was collected from the deepest part of Lake Remoray using a gravity corer (UWITEC, 90 mm of diameter) during the autumn of 2013 (Fig. 1b). Sediment dating was performed using a radionuclide method (^{210}Pb, ^{137}Cs and ^{241}Am) and two additional radiocarbon dates (leaves; Beta Analytic, USA). Age–depth was modeled using a simple linear interpolation (Clam package for R; Blaauw 2010).
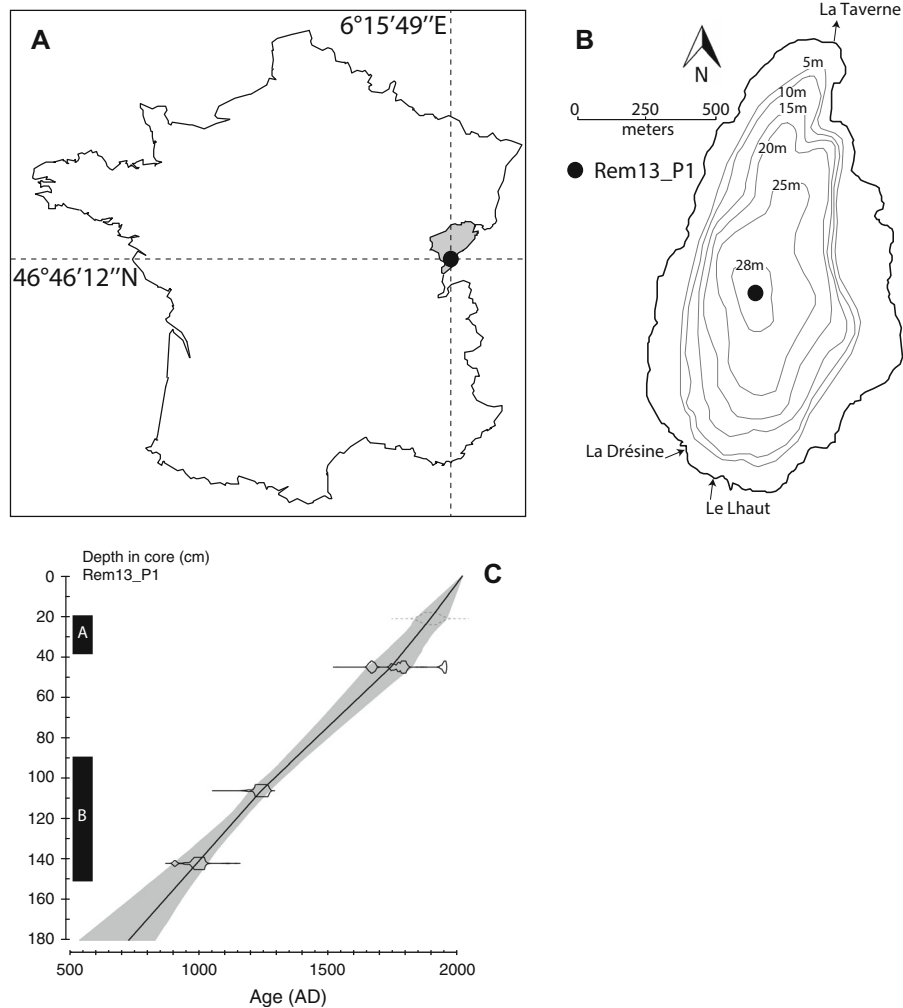
Fig. 1 a Location of Lake Remoray in the Jura Mountains, b bathymetry of Lake Remoray and c age-depth model of the Rem13_P1 core. *Dashed line* indicates radionuclides dating method and *full lines* indicate radiocarbon dates. Samples position in the core: A from 20 to 37 cm; B from 90 to 150 cm

Subfossil chironomid and organic matter sampling

Because genus belonging to the Chironomini tribe has a high affinity to the MOB development (van Hardenbroek et al. 2012), carbon isotope analyses of *Sergentia coracina*-type remains were considered. The first 176.5 cm were cut with a sample thickness of 2, 3 and 4 cm until a depth in the core of 26, 50 and 176.5 cm, respectively (except a sample of 3.5 cm thicknesses between 162 and 165.5 cm). Sediment samples were pre-treated with NaOH (10 %) and HCl (10 %) solutions, following the recommendation of van Hardenbroek et al. (2009). Then, samples were

sieved through a 100 µm mesh. *S. coracina*-type HC belonging to instar 4 were carefully hand-picked under a stereomicroscope. HC were then pooled to reach the minimal mass for $\delta^{13}C$ analysis (20 µg for approximately 10 HC, hereafter "$\delta^{13}C_{HC}$") and introduced into tin capsules. According to the minimal mass required for the analyses and HC concentration, 38 analyses were performed for carbon isotopes.

