



Stable isotope analyses of lacustrine chitinous invertebrate remains: Analytical advances, challenges and potential

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ABSTRACT

This review examines recent developments in the application of stable isotope analyses ($\delta^{18}\text{O}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, δD) to lacustrine invertebrate remains. These remains are ubiquitous in lacustrine sediments and thus provide an opportunity to measure changes in stable isotope ratios across a range of timescales and environments and allow interpretive power beyond taxonomic studies. To date they have been relatively understudied in comparison to carbonate fossils and offer both opportunities and challenges and we explore both themes in this review. This review will explore improvements to analytical instrumentation and the opportunities that this presents, it will look at a range of new studies of the modern lacustrine environment and how these studies allow a more nuanced palaeoenvironmental approach. We review recent studies that have used these advancements in understanding to help to reveal new knowledge of past climates, environments and ecology. In addition, we explore new studies that help to elucidate the role of methane-derived carbon to lacustrine food webs and the drivers behind this, including new data to estimate the contribution of methane derived carbon to an arctic lake. We conclude that major progress is currently being made in invertebrate-isotope analyses, and we expect this to continue apace.

1. Introduction

In 2018, van Hardenbroek et al. (2018a) explored the state of understanding and challenges of analysing stable isotope ratios on a range of organic and inorganic fossils preserved in lake sediments. Their work included a detailed analysis of lacustrine invertebrate remains. This review serves as an update and extension of that review and focuses on new developments of stable isotope analyses of a range of lacustrine invertebrate remains (including the head capsules of chironomid larvae, cladoceran exoskeletons and resting stages, beetle elytra, bryozoan

resting stages, Fig. 1). The reason that lacustrine invertebrate remains are preserved relatively well is that they are predominantly composed of chitin and protein (Stankiewicz et al., 1996, 1998). These materials create very robust exoskeletons, which remain well preserved in lake sediments (see King et al., 2024 for review). Under certain conditions, such as reducing conditions in bottom waters for example, chitinous aquatic invertebrate remains can preserve for hundreds of thousands of years and longer (e.g. Bennike et al., 2023). Opportunities to expand the range of isotopes and reduce the sample size required have come from improvements to instrumentation, through more sensitive Thermal

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Conversion Elemental Analyser isotope ratio mass spectrometry (TC/EA-IRMS). This offers the potential to answer more refined questions that were previously out of reach, and we provide new data that tests the capability of these improvements in EA-IRMS (section 2.3) and explore the potential for taking this forward (section 4). The ability to analyse increasingly smaller samples also brings challenges. The potential impacts of contamination when cleaning/extracting remains from sediment becomes more crucial for example, and we review the recent research focused on sample preparation techniques and any implications for impact on isotope ratios. Further, we discuss the considerations needed to assess the sample size measured and avoid systematic bias. Is smallest always best or does this lead to a biased environmental reconstruction? Isotope research to date has primarily focused on carbon and oxygen, two of the most abundant elements preserved in invertebrate exoskeletons but advances in mass spectrometry have opened up the possibility of analysing the isotopes of less abundant elements and we review the developments that have been made here, including less well-used and understood isotope systems (e.g. sulfur), which may provide new insights to food-web dynamics. We also explore new findings from calibration studies, both in the laboratory and in the field, and discuss new findings surrounding spatial and temporal variations in stable isotope variations in invertebrates within individual lakes and what this means for the future direction of stable isotope studies in this field.

Finally, we turn our attention to palaeoenvironmental reconstructions, describing new applications of stable isotopes to understand past food webs, temperature, hydrology and climate, in some cases in partnership with other techniques including ancient DNA, radiocarbon and biomarker analyses. We review new studies that have used these techniques in combination with stable isotopes or combined these with less frequently used stable isotopes (nitrogen and sulfur for

example) to help to reveal changes in lake palaeoecology. We explore new studies that help to elucidate the role of methane-derived carbon to lacustrine food webs and the drivers behind this, including new data combining radio and stable carbon isotopes to estimate the contribution of methane derived carbon to an arctic lake.

2. Methods: improvements in preparation and mass spectrometry

2.1. Preservation and chemical pretreatments

Several studies have tried to assess the potential impacts of degradation and decomposition on the chemical structure and stable isotope composition of chitinous aquatic invertebrate remains. Verbruggen et al. (2010a) used Curie Point Pyrolysis to study the chemical composition of fresh chironomid head capsules and fossil head capsules that were ca. 16,000 years old. They showed that the ancient head capsules still contained both chitin and protein, as with fresh head capsules. These results indicated that decomposition and other chemical alterations did not fundamentally change the chemical composition of chironomid remains in late Quaternary lake sediment records. Perga (2011) assessed early diagenetic effects on cladoceran remains in incubation experiments with lake water and sediments. The experiments focused on the exoskeletons of *Daphnia*. The results indicated that most of the early diagenetic effects take place in the water column and that these influenced both the carbon and nitrogen isotopic composition of the exoskeletons. Whereas changes in $\delta^{13}\text{C}$ values were relatively minor ($<+1\%$), changes in $\delta^{15}\text{N}$ values were more substantial ($+2\text{--}5\%$). In contrast to incubation in lake water, incubation in sediment did not appear to lead to further changes in the $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values. *Daphnia* exoskeletons often preserve poorly and appear exceptionally susceptible

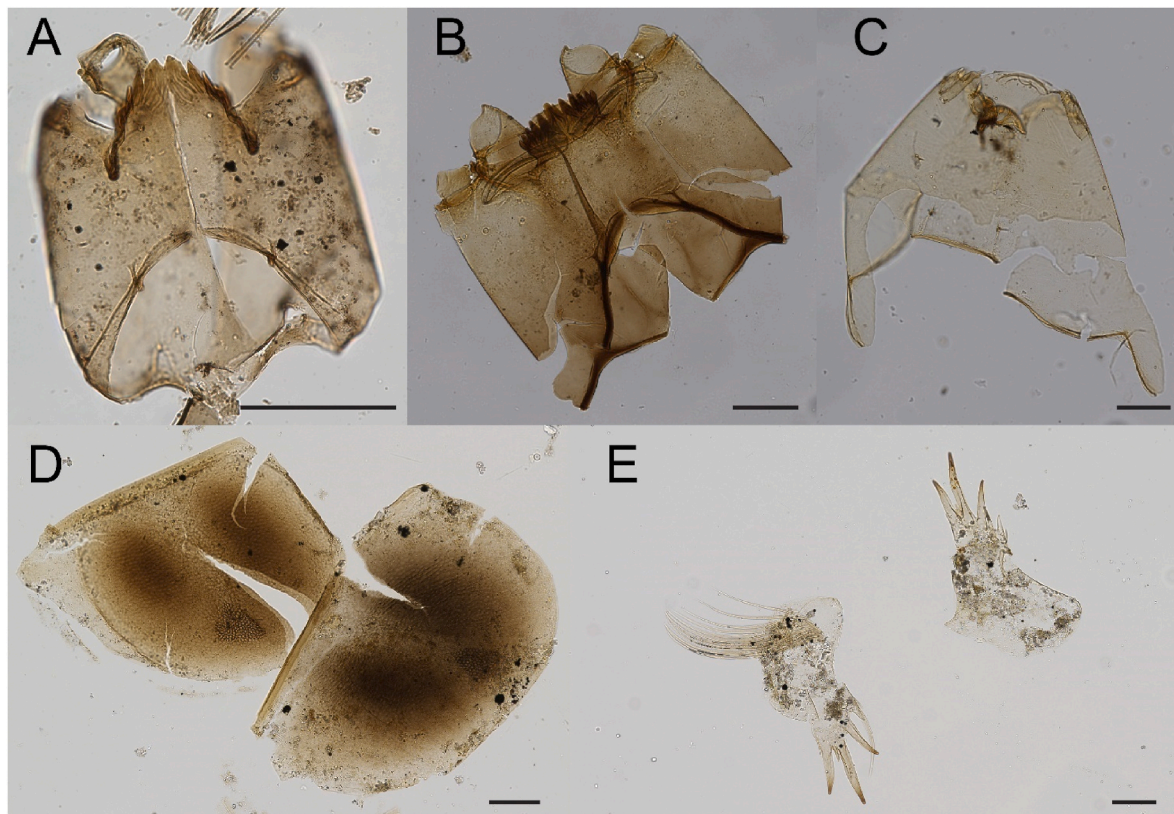


Fig. 1. Examples of chitinous aquatic invertebrate remains from lake sediments. A-C Head capsules of chironomid larvae (A: *Parametrioecnemus/Paraphaenocladius*, B: *Micropsectra radialis*-type, C: *Procladius*), D *Daphnia* ephippia, E mandibles of Chaoboridae (*Chaoborus flavicans*-type). Scale bars in A-E are 100 μm . Photo credits Oliver Heiri.heiri

to microbial degradation. Whether the stable isotope composition of more robust exoskeletons of other cladoceran groups (e.g. *Bosmina*, Chydoridae) or cladoceran ephippia are similarly affected by early diagenesis and degradation as *Daphnia* exoskeletons presently remains unknown. In their oxygen isotope study of chironomid remains from Hawes Water, Lombino et al. (2021b) examined the macromolecular structure of the remains and found that they were characteristic of the formation of aliphatic (geo)polymers during diagenesis, via the polymerization of liberated lipid molecules. They also found that pretreatment had the effect of relatively depleting the proteins and chitin further. An estimation of the impact of diagenesis on the Hawes Water chironomid remains calculated the average inferred diagenetic fractionation to be 1.0035 ± 0.0008 (1 SD) equivalent to an inferred $+3.5 \pm 0.8\text{‰}$ change in $\delta^{18}\text{O}_{\text{chironomid}}$ values.