Sedimentary organic matter was also analyzed on the same samples that received HC analysis (38 samples). Prior to $\delta^{13}C$ analysis, the samples were treated to remove any carbonate content, following recommendations by Belle et al. (2014): fumigation for

6 h under vacuum desiccation in a 12 M HCl solution. Approximately 6 mg of pre-treated sediment was introduced into tin capsules for the analysis of sedimentary organic matter $\delta^{13}C$ (hereafter "$\delta^{13}C_{OM}$").

The temporal evolutions of $\delta^{13}C_{HC}$ and $\delta^{13}C_{OM}$ allow the definition of clusters with similar trend. Hydrogen stable isotopes were performed in five samples within these homogeneous zones. For HC δ^2H analysis, the minimal mass of 0.3 mg (requiring between 150 and 200 subfossil HC) was introduced into silver capsules for (hereafter) "δ^2H_{HC}". The sedimentary organic matter δ^2H analysis was performed on the same samples as the HC hydrogen analysis (five samples), and approximately 1 mg of sediment without pre-treatment was introduced into silver capsules (hereafter "δ^2H_{OM}").

aDNA of methanotroph analysis

Methanotroph aDNA was analyzed from 24 samples (1 cm thickness) taken from the Rem13_P1 core. The aDNA extraction was performed on approximately 300 mg of wet sediment using PowerSoil® DNA Isolation Kit, followed by purification (PowerClean® DNA Clean-Up Kit; from MO BIO Laboratories, Inc.). Primer sequences of methanotroph type I and methanotroph type II were the sequences described by Belle et al. (2014), and total bacteria primers sequences were: total bacteria sens: 5′-GATACCCTGGTAGTC CACGC-3′; total bacteria reverse: 5′-GGCCCCCGTC AATTCCTT-3′. Quantitative polymerase chain reaction (qPCR) conditions were performed, following recommendations by Belle et al. (2014). The sum of methanotroph quantities were expressed relative to total bacteria according to Livak and Schmittgen (2001).

Stable isotope analysis

Carbon isotope analyses were conducted with an Isotope Ratio Mass Spectrometer interfaced with an Elemental Analyzer (EA-IRMS) at INRA Nancy (Champenoux). The results are expressed as the delta notation with Vienna Pee Dee Belemnite as the standard: $\delta^{13}C$ (‰) $= (R_{sample}/R_{standard} - 1) \times 1000$ (‰), where $R = {}^{13}C/{}^{12}C$. Replication of sample measurements from standards gave analytical errors (1σ) of ± 0.12 ‰ ($n = 7$) for HC analyses.

For hydrogen isotope analyses, chitin samples were introduced (approximately 1 mg for larvae and sedimentary organic matter and 0.3 mg for HC) into silver capsules. According to Wassenaar and Hobson (2003), the capsules were left open for a period ≥ 7 days to allow the exchangeable hydrogen in the sample to fully equilibrate with the moisture in the laboratory air. The analytical process also included analysis of standards (BWB-II, whale baleen; and RSPB EGG, egg shell membrane standard with non-exchangeable δ^2H values of -108 ± 4 and -99 ‰, respectively), which allowed the correction of the influence of exchangeable hydrogen pollution by laboratory moisture. This correction assumed that the standards and our samples (HC, larvae, sedimentary organic matter, and food sources) should contain the same proportion of exchangeable hydrogen. Analyses were conducted on an EA-IRMS at Iso-Analytical (UK). The results are expressed as the delta notation with Vienna Standard Mean Ocean Water as the standard: δ^2H (‰) $= (R_{sample}/R_{standard} - 1) \times 1000$ (‰), where $R = {}^2H/{}^1H$. Replication of sample measurements from standards gave analytical errors (1σ) of ± 1.16 ‰ ($n = 14$) for solid analyses and ± 1.68 ‰ ($n = 8$) for water analyses.