Several studies have assessed the effects of chemical pretreatment on chitinous remains of aquatic invertebrates. Pretreatment is a necessary step to disaggregate and systematically remove impurities from the sample prior to analysis. Typically, this comprises an initial addition of potassium hydroxide (KOH) to deflocculate sediment organic material prior to sieving and can be followed by a combination of acids/alkaline steps to remove carbonates (Hydrochloric acid (HCl)), silicates (hydrofluoric acid (HF)) plus sodium chlorite and glacial acetic acid steps to further purify and to remove waxes, oils, and resins (see Verbruggen et al., 2010a for further details). van Hardenbroek et al. (2010) examined the effects of processing samples with 10% KOH (for 2h), 10% HCl (2h), 40% HF (18h) and demonstrated that there was no measurable effect on $\delta^{13}\text{C}$ values. However, a combination of boiling, accelerated solvent extraction and heavy chemical oxidation of chironomid head capsules resulted in a minor (0.2‰) but noticeable effect on the $\delta^{13}\text{C}$ values of the exoskeletons. All measurements, regardless of chemical treatment, fell within a narrow range of $\pm 0.5\text{‰}$ from the average, indicating only very minor effects of pretreatments on $\delta^{13}\text{C}$ values of chironomid exoskeletons. Verbruggen et al. (2011) exposed chironomid head capsules to 10% KOH at room temperature (2h) and at 70 °C (1h) and to 28% KOH (24h) at 100 °C to test the effects of pre-treatments on $\delta^{18}\text{O}$ values of chironomid head capsules. Additional samples were exposed to 30% HCl followed by 40% HF and in a final experiment to 10% KOH followed by accelerated solvent extraction and then exposure to sodium chlorite and glacial acetic acid, a treatment that had previously been used to purify cellulose. Scanning Electron Microscope photographs clearly showed that the treatments changed the microstructure of the head capsules, preferentially dissolving the chitin or protein moieties of the cuticles. Curie Point Pyrolysis confirmed that treatment by strong acids reduced the chitin in the cuticles, whereas treatment with alkali or sodium chlorite strongly reduced the protein contents. Different treatments also significantly affected the $\delta^{18}\text{O}$ values of the head capsules leading to variations of up to 6‰ between the different treatments. Verbruggen et al. (2011) recommend not to use acid or strong alkali treatments for sample pretreatment of fossil chironomid remains intended for oxygen isotope analysis. In a further study, Verbruggen et al. (2011) showed that exposure of head capsules in acidified, isotopically light or heavy water led to increasing differences in $\delta^{18}\text{O}$ values between these treatments with time, suggesting exchange of oxygen between water and the cuticles. Verbruggen et al. (2011) recommended treatments that eliminate carbonates at neutral pH (e.g. in a buffered NH_4Cl solution) rather than in acid solutions for cleaning samples of chitinous invertebrates from carbonate particles before $\delta^{18}\text{O}$ analysis. The effect of NH_4Cl treatment on $\delta^{18}\text{O}$ values of chironomid head capsules, beetle elytra and crab chitin standard were also tested by Clarke et al. (2019), who found CaCO_3 was effectively removed and no significant effect on $\delta^{18}\text{O}$ values of invertebrate remains or chitin standard was evident after up to 24h in NH_4Cl . To our knowledge the effects of pretreatment on the hydrogen isotopes of invertebrate remains have not yet been investigated but are likely to be complex (Wassenaar and Hobson, 2000).

2.2. Mass spectrometry

The low, and variable, mass of chironomid head capsules and other invertebrate remains provides a challenge for mass spectrometry. In 2010, Verbruggen et al. (2010a) suggested a minimum weight of 40–50 μg was required for $\delta^{18}\text{O}_{\text{chitin}}$ measurement based on replicate measurements of *Chironomus riparius* head capsules. Similarly, van Hardenbroek et al. (2010) determined a minimum sample size of 20 μg was needed for reliable $\delta^{13}\text{C}_{\text{chitin}}$ measurements on EA-IRMS instrumentation. These requirements have resulted in the need to combine many head capsules together to arrive at a sample size large enough to meet lower detection limits, and thus restricting the resolution of the analyses. In their 2018 review paper on lacustrine organic and inorganic fossils, van Hardenbroek et al. provided an overview of the mean weight of individual remains, to assess the minimum number of remains required for stable isotope measurements (Table 1). This illustrates both inter and intra-taxon variability in mass which creates further challenge to designing robust analytical programs.

In the last 5 or so years, new developments in EA-IRMS have allowed the lower detection limits for organic material to decrease significantly.

Table 1

Mean weight and standard deviation of individual invertebrate remains. Data are derived from surface and down core sediments after chemical processing with 10 % HCl and 10 % KOH (van Hardenbroek et al., 2010, 2012, 2013; van Hardenbroek, Heiri and Wooller unpublished). Note that values are means for samples containing different sizes of remains, including head capsules from different larval instars. Originally included in the supplementary data of van Hardenbroek et al., (2018a).

	Surface samples			Down core samples		
	weight (μg)	SD	n	weight (μg)	SD	n
Chironomid head capsules						
<i>Chironomus anthracinus</i> -type	2.5	3.4	18			
<i>Chironomus plumosus</i> -type	2.8	2.7	14			
<i>Chironomus</i> spp.	2.1	2.1	81	1.2	0.4	30
<i>Dicretodipes</i>	1.2	0.7	6			
<i>Endochironomus</i>	1.0	0.5	6			
<i>Glyptodipes</i>	2.0	2.3	6			
<i>Microdipes</i>	1.0	0.4	5			
<i>Polypedilum</i>	0.6	0.2	5			
<i>Sergentia</i>	0.9	0.3	1			
Chironomini (including all taxa)	1.6	2.2	241	1.0	0.4	76
<i>Coryocera ambigua</i>				1.3	0.1	7
Tanytarsini	0.6	0.5	122	0.5	0.2	39
Orthocladinae	0.7	0.5	105	0.9	0.5	42
Diamesinae	1.9	0.9	3			
Tanytopodinae	1.0	0.7	89	1.0	0.3	17
Cladoceran ephippia						
<i>Daphnia</i> spp.	2.8	2.9	148	2.8	2.7	51
<i>Simocephalus vetulus</i>	2.8	1.4	11			
<i>Ceriodaphnia</i> spp.	0.8	0.7	45			
<i>Leydigia</i>	1.1	0.8	5			
Chydorid	0.9	0.5	26			
Bryozoan statoblasts						
<i>Plumatella</i> spp.	1.2	0.7	116	0.6	0.3	23
<i>Cristatella mucedo</i>	30.3	28.1	66	21.0	8.1	15
<i>Lophopus crystallina</i>	4.6		1			
<i>Pectinatella magnifica</i>	18.1		2			
Other remains						
Ephemeroptera (mandible)	0.6	0.3	32			
Turbularia (cocoon)				1.2	0.2	18
Ostracoda (shell lining)	1.8	1.5	56			
Trichoptera (frontoclypeus/mandible)	2.2	3.7	32			
<i>Sialis</i> (frontoclypeus/mandible)	2.5	1.6	23			
<i>Chaoborus</i> (mandible)	1.5	1.0	37			
<i>Chaoborus</i> (thoracic horn)	0.9	0.2	7			

Sayle et al. (2019) demonstrated increases in sensitivity of up to $\times 30$ in sequential measurements of $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ values in archaeological bone collagen. The analytical capacity of the latest generation of EA-IRMS far exceeds the capabilities of the equipment available for earlier researchers (e.g. Verbruggen et al., 2010a), and potentially would allow $\delta^{18}\text{O}_{\text{chitin}}$ measurement on single-taxa or single head capsules. Laser ablation-based techniques have also been able to significantly improve lower detection limits. Schilder et al. (2018) used LA/nC/GC/IRMS for $\delta^{13}\text{C}$ analysis of individual *Daphnia* ephippia. Previous methods would analyse approximately 20 ephippia together as a means of averaging the signal produced and to reach the minimum sample weight needed. When they analysed individual ephippia, Schilder et al. (2018) found large $\delta^{13}\text{C}$ within-sample variability, interpreted as due to seasonal variation in the incorporation of methane-derived carbon. This method can potentially be applied to other chitinous remains (of chironomids and other insect larvae, bryozoans). One consequence of analysing smaller samples is the need to reduce signal noise or background, including consideration of the silver capsules used to contain the samples for analysis (e.g. Wang et al., 2008). An additional concern is that often, multiple determinations of single specimens can produce a wide range of variability, reflecting seasonal and micro-habitat variations (e.g. Schilder et al., 2018). This then necessitates analysing a large number of individuals to get a reliable mean \pm SD value for a sample, effectively rendering the approach unviable. However, it also may allow a much greater understanding of within-lake dynamics and open wider research avenues, improving interpretations. The implications and opportunities afforded by analysing single specimens will depend on a number of issues such as habitat (lake dynamics), seasonality of food resources, variability and range of isotopic change. There is also evidence of variation in $\delta^{13}\text{C}$ values from different chironomid instar larvae stages (Frossard et al., 2013), which also needs to be considered (section 2.4.1). Below we provide new data on an experiment where we explore the potential for analysing $\delta^{18}\text{O}$ values on single chironomid head capsules.

2.3. Determination of minimum head capsule numbers including oxygen isotope analyses on very small samples

Only three $\delta^{18}\text{O}_{\text{chitin}}$ Last Glacial/Interglacial Transition (LGIT) records derived from chironomid head capsules are presently available (Verbruggen et al., 2011; Lombino, 2014; King et al., 2021). The $\delta^{18}\text{O}_{\text{chitin}}$ measurements for all of these records were performed on samples that contained a large number of head capsules. To reach this number, head capsules from different chironomid taxa had to be combined into a single measurement, which potentially led to a mixed signal as different taxa are thought to behave differently in terms of their isotopic fractionation (Chang et al., 2017). Single-taxon or even single-head capsule $\delta^{18}\text{O}_{\text{chitin}}$ measurements could be used to circumvent this potential problem, but it is currently unclear what number of head capsules is needed to obtain a reliable $\delta^{18}\text{O}_{\text{chitin}}$ measurement, which will ultimately rely on delivering sufficient mass of oxygen to produce a reliable and precise $\delta^{18}\text{O}$ value.

It has so far proven difficult to determine the exact number of head capsules needed for reliable $\delta^{18}\text{O}_{\text{chitin}}$ measurement for several reasons. First, there is great variability in the size of head capsules between different morphotypes (Table 1). Second, head capsule size may change depending on food availability and climatic conditions – temperature is known to impact the growth rates of chironomids, with larger species affected disproportionately more with increasing temperature (Eggermont and Heiri, 2012; Wonglersak et al., 2021). Change in mean regional temperature affects several aspects of the ecology and life history of species, including their abundance, distribution and body size (e.g., Halpin, 1997; Walther et al., 2002; Thomas et al., 2004). It is generally known that the developmental rate of early life stages in ectotherms depends on environmental temperature, known as the temperature-size rule (TSR) (Atkinson, 1994), which ultimately affects

the final adult body size. Third, chironomid larvae go through different growth stages (instars), and the number of head capsules derived from second and third (smaller) or fourth (larger) instars can vary between samples. Because of the variability in the size and weight of individual head capsules, it is more useful to state the amount of sample needed for $\delta^{18}\text{O}_{\text{chitin}}$ measurement in terms of weight (μg) (Table 1).

Here we present results of an investigation into the minimum amount of sample required for $\delta^{18}\text{O}_{\text{chitin}}$ analysis using the Thermo Fisher Iso-Link EA as available in the British Geological Survey (BGS; Keyworth, UK). In our experiment, we conducted repeat measurements on samples of incremental weights using two materials: laboratory grown *Chironomus* head capsules and a cellulose standard (IAEA-CH-3). The cellulose standard was weighed out at 2 μg increments between 0 and 60 μg , 5 μg increments between 60 and 150 μg and 10 μg increments between 150 and 300 μg . A total of 56 cellulose samples were analysed. Laboratory grown *Chironomus* were grouped into samples of 1, 5, 10, 15, 20, 30 and 40 head capsules and weighed prior to analysis. It is of note that head capsules from laboratory grown chironomid larvae tend to be higher in mass (up to 4x; Table 1) than fossil head capsules as preserved in LGIT lake sediments. This difference in size may be due to the unlimited resources and no competition that the laboratory grown *Chironomus* experienced compared to those growing in a natural environment. A total of 40 samples containing head capsules from laboratory grown chironomid larvae were analysed. Isotope values were calibrated to the V-SMOW scale using IAEA-601 (23.14 ‰) and IAEA-602 (71.28 ‰).

The results (Fig. 2) show that there is a linearity effect on the $\delta^{18}\text{O}$ values for all materials tested. To determine the minimum amount of material needed for reliable $\delta^{18}\text{O}_{\text{chitin}}$ measurements we first determined the average $\delta^{18}\text{O}_{\text{chitin}}$ value and the standard deviation (SD) for chironomid head capsule samples larger than 30 μg , resulting in a mean $\delta^{18}\text{O}$ value ($\delta^{18}\text{O}_{\text{ave}}$) of 17.4‰ ($\pm 0.63\text{‰}$). We then determined at what sample-size the $\delta^{18}\text{O}_{\text{chitin}}$ measurements for the first time equaled $\delta^{18}\text{O}_{\text{ave}}$ value as well as when they first equaled $\delta^{18}\text{O}_{\text{ave}}$ value minus 1SD. The results showed that a sample weighing 32 μg yielded a value higher than $\delta^{18}\text{O}_{\text{ave}}$ value, and that even a sample with a mass as low as 26 μg produced a $\delta^{18}\text{O}$ value that fell within the $\delta^{18}\text{O}_{\text{ave}}$ value -1SD range. When looking at the number of head capsules included in these samples, samples containing fewer than 10 large HCs ($>2 \mu\text{g}$) were unlikely to yield reliable measurements, whereas most samples including 10–15 large head capsules yielded results that fall within the mean $\pm 1\text{SD}$. We conclude that using the latest generation of EA-IRMS allows for samples as small as ca. 30 μg of head capsule material to be measured and that samples as small as 25 μg can at times produce reliable results, which is a marked reduction of the amount needed previously (Verbruggen et al., 2010a). Using a sample size of ca 25–30 μg and containing 10–15 large HCs means that taxon-specific isotopic measurements are now within reach, overcoming the complexity of interpreting $\delta^{18}\text{O}_{\text{chitin}}$ measurements on samples composed of a mix of taxa. The problem of mixed-signals applies to all chitin-isotope studies and more comparable tests are needed to determine "how low you can go" using the latest EA-IRMS, something that we explore in more detail in section 4.

2.4. Experimental studies

Experimental studies continue to play an important role in developing isotope-based approaches analysing chitinous invertebrate remains. Such studies have been designed to assess the effects of food and water sources with different isotopic composition on the isotopic signature of invertebrates and their fossilizing body parts, the effects of degradation and fossilization processes on the invertebrate remains, the influence of sample preparation techniques on isotope values (see Section 2.1), and to study changes in isotopic values of invertebrate remains along an environmental gradient (e.g., temperature). Below we separate these studies into laboratory and field experiments.

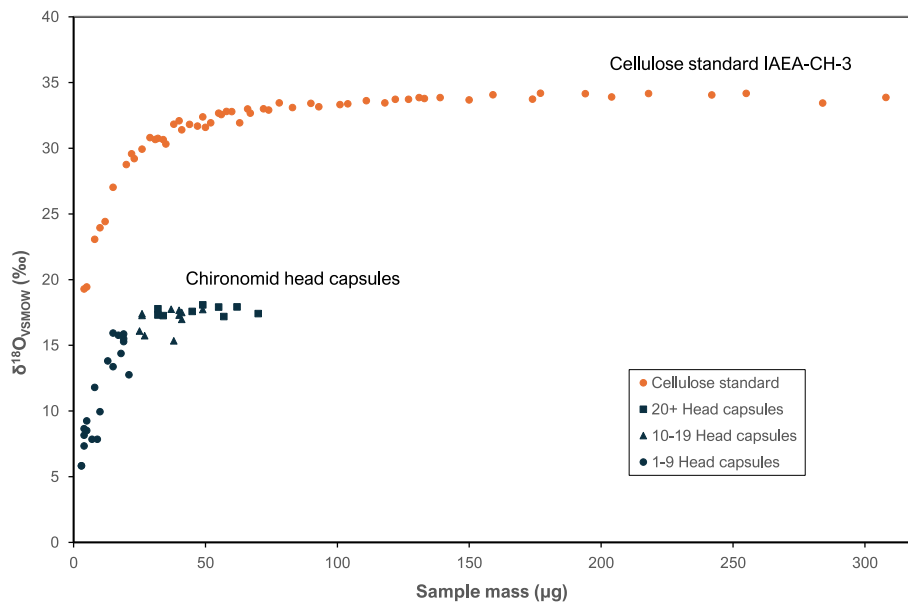


Fig. 2. $\delta^{18}\text{O}$ values of IAEA CH3 – cellulose standard material (orange circles), and lab-grown chironomid head capsules (blue symbols) against total mass of the sample. Symbols for the lab grown chironomid head capsules indicate the number of HCs used in an individual sample: circle = 1–9 hcs; triangle = 10–19 hcs; square = 20+ hcs. Contains BGS Isotope data © UKRI.