Rearing data analysis

The contribution of hydrogen from food to the chironomid larvae biomass was assessed by the use of the Bayesian mixing model proposed by Solomon et al. (2009):

$$\delta^2H_{larvae} = \omega_{water} \times \delta^2H_{water} + (1 - \omega_{water}) \times \sum_{j=1}^{j=n} \left(\phi_{food\,j} \times \delta^2H_{food\,j} \right)$$

In the equation, δ^2H_{larvae}, δ^2H_{water} and $\delta^2H_{food\,j}$ are the hydrogen isotopic signatures of chironomid larvae, water and food measured for each experimental condition and $\phi_{food\,j}$ is the proportion of the food source j in the diet of chironomid larvae. In our rearing experience, $\phi_{food\,j}$ is assumed to be 100 % because larvae were fed with a unique food source per experimental condition; The proportion of the H derived from food can be calculated with the proportion of H in chironomid larvae tissues that is derived from water ($1 - \omega_{water}$). Student's t test was

performed to assess the significance of the difference in the contribution of hydrogen derived from the diet to the chironomid biomass.

Paleolimnological data analysis

We quantified the contribution of carbon originated from MOB to the chironomid biomass by the use of a simple two-source isotope-mixing model (Phillips and Gregg 2001) modified to take into account the difference in $\delta^{13}C$ values between chironomid larvae and their HC (−0.9 ‰; Frossard et al. 2013).

$$\delta^{13}C_{larvae} = \delta^{13}C_{HC} + 0.9 = (\phi_{OM} \times \delta^{13}C_{OM}) + (\phi_{MOB} \times \delta^{13}C_{MOB}) + 0.9$$

In this equation, trophic fractionation between food and chironomid larvae is assumed to be negligible (Frossard et al. 2013). The $\delta^{13}C$ values of sedimentary organic matter ($\delta^{13}C_{OM}$) were assumed to represent those of a mixture of all carbon sources available for chironomid larvae, whereas the $\delta^{13}C$ values of MOB ($\delta^{13}C_{MOB}$) were obtained from the literature and ranged from −90 to −60 ‰ (Eller et al. 2007; Jones et al. 2008).

The temporal evolution of paleolimnological data (MOB proportion, $\delta^{13}C_{HC}$, $\delta^{13}C_{OM}$, and MOB contribution) was smoothed using a local polynomial regressions (loess parameters: size of the neighborhood = 40 % of the data, degree = 2). All statistical analyses and graphical display were performed using R.2.15.1 statistical software (R Core Team 2012).

Results

Rearing experiment

δ^2H values of food sources and water

Tetramin, Oat and G. densa have 42, 48 and 40 % carbon content, respectively (Goedkoop et al. 2006; Doi et al. 2007; this study). The δ^2H values of the three food sources significantly differed (Kruskal–Wallis, $p < 0.05$), with values for Tetramin, Oat and G. densa of −126.6 ± 1.1, −104.2 ± 4.9 and −140.5 ± 4.9 ‰, respectively. The δ^2H values of water in the experiment beakers were −58.4 ± 1.2, −56.2 ± 0.4 and −54.7 ±
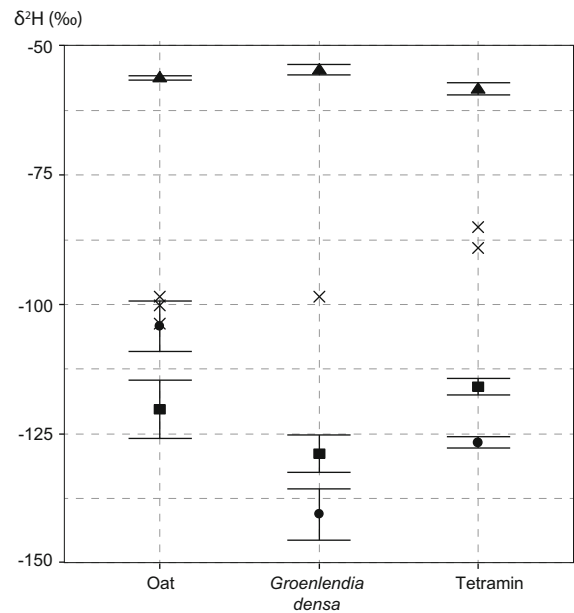
Fig. 2 δ^2H values of chironomid feeding experiment. *Squares* indicate larvae, *cross* HC, *triangle* water and *full circle* food

1 ‰ for Tetramin, Oat and G. densa feeding conditions, respectively (Fig. 2). There was a significant effect of the rearing conditions on the δ^2H values of the water (Kruskal–Wallis, $p < 0.05$).