2.4.1. Culture experiments

A number of studies have cultivated and examined organism groups such as chironomids, cladocerans and bryozoans in the laboratory and assessed how different food sources and the culture water affect their isotopic composition. These studies have been summarised in Table 2. In these experiments the isotopic composition of the water or of the food sources, or both, were manipulated and the isotopic composition of the

entire organisms or their fossilizing structures (e.g., chitinous exoskeletons, chitinous valves of ephippia or statoblasts) were measured. For example, Heiri et al. (2012) and Frossard et al. (2013) cultivated chironomid larvae under constant conditions but with food with different $\delta^{13}\text{C}$ values. They showed that food source $\delta^{13}\text{C}$ values essentially determine the $\delta^{13}\text{C}$ values of the whole chironomid larvae, with a small difference (mostly in the range of 1–2‰) between food and

Table 2

Summary of offsets measured from cultivated isotope experiments in four key areas: 1) isotopic fractionation effects (e.g. between food and invertebrate tissue), 2) intra and inter-individual variation in isotope values, 3) diagenesis and 4) pretreatment effects.

Author(s)	Organism	Isotope element	Offset	Conditions
Perga (2009)	cladoceran exoskeletons	$\delta^{13}\text{C}$	No offset	Offset between cladoceran exoskeletons and samples consisting of the entire organisms.
Van Hardenbroek et al. (2010)	chironomid head capsules	$\delta^{13}\text{C}$	$\pm 0.5\%$ from the average	Chemical pretreatment effects.
Perga (2011)	exoskeletons of <i>Daphnia</i>	$\delta^{13}\text{C}$	<1‰	Preservation - early diagenetic effects.
Schilder et al. (2015a)	Cultivated ephippia valves	$\delta^{13}\text{C}$	Up to 0.2‰ higher	Offset between fossilizing ephippia valves of <i>Daphnia</i> .
Heiri et al. (2012), Frossard et al. (2013)	cultivated chironomid larvae	$\delta^{13}\text{C}$	1–2‰	Offset between food and chironomid body tissue.
Heiri et al. (2012), Frossard et al. (2013)	cultivated chironomid larvae	$\delta^{13}\text{C}$	0–2‰	Offset between older instar larvae compared to the rest of the larvae.
Van Hardenbroek et al. (2016)	bryozoan <i>Plumatella</i>	$\delta^{13}\text{C}$	Within $\pm 1.5\%$	Offset between soft tissue and fossilizing statoblast or sessoblast valves.
Heiri et al. (2012)	cultivated chironomid larvae	$\delta^{15}\text{N}$	Mostly within $\pm 1\%$	Offset between larval head capsules and the larval bodies.
Heiri et al. (2012)	cultivated chironomid larvae	$\delta^{15}\text{N}$	Up to 3‰ difference	Offset between food ingested by the larvae and the larval bodies – variance observed due to food $\delta^{15}\text{N}$ value.
Wang et al. (2009)	cultivated chironomid larvae	$\delta^{15}\text{N}$	2‰ higher	Offset between food ingested by the larvae and the larval bodies.
Schilder et al. (2015a)	Cultivated ephippia valves	$\delta^{15}\text{N}$	systematically lower ($1.6 \pm 0.4\%$)	ephippia valves compared to the bodies of <i>Daphnia</i> .
Schilder et al. (2015a)	Cultivated ephippia valves	$\delta^{15}\text{N}$	3‰	Offset between food and ephippia body tissue.
Perga (2011)	exoskeletons of <i>Daphnia</i>	$\delta^{15}\text{N}$	2–5‰	Preservation - early diagenetic effects.
Perga (2009)	cladoceran exoskeletons	$\delta^{15}\text{N}$	7.9‰ lower compared with the samples of entire <i>Daphnia</i> .	Offset between cladoceran exoskeletons and samples consisting of the entire organisms.
Schilder et al. (2015a)	Cultivated ephippia valves	$\delta^{18}\text{O}$	No offset	Offset between ephippia valves compared to the bodies of <i>Daphnia</i> .
Clarke et al. (2019)	Beetles	$\delta^{18}\text{O}$	$\sim 2\%$	Offset between fragments of modern beetle exoskeletons (head, pronotum, elytron, metasternum, abdomen, leg).
Verbruggen et al. (2011)	chironomid head capsules	$\delta^{18}\text{O}$	up to 6‰ between the different treatments.	Chemical pretreatment effects study.
Verbruggen et al. (2011)	chironomid head capsules and cladoceran ephippia	$\delta^{18}\text{O}$	ca. 1–2‰	offset between head capsule and ephippia (higher).
Gröcke et al. (2010)	Beetles	δD	7–9‰	Offset between different beetle fragments.

chironomid body tissue for later instar larvae. The experiments also revealed that $\delta^{13}\text{C}$ values of the fossilizing body part of the larvae, the head capsule, are slightly lower for older instar larvae (ca. 0–2‰ in most cases) compared to the rest of the larvae, but also strongly determined by the $\delta^{13}\text{C}$ values of the food. However, Frossard et al. (2013) also showed that these offsets between soft tissue and head capsules may be a bit larger for first and second instar larvae, although usually <3‰. For most fossil samples measured for $\delta^{13}\text{C}$ values, the focus will be on 3rd and 4th instars, so that this difference, whilst observed in under laboratory settings, is unlikely to make a major impact on palaeoecology. Experiments on cladocerans (Schilder et al., 2015a) showed that $\delta^{13}\text{C}$ values of the fossilizing ephippia valves of *Daphnia* are very similar to each other but on average slightly (0.2‰) higher than $\delta^{13}\text{C}$ values of the entire *Daphnia* body. The experiments also showed that varying the $\delta^{13}\text{C}$ values of the food ingested by the *Daphnia* affected their $\delta^{13}\text{C}$ values, although temperature also influenced the results. The latter effect was assigned to respired carbon possibly affecting the experiment, and it remained unclear whether a similar temperature influence should also be expected for cladoceran populations in natural environments. van Hardenbroek et al. (2016) showed in a culturing experiment with the bryozoan *Plumatella* that zooids (consisting of soft tissue) were close in their $\delta^{13}\text{C}$ values (within 1.5‰) to the $\delta^{13}\text{C}$ values of the fossilizing statoblast or sessoblast valves and within 2‰ of the particulate organic matter that the zooids fed on. Overall, these experiments agree with similar studies that indicate a $\delta^{13}\text{C}$ value increase of between 0 and 1‰ for animals relative to their food sources (DeNiro and Epstein, 1978; McCutchan et al., 2003). They also show that for chitinous invertebrate remains only relatively minor offsets in $\delta^{13}\text{C}$ values relative to the isotopic signature of the entire organisms should be expected, thus allowing studies of changes in the food web based on fossil remains.