δ^2H offset between larvae and HC

Unfortunately, the very low hydrogen content in a few samples (<10 µg) has prevented the obtention of the δ^2H values for 2 HC samples from G. densa feeding experiments and 1 HC sample from Tetramin as food. Nevertheless, all the 6 offsets calculated between larvae and their HC at the fourth instar were very negative and ranged from −32 to −13.5 ‰ (Table 1; Fig. 2). These offsets show that the HC were significantly enriched in ^2H compared to the whole larvae.

Contribution of hydrogen from food to the chironomid biomass

The δ^2H values of larvae were −115.9 ± 1.6 ‰ from the Tetramin feeding experiment, whereas Tetramin and water exhibit δ^2H values of −126.6 ± 1.1, and −58.4 ± 1.2 ‰, respectively. Applying the equation proposed by Solomon et al. (2009), the mean

Table 1 Summary of the δ^2H offset and diet contribution values observed in the three rearing conditions

Food source	Offset (‰)	Diet contribution (%)
Oat	−13.5	Na
	−25.7	Na
	−19.1	Na
Tetramin	Na	82.6
	−28.26	87.2
	−31.2	82.7
Groenlandia densa	Na	80.2
	−27.8	88.5
	Na	91

proportion of H in larval tissues originated from Tetramin was 84.2 ± 2.7 % $(1 - \omega_{water} = (\delta^2H_{larvae} - \delta^2H_{water})/\delta^2H_{food} - \delta^2H_{water}) \times 100$, in triplicate. In the experiment with Groenlandia as food, the mean proportion of larval H originated from food was 86.6 ± 5.6 % (Fig. 2). These values were not significantly different (Student's t test, $p > 0.05$), and the average value was 85.4 ± 4.1 %. In the Oat experiment, we observed unexpected results. The mean δ^2H value of larvae was lower (-120.2 ± 5.6 ‰) than the δ^2H values of both water (-56.2 ± 0.4 ‰) and Oat (-104.2 ± 4.9 ‰) (Fig. 2). Consequently, the larvae have mainly fed on an alternative (or complementary) food with a lower δ^2H value than Oat.

Application to Lake Remoray

### MOB abundance

The sediment core collected in the deepest part of Lake Remoray covered the last 1500 years (Fig. 1c). The MOB aDNA is in relative high proportion, and the average value is around 15 % of the total bacteria aDNA (Fig. 3a). The temporal evolution reveals values ranging from 5 to 34.5 %. There is no statistical difference in the MOB proportion between A and B samples (Student's t test, $p < 0.05$).

In situ variability of $\delta^{13}C$ and δ^2H values of subfossil chironomid

The $\delta^{13}C$ values of the chironomid remains (HC) are very negative all along the sediment core (ranging from -43.9 to -38 ‰; Fig. 3b), and much more depleted in ^{13}C than the sedimentary organic matter (ranging from -33.4 to -28.1 ‰; Fig. 3b). Thus, the differences between the $\delta^{13}C_{OM}$ and $\delta^{13}C_{HC}$ values are very high and range from 6.5 to 17 ‰ (Fig. 3b). A simple two-source isotope-mixing model ($\delta^{13}C$ of sedimentary organic matter and $\delta^{13}C$ of MOB with a value of -90 and -60 ‰) was performed to estimate the contributions of MOB to the chironomid biomass along the core. These estimations showed a temporal trend with values ranging from 10 to 27 % with MOB