Heiri et al. (2012) also reported on $\delta^{15}\text{N}$ values of cultivated chironomid larvae. The results showed that, again, larval head capsule $\delta^{15}\text{N}$ values are strongly determined by the $\delta^{15}\text{N}$ values of the food ingested by the larvae and similar (mostly within $\pm 1\%$) to the $\delta^{15}\text{N}$ values measured for the larval bodies. However, when fed on food sources that were significantly higher in nitrogen isotope value ($\delta^{15}\text{N}$ values above 8‰ AIR) the $\delta^{15}\text{N}$ values of the larvae were slightly (up to 3‰) lower and a similar pattern was observed when food had very low $\delta^{15}\text{N}$ values (i.e. chironomid $\delta^{15}\text{N}$ values were up to 3‰ higher than the food. Wang et al. (2009) found ca. 2‰ higher $\delta^{15}\text{N}$ values for chironomid larvae than the food (*Spirulina* algae) provided to the larvae in another culturing experiment. $\delta^{15}\text{N}$ values were not varied systematically in the experiments with *Daphnia* of Schilder et al. (2015a), but $\delta^{15}\text{N}$ values of *Daphnia* body tissue was ca. 3‰ higher than the food source. Furthermore, the cultivations by Schilder et al. (2015a) suggest that the $\delta^{15}\text{N}$ values of the fossilizing ephippia are systematically lower (1.6 \pm 0.4‰ 1SD) than those of the bodies of the examined *Daphnia*. Perga (2009) examined offsets in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between cladoceran exoskeletons and samples consisting of the entire organisms. She showed that, for *Bosmina* and *Daphnia*, $\delta^{13}\text{C}$ values of the exoskeletons were closely correlated with and very similar to the samples consisting of complete specimens including soft tissues. In contrast, $\delta^{15}\text{N}$ values of exoskeletons correlated strongly with samples of complete specimens but were considerably (by -7.9%) lower compared with the samples of entire *Daphnia*. Though more limited data are available for $\delta^{15}\text{N}$ values than for $\delta^{13}\text{C}$ values, available experiments suggest that offsets between food and aquatic invertebrate tissue may be more variable for nitrogen than for carbon, which may be due to the influence of microbial activity on the $\delta^{15}\text{N}$ value of the food (Goedkoop et al., 2006). However, the results also suggest that changes in food source $\delta^{15}\text{N}$ values should lead to changes in $\delta^{15}\text{N}$ values of invertebrate tissue and their fossilizing structures (e.g. ephippia valves, chironomid head capsules) (Table 2). Further studies on nitrogen isotopes would help to better understand the factors determining the variability in offsets.

Wang et al. (2009) examined oxygen and hydrogen isotope ratios of chironomid larvae using cultivating experiments. Chironomids were

cultivated with an identical diet but in two isotopically distinct waters. Using a two-endmember mixing model, Wang et al., 2009 estimated that 69% of the oxygen and 31% of the hydrogen in the chironomid larvae are derived from habitat water, with the rest presumably provided by the food ingested by the larvae. That a major part of the hydrogen in chironomid tissue originates from the food ingested by larvae was confirmed in cultivation experiments by Belle et al. (2015b), although these authors also indicated that further experiments are necessary to better constrain the influences on δD values of chironomids and their remains in lake sediments (see Section 4). Schilder et al. (2015a) examined oxygen isotopes in *Daphnia* in an experimental culturing setup and showed that a change in $\delta^{18}\text{O}$ values of the culture water led to a shift in $\delta^{18}\text{O}$ values of the cultured *Daphnia*. However, they also concluded that only part (in their experiment estimated to 56–69%) of the oxygen incorporated into *Daphnia* biomass originated from the culture water, as expected based on earlier studies on the importance of the isotopic composition of water for invertebrate tissues (e.g., Soto et al., 2013; Nielson and Bowen, 2010; Wang et al., 2009). Schilder et al. (2015a) also showed that ephippia valves were very similar in their $\delta^{18}\text{O}$ values to the $\delta^{18}\text{O}$ values of the entire *Daphnia*, indicating that the fossilizing valves carry the $\delta^{18}\text{O}$ signature of planktonic cladocerans. Whereas experiments with aquatic invertebrates suggest relatively constant offsets between $\delta^{13}\text{C}$ values of food sources and invertebrates, the available studies examining $\delta^{18}\text{O}$ and δD values in cultures indicate that, for oxygen and hydrogen, isotopic values are determined by the joint effects of food and the ambient water which in a lake, for example, would vary under changing climatic conditions. Available studies with *Daphnia* indicate that offsets between soft tissue and the fossilizing, sclerotized exoskeleton are relatively small and constant, a finding that should be verified also for other aquatic invertebrate groups with fossilizing exoskeleton structures. Clarke et al. (2019) found that different fragments of modern beetle exoskeletons (head, pronotum, elytron, metasternum, abdomen, leg) did not vary significantly in $\delta^{18}\text{O}$ values. They observed a variability in $\delta^{18}\text{O}$ values between body parts of $\sim 2\%$ (0.1–0.7‰ SD). Variations in $\delta^{18}\text{O}$ values within individual elytron of very large beetle specimens were minimal (SD of 0.2–0.5‰), and for fossil specimens this within-elytron variability was slightly larger (SD up to 1.2‰). This is similar to findings on the δD values of beetle fragments (Gröcke et al., 2010), which indicated a variability (SD) of 7–9‰ in beetle body parts. Taken together, this work indicates that any combination of beetle fragments could be used for $\delta^{18}\text{O}$ or δD analysis in palaeoenvironmental studies as long as expected shifts are greater than $\sim 1\%$ for $\delta^{18}\text{O}$ and $\sim 9\%$ for δD .

The fact that the oxygen-isotope ratio of the host water and diet appears to be the dominant control on $\delta^{18}\text{O}_{\text{cuticle}}$ has led to the assumption that the fractionation of oxygen isotopes between cuticle and water ($\alpha_{\text{cuticle-H}_2\text{O}}$) is independent of temperature. An experiment by Lombino et al. (2021a) is one of only a handful of studies to question this long-standing assumption. Lombino et al. (2021a) cultured chironomid larvae in the laboratory with water with a known oxygen-isotope composition and a stable and constant food source across a range of temperatures (5–25 °C). The results, although small in scale, did show a measurable effect of temperature on the fractionation effect. A few other studies have also hinted at such a relationship (Wang et al., 2009; Soto et al., 2013), but with differences to Lombino et al. (2021a), likely attributed to species specific variation in response. This clearly requires further consideration to fully understand the implications for reconstructions of past water temperature and is especially significant when $\delta^{18}\text{O}_{\text{chironomid}}$ and $\delta^{18}\text{O}_{\text{carbonate}}$ data from lakes are combined to reconstruct water temperature. Verbruggen et al. (2010b) found differences in the amplitude of variations in bulk carbonate and chironomid $\delta^{18}\text{O}$ from their study of Lateglacial and early Holocene sediments from Rotsee (Switzerland). Here it wasn't clear if this was due to differential temperature effects on oxygen isotope fractionation during the formation of carbonates and chironomid head capsules, or of seasonal changes of lake water $\delta^{18}\text{O}$. These studies have highlighted a

significant gap in our knowledge in terms of the significance of the influence of temperature on oxygen isotope fractionation effects.

2.4.2. Field experiments

Several authors have examined the response of the stable isotope composition of chitinous invertebrate remains to potential environmental drivers by examining changes in their isotopic composition across different ecosystems and along environmental gradients. For example, Verbruggen et al. (2011) isolated chironomid head capsules and cladoceran ephippia from surface sediments in lakes ($n = 31$) sampled on a north to south gradient across Europe. The results showed that aquatic invertebrate $\delta^{18}\text{O}$ values changed consistently and linearly with changing lakewater $\delta^{18}\text{O}$ values. However, they also revealed a systematic offset of ca. 1–2‰ between head capsule and ephippia $\delta^{18}\text{O}$ values, with the ephippia consistently having higher $\delta^{18}\text{O}$ values. Similar studies were presented by Mayr et al. (2015) who examined chironomid $\delta^{18}\text{O}$ values in seven Patagonian lakes and compared them with $\delta^{18}\text{O}$ values of the lakewater and by Chang et al. (2016, 2017) who compared chironomid $\delta^{18}\text{O}$ and δD values with those of lakewater. These studies all showed a close correlation between chironomid and lakewater $\delta^{18}\text{O}$ values consistent with results on culture experiments by Wang et al. (2009). Verbruggen et al. (2011), Mayr et al. (2015) and Chang et al. (2016, 2017) all describe linear regression equations that relate chironomid $\delta^{18}\text{O}$ to lakewater $\delta^{18}\text{O}$ values and Chang et al. (2016) also describe linear regressions between chironomid δD to lake water δD values. van Hardenbroek et al. (2016) examined whether δD values of bryozoans and their fossilizing statoblast valves were related to lakewater δD values in 23 lakes and ponds and one stream in Central and Northwest Europe. The authors found that bryozoans statoblasts were systematically related in their δD values to those of the lake water, particularly for the genus *Plumatella*. However, no clear relationship was observed for the bryozoan zooids (consisting of soft tissue). They suggested that this may be due to the possible contamination of zooid samples with other material (attached organic material and undigested particles in the guts), the more diverse chemical composition (higher lipid content in zooids), and different turnover rates of zooids compared with statoblasts.

Several authors have suggested that chironomid head capsules may contain methane-derived carbon in lakes where methane is an important food source for the aquatic foodweb, since chironomid larvae may feed on methane oxidizing bacteria where these are abundant (Grey et al., 2004a,b; van Hardenbroek et al., 2010; Deines et al., 2007). Field studies examining living larvae have shown that their tissue may become extremely low in $\delta^{13}\text{C}$ where methane-derived carbon is incorporated into chironomid biomass (Jones et al., 2008). van Hardenbroek et al. (2010) were able to demonstrate in culturing experiments with labelled methane that the carbon isotope signature of methane was taken up by chironomid larvae and incorporated in their fossilizing head capsules. Several later studies have assessed whether the carbon isotope composition of remains or resting stages of chironomids or other invertebrates that are known to feed on methane oxidizing bacteria are correlated with in-lake methane concentrations when lakes with varying methane availability are examined. van Hardenbroek et al. (2012) reported a strong negative correlation between $\delta^{13}\text{C}$ values of the head capsules of the chironomid group Chironomini and methane flux at the lake surface in seven Swedish lakes, whereas other chironomid groups showed no relationship (Orthocladinae) or a weaker and non-significant negative relationship (Tanytarsini, Tanytopodinae). Such observations add support to the need to thoroughly assess the environmental setting of each study site (section 2.2 & 2.3). A similar pattern was also observed when chironomid head capsule $\delta^{13}\text{C}$ values were compared with methane flux from the sediment within two of the studied lakes, with a strong negative relationship apparent for Chironomini, whereas other chironomid groups did not show a significant correlation. These data were combined with additional $\delta^{13}\text{C}$ measurements on chironomid head capsules from surface sediments from ten thermokarst lakes in Siberia by van

Hardenbroek et al. (2013). The combined dataset showed significant negative correlations between methane flux at the lake surface and $\delta^{13}\text{C}$ values of head capsules of *Chironomus*, Chironomini and Tanytarsini, whereas the relationship for other chironomid groups was not significant.

van Hardenbroek et al. (2013) also reported $\delta^{13}\text{C}$ values of *Daphnia* ephippia in their study lakes and showed that these also correlated negatively with methane flux measurements. In a more detailed assessment, Schilder et al. (2015b) compared $\delta^{13}\text{C}$ values of *Daphnia* ephippia in surface sediments of 15 small lakes in Europe and reported strong and negative correlations with methane concentrations in lake surface and deepwater samples. Relatively low $\delta^{13}\text{C}$ values were only found in lakes with high methane concentrations in the anoxic deepwater layers. Van Hardenbroek et al. (2018b) showed that a strong, negative correlation is not only apparent between methane concentrations and $\delta^{13}\text{C}$ values of *Daphnia* ephippia in lake surface sediments but also with $\delta^{13}\text{C}$ values of planktonic cladoceran ephippia in flotsam collected from the water surface of small lakes. This confirmed the findings of Schilder et al. (2015b) that methane-derived carbon can be incorporated by planktonic, filter-feeding cladocerans and can lead to strongly negative $\delta^{13}\text{C}$ values of their fossilizing ephippia valves.

The incorporation of methane-derived carbon varies seasonally, and appears to be strongest during autumn turnover, when built-up CH_4 in the hypolimnion is mixed with oxygen-rich waters and incorporated into biomass of methane oxidizing bacteria, making it available for *Daphnia* (Taipale et al., 2007; Morlock et al., 2017; Essert et al., 2022a). A similar seasonal pattern has been observed for $\delta^{13}\text{C}$ values of chironomid larvae collected at different times of the year (Grey et al., 2004b; Yasuno et al., 2012) and lower $\delta^{13}\text{C}$ values in copepods during the winter season have also been observed (Santer et al., 2006). A recent study of 11 lakes by Essert et al. (2022b) demonstrated how $\delta^{13}\text{C}$ values of *Daphnia* differed under stratified (summer) conditions compared with mixed (winter) conditions, and how this difference was correlated significantly with indicators for stratification strength and with summer deep water CH_4 and CO_2 concentrations. The $\delta^{13}\text{C}$ values of ephippia in surface sediments in these 11 lakes were also lower for lakes with higher CH_4 concentrations, but this relationship was not significant (Essert et al., 2023), which was explained by the timing of ephippia production and lake mixing: if lake mixing happened early (October/November) the $\delta^{13}\text{C}$ values of ephippia were closer to $\delta^{13}\text{C}$ values of *Daphnia* in summer, reflecting $\delta^{13}\text{C}$ values of phytoplankton. If lake mixing happened late (December/January) the $\delta^{13}\text{C}$ values of ephippia were closer to $\delta^{13}\text{C}$ values of *Daphnia* in winter, indicating $\delta^{13}\text{C}$ values of methane-derived carbon (Essert et al., 2023).

These studies convincingly show that $\delta^{13}\text{C}$ values of chitinous invertebrate remains of some planktonic cladocerans and chironomid groups that feed on methane oxidizing bacteria (MOB) can significantly lower in isotope value with higher availability of methane in the habitats these groups live in. However, robust numerical relationships between methane concentrations and $\delta^{13}\text{C}$ of these invertebrate groups have not yet been developed.

2.5. Spatial and temporal variations in the isotope composition of invertebrates and their remains

Based on experimental and field data in section 2.4 (summarised in Table 2), it can be concluded that the stable isotope composition of invertebrate remains preserved in lake sediments primarily reflects the stable isotope composition of the living organism, which in turn reflects the stable isotope composition of the diet and/or water in their environment. The next question when using invertebrate remains (and their stable isotope composition) to infer environmental change, is which moment in space and time is represented by the invertebrate remains? How much spatial and temporal variation exists in the stable isotope composition of invertebrate remains, and what taphonomic processes cause these variations?

2.5.1. Within-lake variability

The within-lake variability of stable isotopes of invertebrate remains has only been investigated in a few studies to date. In some lakes, limited spatial variation of only 5–6‰ in $\delta^{13}\text{C}$ values has been observed, but in others this variation is > 40‰ (see also Fig. 3). For example, Essert et al. (2022a) found a very small, non-significant spatial difference in $\delta^{13}\text{C}$ of *Daphnia* ephippia in 30 surface sediment samples taken across an area of ca 1200 × 500 m in the deepest area (>20m depth) of Lake Remoray (range –38.6 to –34.9‰, mean –37.1 ± 0.8‰). Essert et al. (2022a) also found that no significant spatial variation affected $\delta^{13}\text{C}$ values of living *Daphnia* and seston in the water column in the four studied lakes. In contrast, Belle et al. (2015a) analysed $\delta^{13}\text{C}$ values of *Chironomus anthracinus*-type head capsules in a 2 × 2 m area in the deepest part (26 m water depth) of Lake Grand Maclu, France. They found a range in $\delta^{13}\text{C}$ values of –47.7 to –36.0 ‰ (mean –41.5 ± 2.4‰). The study by Belle et al. (2015a) also reported some spatial autocorrelation between $\delta^{13}\text{C}$ values of head capsules, but this was not observed for bulk sedimentary organic matter (SOM), for which the range of $\delta^{13}\text{C}$ values was much smaller (–35.2 to –33.4‰, mean –34.9 ± 0.43‰).

Previously unpublished data are presented here in Figs. 3 and 4, showing $\delta^{13}\text{C}$ values of remains from different invertebrate taxa along transects from littoral to profundal habitats in four lakes in Germany and The Netherlands (Holzsee, Plußsee, Schöhsee, De Waay; Rinta et al., 2017). Most living chironomid larvae are found in shallow areas (up to ~3 m depth). Only *Chironomus* larvae, adapted to surviving low-oxygen conditions (Brodersen et al., 2004), were observed at depths up to 15 m (Fig. 3). Head capsules are found at greater depths, indicating transport of these remains from shallow to deep areas, a pattern well known from taphonomic studies (e.g., van Hardenbroek et al., 2011). Overall, $\delta^{13}\text{C}$ values of living invertebrates and their fossilizing remains are within the same range (Fig. 4), but there are only a few clearly observable patterns in the $\delta^{13}\text{C}$ values. The smallest range in $\delta^{13}\text{C}$ was observed in Orthoclaadiinae (–30.5 to –25.6‰), which are mostly collector-gatherers and scrapers, whereas larger ranges were observed in Tanytarsini

(collector-filterers and gatherers, –31.8 to –24.1‰), and Tanytarsini (predators, –32.2 to –24.1‰). The largest range of $\delta^{13}\text{C}$ values was observed in Chironomini (collector-gatherers and burrowers, –40.5 to –25.6‰). The lowest values were found in remains of Chironomini, specifically *Chironomus* (range –40.5 to –25.8‰), as well as *Daphnia* (range –41.5 to –30.2‰), and of these, the lowest values were found at greater depths (Fig. 3). Exceptionally low $\delta^{13}\text{C}$ values for living larvae of *C. plumosus* were found in the deepest part of Holzsee (–60.0‰), but such low values were not found in remains of *C. plumosus*-type, which showed higher values of –38.9‰. This is likely the result of the fact that a single measurement of remains contained between 5 and 40 individual head capsules, resulting in a $\delta^{13}\text{C}$ value that provides a long-term average, integrating over several seasons or years. More detail on seasonal variation in stable isotope values will be discussed in section 2.5.2. In Schöhsee and De Waay, remains of *Chironomus* larvae living at greater depths had lower $\delta^{13}\text{C}$ values than any living larvae (Fig. 3) collected in shallower areas, suggesting a ^{13}C -depleted carbon source in deeper parts of the lake (away from the littoral) during part of the year.