Fig. 3 MOB aDNA proportion (**a**), $\delta^{13}C_{OM}$ (**b**), $\delta^{13}C_{HC}$ (**b**), and MOB contribution (with $\delta^{13}C$ value of -60 ‰) to the chironomid biomass (**c**). In the **b**, *squares* correspond to sedimentary organic matter results, whereas *diamonds* to the HC values
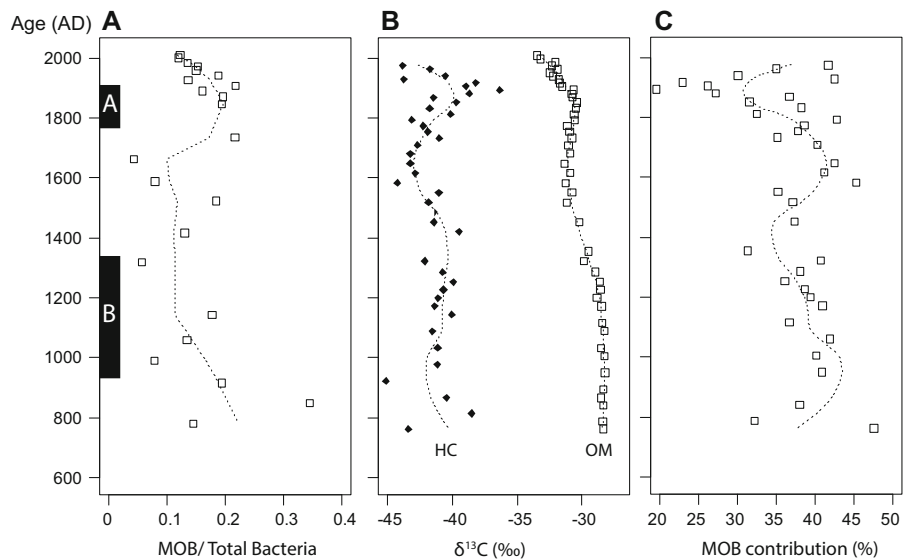
Table 2 Stable carbon and hydrogen data for the Remoray core

	Sample	$\delta^{13}C$ (‰)	δ^2H (‰)
OM	A	-30.6 ± 0.14	-103.2
	B	-28.6 ± 0.48	-98.2 ± 4.51
HC	A	-40.2 ± 2.24	-107.5
	B	-40.9 ± 0.72	-107.8
Larvae calculated	A	-41.1 ± 2.24	-131.6 ± 6.1
	B	-41.8 ± 0.72	-132.1 ± 6.6
Assimilated mixture	A	-41.1 ± 2.24	-142.6 ± 7.8
			-186.5 ± 12.4
	B	-41.8 ± 0.72	-143 ± 7.8
			-187.1 ± 12.4

Isotopic values of larvae were calculated using an offset of -0.9 ‰ for $\delta^{13}C$ (Frossard et al. 2013); and offsets ranging from -31.2 to -13.5 ‰ for δ^2H. The contribution values of diet-derived hydrogen are 53 and 85 %
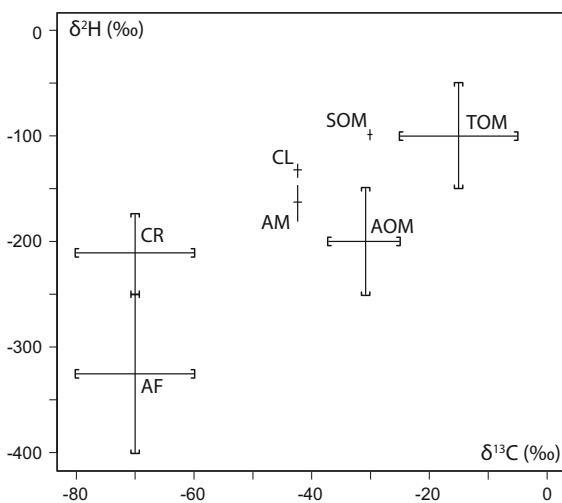
Fig. 4 Biplot representing $\delta^{13}C$ and δ^2H values of the isotopic baseline of potential organic matter available for the chironomid feeding. CR corresponds to the CH_4 isotopic values from CO_2 reduction and AF from acetate fermentation (Whiticar et al. 1986; Whiticar 1999). AOM corresponds to aquatic organic matter (Vuorio et al. 2006; Hondula et al. 2013); and TOM to terrestrial organic matter (Meyers and Ishiwatari 1993; Doucett et al. 2007); SOM to sedimentary organic matter (data from this study); AM to assimilated mixture (calculated values from this study) and CL to chironomid larvae (deduced values from HC of this study)

values of -90 ‰; and from 20 to 54 % with MOB value of -60 ‰ (Fig. 3c).