Van Hardenbroek et al. (2012) studied transects in two lakes in Sweden and, like in the data presented in Figs. 3 and 4, observed no clear spatial pattern in $\delta^{13}\text{C}$ of chironomid remains in relation to depth or to $\delta^{13}\text{C}$ of SOM. Van Hardenbroek et al. (2012) did, however, observe a correlation between $\delta^{13}\text{C}$ of Chironomini and methane fluxes, a pattern that will be discussed in more detail in section 3.4. It is interesting to consider spatial variations in $\delta^{13}\text{C}$ values over time here. Only very few studies have compared temporal trends in $\delta^{13}\text{C}$ values in multiple dated sediment cores from the same lake, but this is an excellent way to study the spatial extent of how eutrophication and hypoxia affect lake ecosystems. For example, Frossard et al. (2014, 2015) found decreasing $\delta^{13}\text{C}$ values in chironomid and cladoceran remains in response to eutrophication in Lake Annecy and Lake Bourget. The decrease in $\delta^{13}\text{C}$ values was observed in recent sediments from the lakes and dating suggests that the decrease started in deep-water locations, followed later by shallower locations, suggesting a gradual expansion of hypoxia from

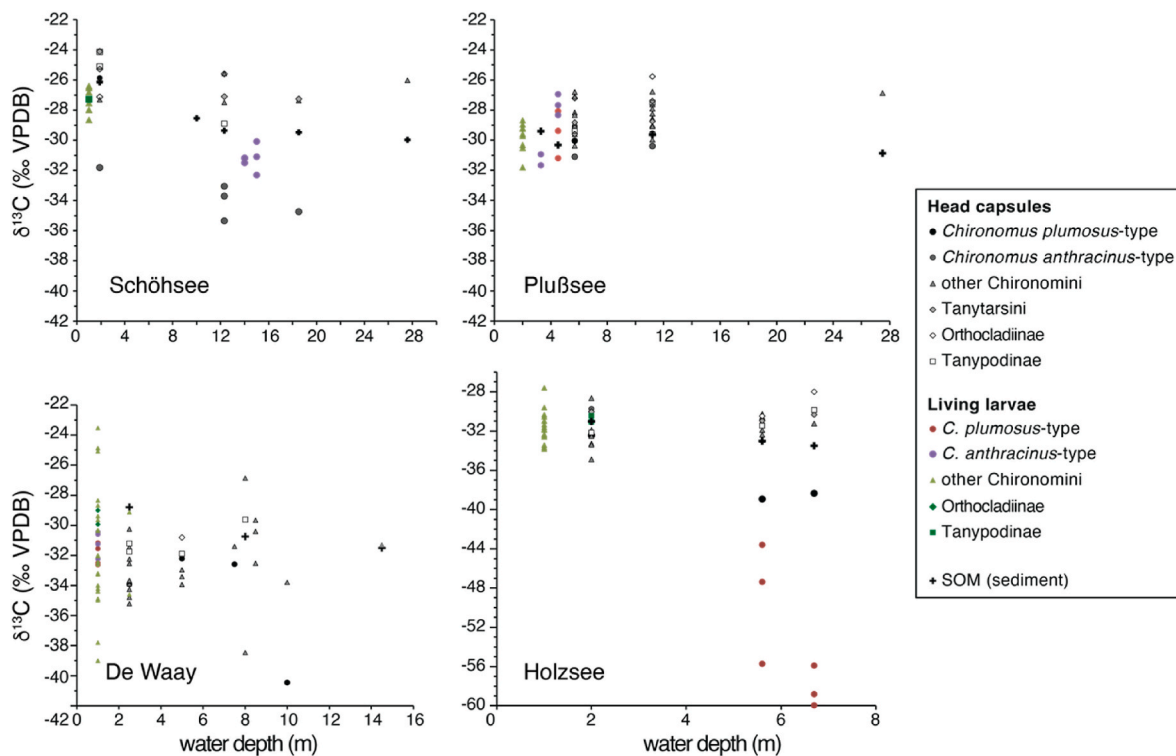


Fig. 3. $\delta^{13}\text{C}$ values of living chironomid larvae (coloured symbols) and head capsules (symbols in greyscale) from lakes Holzsee, Schöhsee, Plußsee, and De Waay. Tissues of living larvae analysed at the UC Davis Stable Isotope Facility and tissues of head capsules analysed at the Alaska Stable Isotope Facility, UAF. Replicate measurements on standard materials indicate an analytical error smaller than 0.2‰.

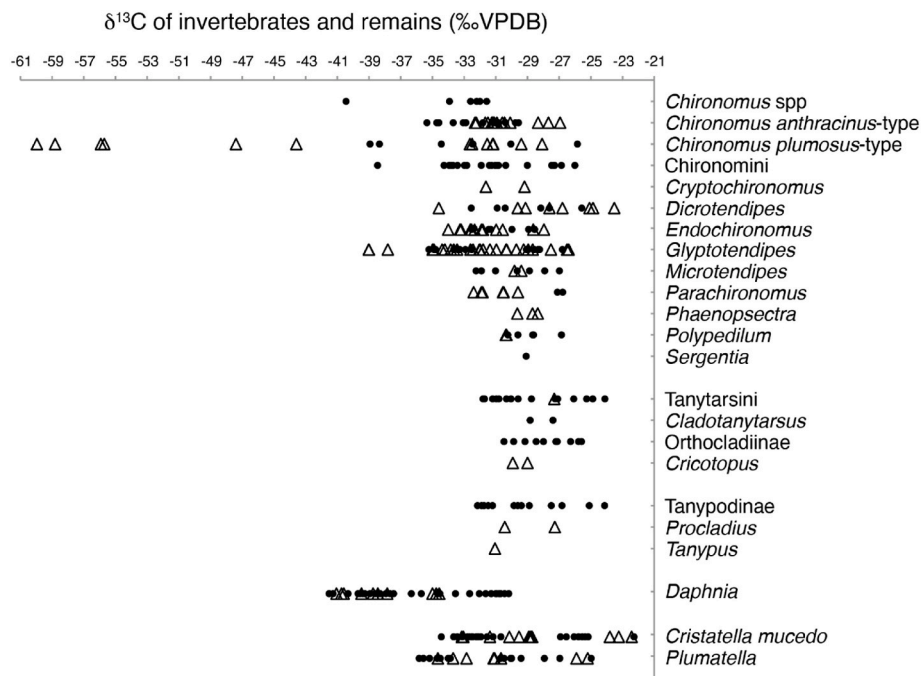


Fig. 4. $\delta^{13}\text{C}$ values of living chironomid larvae and other aquatic invertebrates (open triangles) and head capsules and fossilizing structures (closed circles) from lakes Holzsee, Schöhsee, Plußsee, and De Waay. Tissues of living larvae and aquatic invertebrates were analysed at the UC Davis Stable Isotope Facility and head capsules and other fossilizing structures tissues at the Alaska Stable Isotope Facility, UAF. Replicate measurements on standard materials indicate an analytical error smaller than 0.2‰.

profundal to sublittoral zones of the study lakes with ongoing eutrophication. A similar observation was made by Schilder et al. (2017), in lake De Waay, where the $\delta^{13}\text{C}$ values of aquatic invertebrates became progressively lower with increasing eutrophication and hypoxia, first in remains of *Daphnia*, indicating deep water hypoxia, and only later in sediment-dwelling *Chironomus*, indicating hypoxia in the sublittoral.

Spatial variation of methane production and consumption in lakes can lead to spatial variability of $\delta^{13}\text{C}$ values of invertebrates with lower $\delta^{13}\text{C}$ values observed in lake areas where a strong oxycline is present (Deines and Grey, 2006) or where dense macrophyte cover causes hypoxia (Agasild et al., 2013). The same pattern of lower $\delta^{13}\text{C}$ values in areas where methane-derived carbon is more abundant can be observed in the remains of aquatic invertebrates (van Hardenbroek et al., 2012; Belle et al., 2015a). Interesting examples of spatial variability of methane-derived carbon are provided by Frossard et al. (2014, 2015), who observed an expansion (to shallower depths) of the anoxic hypolimnion with ongoing eutrophication. As a result, chironomid head capsule $\delta^{13}\text{C}$ values became progressively lower, a process that was first observed in a sediment core taken in the deepest part of lakes, followed a few decades later in cores from shallower areas. This could indicate a gradual expansion of hypoxia from profundal to sublittoral zones of the study lakes in response to eutrophication (Frossard et al., 2014, 2015).