Only two samples (among five) were analyzed for the hydrogen stable isotope and determined by the HC concentration in the Rem13_P1 core (A from 20 to 57 cm; and B from 90 to 150 cm). The δ^2H_{OM} values were -103.2 and -98.2 ± 4.51 ‰, and the δ^2H_{HC} values were -107.5 and -107.8 ‰ for samples A and

B, respectively. The $\delta^{13}C$ values ($\delta^{13}C_{HC}$, $\delta^{13}C_{OM}$), and δ^2H_{OM} were then averaged for comparison in order to cover the range of the two HC δ^2H samples.

It is possible to estimate the isotopic values of larvae using the offsets between larvae and HC (Frossard et al. 2013 for $\delta^{13}C$; for δ^2H offset see part "δ^2H offset" section below). These offsets are -0.9 ‰ for the $\delta^{13}C$ values and ranged from -32 to -15.3 ‰ for δ^2H values. According to these results, the evaluation of the larvae isotopic signatures from sample A is -41.1 ± 2.24 ‰ for $\delta^{13}C$ and -131.6 ± 6.1 ‰ for δ^2H (Table 2). For sample B, the $\delta^{13}C$ and δ^2H values of larvae are evaluated about -41.8 ± 0.72 and -132.1 ± 6.6 ‰, respectively.

The trophic fractionation between chironomid larvae and their source is considered to be negligible for carbon isotopes (Heiri et al. 2012; Frossard et al. 2013), and the "assimilated mixture" $\delta^{13}C$ values are -41.1 ± 2.24 and -41.8 ± 0.72 ‰ for the two samples (Table 2; Fig. 4). The evaluation of the $\delta^2H_{assimilated\ mixture}$ is calculated by the following equation:

$$\delta^2H_{assimilated\ mixture} = \left(\delta^2H_{larvae} - \left(\omega_{water} \times \delta^2H_{water}\right)\right) / \left(1 - \omega_{water}\right).$$

Based on the results from this study, $(1 - \omega_{water})$, which is the proportion of hydrogen in chironomid larvae derived from the diet, is approximately 85 % (Fig. 2b). Because lower values of this proportion are reported in the literature (Wang et al. 2009; Soto et al. 2013), the lowest value of 53 % has been also taken into account to include the uncertainty of our results in the calculated δ^2H of assimilated mixture. In the equation, the δ^2H of water has been assumed to be constant and at about -70 ‰ in Jura lakes (δ^2H value of water from Lake

Saint-Point hypolimnion; Leroux 2010). The calculated δ^2H values for the assimilated mixture ranged between -186.5 ± 12.4 and -142.6 ± 7.8 ‰, and between -187.1 ± 12.4 and -143 ± 7.8 ‰ for the A and B samples, respectively (Table 2).

Discussion

Rearing experiment

δ^2H offset

The study analyzing the difference between larvae and their HC at the fourth instar revealed a range of δ^2H offset between -31.2 and -13.5 ‰ (Fig. 2a). The significant depletion in ^{2}H of larvae compared to their HC could be explained by the difference in chemical composition of these tissues. In fact, the exoskeleton of an arthropod, including chironomid HC, is mainly composed of chitin, a polysaccharide chain. The whole body of larvae corresponds to two biochemical matrices: exoskeleton and soft tissues (including muscle, fatty tissues…). The chemical composition of soft tissue is more complex than that of exoskeleton. The main components of the soft tissues include proteins, lipids, carbohydrates and chitin (approximately 50, 14, 23 and 4 % of dry mass, respectively, Yurkowski and Tabachek 1979). It is widely recognized that lipids exhibit very low δ^2H values (Estep and Hoering 1980; DeNiro and Epstein 1978). The higher lipid content in larval tissues may induce the depletion of approximately -25 ‰, when compared to the δ^2H values of HC. Several studies have also highlighted the influence of the lipid content on explaining the differences in δ^2H values between tissues in the same animal (Hobson et al. 1999; Graham et al. 2014). Moreover, Soto et al. (2013) have shown that lipid extraction before deuterium analysis of chironomid larvae induces an increase in the δ^2H values, and they have suggested extracting the lipid before bulk tissue analysis. In this study, the δ^2H analysis was performed on bulk tissue for both HC and larvae. It is possible that a change in the lipid content in larvae can induce a change in the offset value. Nevertheless, the correction of the HC δ^2H value by an offset, ranging from -31.2 to -13.5 ‰, can be proposed as first values to provide a reliable estimation of the larval δ^2H value.

Contribution of the hydrogen from diet to the chironomid larvae

This experiment was designed with three isotopically distinct foods (with a range of -36 ‰) and growing water with a low δ^2H variation (<3 ‰). This configuration allows the assessment of the contribution of hydrogen derived from food, which is transferred to the chironomid biomass assuming that this contribution is not food-dependent. In fact, Solomon et al. (2009) have shown that this contribution depends on the trophic level of the consumer which is constant in our rearing experiment. Low δ^2H variation for water and highly variable δ^2H of food mimic natural conditions were diet sources that can strongly vary over time (Grey et al. 2004) and space (Belle et al. 2015) for each consumer. This contribution reached approximately 85 % (Fig. 2b; 85.4 ± 4.1 %) for two of the three treatments (Tetramin and *G. densa*). Prior to this study, two estimates were reported for the contribution of diet to the chironomid larvae δ^2H values. Wang et al. (2009) found that 69 % of hydrogen in chironomid was derived from the diet, whereas Soto et al. (2013) estimated this contribution at 53 %. Applying to *C. riparius* larvae, the formula proposed by Solomon et al. (2009) to calculate this contribution from the trophic level of the consumer leads to a contribution of 75 % considering the trophic level equal to 1 (after producers). The range of values is large, and many uncertainties are still unresolved (e.g., influence of the temperature on this proportion, etc.). In addition, other factors could affect the experimental conditions in the growing media (evaporation, algal development, bacterial development, etc.).

The Oat experiment illustrates well the difficulties in maintaining constant the "iso-integrity" of food available for chironomid larvae. Microbial colonization, enhanced by the high mineral element content of Oat, could induce an alteration of the δ^2H of food (Goedkoop et al. 2006) or can be preferentially assimilated by larvae (Akerblom and Goedkoop 2003). In this way, biofilm formations were seen in the growing media during the chironomid development. It is impossible on the basis of available data to better understand what happened in the growing media. However, these results could provide evidence of the selective feeding behavior of chironomid larvae.

These uncertainties encouraged us to take into account the lowest and the highest values of

contribution (53 and 85 %) to reconstruct the δ^2H signature of assimilated mixture by chironomid larvae from the A and B samples in the Remoray's core.

Past CH_4 cycle in Lake Remoray

MOB contribution to the chironomid biomass

qPCR analysis of aDNA allows highlighting the very high contribution of MOB to the total bacteria aDNA extracted from sediment samples. The relative proportion reaches approximately 13 % (Fig. 3a). The living MOB community mainly grows at the sediment/water interface (Rahalkar et al. 2009; Schubert et al. 2011; Ho et al. 2011), and this development is limited by the availability of CH_4 (Hershey et al. 2015). As a consequence, results from the present study suggest that both CH_4 production and CH_4 oxidation rates were very high in Lake Remoray over the studied period. In addition, the availability of the MOB should significantly influence the chironomid paleodiet (Belle et al. 2014).

Carbon isotope analysis provides evidence of the incorporation of a very ^{13}C-depleted organic matter into chironomid biomass because the $\delta^{13}C$ value of the assimilated mixture appears much lower than that of sedimentary organic matter (−42 vs. −30 ‰; Table 2). The complementary assimilation of CH_4-derived carbon by chironomid larvae is evident (Kohzu et al. 2004; Doi et al. 2006). Moreover, it is widely recognized that MOB can contribute significantly to the chironomid biomass (Jones et al. 2008 and references therein). The results from the mixing model suggest a variable contribution to the chironomid biomass, but these values are always superior to 10 % (from 10 to 27 % with MOB values of −90 ‰; and from 20 to 54 % with MOB value of −60 ‰). Despite the uncertainty about the MOB contribution to the chironomid biomass, the results clearly show that transfer of biogenic CH_4 into the benthic food web of Lake Remoray is important all along the studied period (the last 1500 years).

Methanogenesis pathway and δ^2H

It was very fastidious and time consuming to reach the minimal mass needed for the hydrogen analyses (three of five samples were below the limit of quantification of 10 μg of hydrogen), and this mass required a large

number of HC (more than 150 subfossil HC per sample). The hydrogen isotope analysis could provide complementary information about the methanogenesis pathway in context, where CH_4 oxidation and transfer into the food web was provided by other descriptors (MOB aDNA and $\delta^{13}C_{HC}$ in this methodology).

As concluded by several previous works, it is possible to distinguish the type of substrate (acetate or carbon dioxide) used during methanogenesis. Indeed, acetate fermentation allows the production of very ^2H-depleted CH_4 (currently between −400 and −250 ‰), whereas carbon dioxide reduction produces a less ^2H-depleted CH_4 (currently ranging from −250 to −170 ‰; Whiticar et al. 1986; Whiticar 1999). The trophic fractionation for the hydrogen isotopes is still poorly studied, but it is known that MOB exhibits a higher fractionation for hydrogen than for the carbon (Coleman et al. 1981; Sessions et al. 2002), suggesting that the δ^2H values of MOB should be much more negative than CH_4 δ^2H values. However, Sessions et al. (2002) have also shown that an important part (up to 50 %) of the hydrogen content in lipids of MOB derives from environment water, inducing an opposite effect to the trophic fractionation. These observations make it difficult to reliably estimate the MOB δ^2H values.

However, in the present study, the estimated assimilated mixture was moderately ^2H depleted compared to the sedimentary organic matter (between −186 and −142 ‰ against −100 ‰, Table 2). The present results from the mixing models applied to $\delta^{13}C$ values have shown that this dietary corresponds to a mixture between MOB and sedimentary organic matter (likely a ratio ranging between 10:90 and 54:46; Fig. 3). Nevertheless, the moderately negative signature of the assimilated mixture (between −186 and −142 ‰) may suggest that in Lake Remoray, the dominant methanogenesis pathway may be CO_2 reduction, which seems to coincide with what is known about environmental conditions required for this pathway of methanogenesis. In fact, this hydrogenotrophic pathway of CH_4 synthesis has also been previously reported from deep stratified lakes similar to Lake Remoray (Lojen et al. 1999; Ogrinc et al. 2008). In addition, the catchment area of Lake Remoray is very large and could induce an important flow of terrestrial organic matter with a low index of degradability ($\delta^{13}C$ values of sedimentary organic carbon −28 ‰; Fig. 3c). Hydrogenotrophic pathway seems to correspond to the anoxic degradation of

refractory organic matter under low temperatures (Sugimoto and Wada 1993; Mandic-Mulec et al. 2012), whereas acetoclastic methanogenesis corresponds to degradation of labile organic matter.

Conclusion and future challenges

A rearing experiment was conducted to calibrate the use of hydrogen stable isotopes to reconstruct the past CH_4 cycle in lake. Results show that the contribution of hydrogen derived from diet to the chironomid biomass is important (around 85 %), making the hydrogen stable isotopes an excellent trophic tracer. This value is the highest in the literature and highlights the complexity of the metabolic processes involved in the chironomid feeding behavior. Moreover, the range of offset between larvae and HC seems to vary from -31.2 to -13.5 ‰. The use of the extreme values in paleolimnological studies should allow the estimation of δ^2H value of the chironomid larvae and reflect the uncertainty of this value. More rearing experiments are needed to better estimate these two prerequisites, in particular the use of other food sources (e.g., with MOB culture similar to experiment designed by Deines et al. 2007), and different chironomid species. In addition, complementary studies are needed to assess the potential effect of acid pre-treatment on the HC δ^2H values (similar to Perga 2009). In addition, recent advance in mass spectrometry could allow the work with very low mass of hydrogen inducing the decrease in the HC mass required (desirable for a wide use in paleolimnological studies).

The application in the paleolimnological study of Lake Remoray appears fastidious and time consuming. However, the use of the range of offsets and the range of diet contribution allow the estimation of the δ^2H values of the assimilated mixture by chironomid larvae and these values are not enough negative to highlight the implication of the acetoclastic pathway. In this context, this new paleotracer may provide evidence that CO_2 reduction was the main dominant process of methanogenesis in Lake Remoray over the studied period. In conclusion, hydrogen stable isotope analysis could be useful to distinguish the long-term evolution and the relative contribution of the two pathways of methanogenesis and offers a powerful package to study the past CH_4 cycle with combined analyses of $\delta^{13}C$ and MOB aDNA.

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