Measurements of δD values of invertebrates have also been used to assess the contribution of MOB to their diet, as MOB strongly discriminate against the heavier deuterium, resulting in low δD values of chironomid larvae (Deines et al., 2009; Belle et al., 2015b) but studies are limited in number. Equally, sulfur isotopes may also offer insights to benthic food webs in freshwater lakes (Croisetière et al., 2009; Karube et al., 2012). Onishi et al. (2023) measured sulfur isotopes of sulfide and benthic animals (oligochaete, chironomids, malacostraca and bivalves) in the surface sediments of Lake Biwa, Japan to examine the biogeochemical sulfur cycle. Their calculations estimate that 58%–67% of the total biomass sulfur in the benthic food web of the lake was sulfide-derived sulfur via sulfur-oxidizing bacteria, inferring they are important nutritional resources supporting benthic food webs in the lake ecosystem alongside the contributions from methane oxidation. This

area of food web understanding is very under explored and improvements in analytical techniques may open up new possibilities for research here (Section 4).

2.5.2. Seasonal variability

The long-term patterns in $\delta^{13}\text{C}$ values such as those mentioned above should be interpreted with care. It is easy to assume that the $\delta^{13}\text{C}$ values of invertebrate remains represent the same moment in the annual cycle, but is that correct? In the study of Essert et al. (2022a), which found minimal spatial variability of ephippia $\delta^{13}\text{C}$ values in Lake Remoray, the authors describe a large temporal variability across the seasonal cycle: the $\delta^{13}\text{C}_{\text{Daphnia}}$ values were close to the $\delta^{13}\text{C}_{\text{seston}}$ values during the summer stratification period, resulting in an increase in $\delta^{13}\text{C}$ values, whereas after the autumnal turnover, the $\delta^{13}\text{C}_{\text{Daphnia}}$ values moved away from the $\delta^{13}\text{C}_{\text{seston}}$ values to reach much lower values. During the summer stratification period, the $\delta^{13}\text{C}_{\text{seston-Daphnia}}$ values were, on average, 1‰ for Lake Remoray, -0.4‰ for Lake Bonlieu, -0.2‰ for Lake Longemer, and 1.7‰ for Lake Retournemer, whereas in winter, these values reached 5.6‰ for Lake Remoray, 4.9‰ for Lake Bonlieu, 2.6‰ for Lake Longemer, and 11.3‰ for Lake Retournemer. Recently, Essert et al. (2023) demonstrated that lakes in which turnover happened early (in October) had low $\delta^{13}\text{C}_{\text{ephippia}}$ values indicating that methane-derived carbon was available in the water column at the time of ephippia production in autumn. In contrast, lakes with later turnover (in December) had higher $\delta^{13}\text{C}_{\text{ephippia}}$ reflecting $\delta^{13}\text{C}$ values of phytoplankton only, as no methane-derived carbon was mixed into the water column yet at the time when ephippia were produced (prior to turnover). A study in Lake Gerzensee (Morlock et al., 2017) found a similar pattern, with a large range of $\delta^{13}\text{C}_{\text{Daphnia}}$ values from -44.2‰ in early spring to -29.8‰ in summer. *Ceriodaphnia* ephippia had an even larger range of $\delta^{13}\text{C}$ values (-50.1 to -34.3‰) with the most rapid change for both *Ceriodaphnia* and *Daphnia* happening during autumn overturn. Interestingly, the temporal variability of ephippia $\delta^{13}\text{C}_{\text{Daphnia}}$ values collected from the lake surface (range -41.7 to -38.8‰ throughout the year) was much smaller than that of the living organisms, suggesting that ephippia were produced batch-wise in early summer and/or late

autumn and then floated on the lake surface for several months. The studies by Essert et al. (2022a, 2023) and Morlock et al. (2017) highlight how important it is to consider what moment in the annual cycle is captured by the $\delta^{13}\text{C}$ values of invertebrate remains. Their studies also beg the question if this is a moment that can change from year to year – and what the impact of changes in the timing of seasons and/or ice-cover can have on $\delta^{13}\text{C}$ values of invertebrates from sediment records covering hundreds or thousands of years.

2.6. Summary of uncertainties evident from modern studies

To successfully utilise stable isotope ratios as proxies for palaeoenvironmental change it is necessary to understand the potential uncertainties evident both in nature (within lake, seasonal amongst others) but also from analytical and pre-treatment processes. We have compiled a table listing a summary of available published offsets from modern data sets (Table 2). The data covers four key areas: 1) isotopic fractionation effects (e.g. between food and invertebrate tissue), 2) intra and inter-individual variation in isotope values, 3) diagenesis and 4) pre-treatment effects.

Overall, calibration/modern studies on chitinous invertebrate remains point to only relatively minor offsets in $\delta^{13}\text{C}$ values relative to the isotopic signature of the entire organisms, thus allowing studies of changes in the food web based on fossil remains. Though more limited data are available for $\delta^{15}\text{N}$ values than for $\delta^{13}\text{C}$ values, available experiments suggest that offsets between food and aquatic invertebrate tissue and their fossilizing structures (e.g., ephippia valves, chironomid head capsules) may be more variable for nitrogen than for carbon, however, the results suggest that the $\delta^{15}\text{N}$ values of invertebrate tissue will reflect food source $\delta^{15}\text{N}$ values. For oxygen and hydrogen isotopes, there is less available experimental work, but work on beetles indicates that any combination of beetle fragments could be used for $\delta^{18}\text{O}$ or δD analysis in palaeoenvironmental studies as long as expected shifts are greater than $\sim 1\%$ for $\delta^{18}\text{O}$ and $\sim 9\%$ for δD .

3. Review of recent advances in palaeoenvironmental applications in a range of freshwater invertebrates

Following on from the advances in knowledge made through investigations of modern processes, we discuss new insights from the application of this knowledge to palaeoenvironmental studies.

3.1. Hydrogen and oxygen isotopes

Remarkable insights into past hydrologic changes, climate and temperature have come from the measurements of the δD and $\delta^{18}\text{O}$ values of ice cores (e.g. Lohmann et al., 2024; Thompson et al., 2024). Despite these high resolution palaeoclimatic insights the extent to which this work can be applied is relatively limited by the spatial availability of regions with glacial ice. Researchers have continued to fill in some of the spatial palaeohydrological story by examining and applying δD and $\delta^{18}\text{O}$ analyses of other palaeo records including lakes, peat and other remains (e.g. beetles etc.) (Jones et al., 2020; Wooller et al., 2008). The first application of measuring the $\delta^{18}\text{O}$ of chironomid head capsules was conducted by Wooller et al. (2004) and included a small calibration data set. The study focused on reconstructing past $\delta^{18}\text{O}$ values of lake water in Greenland over the late Quaternary. Subsequently there has been a steady addition of applications of this approach (Wooller et al., 2008; Verbruggen et al., 2010b; Lombino, 2014; Lasher et al., 2017; Luoto et al., 2018; King et al., 2021; Puleo et al., 2022; McFarlin et al., 2023; Raposeiro et al., 2024). In most instances these data have been produced as part of a multiproxy approach, which has included in some cases cutting edge techniques including ancient DNA and biomarker analyses (Graham et al., 2016). Analyses of chironomid $\delta^{18}\text{O}$ values can also be coupled with the generation of past temperatures based on changes in the assemblages of the chironomid taxa present at a site over time (King

et al., 2021). In some instances, measurement of other isotope proxies, including $\delta^{13}\text{C}$ analyses of chironomids can be used to test whether some of the $\delta^{18}\text{O}$ variability is driven by changes in feeding ecology (e.g., King et al., 2021). As these studies grow in number, our understanding of the relationship between isotope proxy and environment improves. A $\delta^{18}\text{O}$ chironomid record from Lake Prata in the subtropical Azores archipelago highlighted that in temperate regions with low thermal variation, the isotopic content of precipitation is only weakly correlated with temperature and here the $\delta^{18}\text{O}$ chironomid record reflects changes in the seasonality of precipitation (Raposeiro et al., 2024). It is clear that we can expect variation in response due to large scale environmental controls and our knowledge will expand as this work continues. There is an extensive review of $\delta^{18}\text{O}$ records from aquatic remains included in van Hardenbroek et al. (2018a) and we refer readers to this paper for a more comprehensive discussion. In terms of new work since van Hardenbroek et al. (2011) review, focus has been on multi-proxy approaches and the inclusion of oxygen and hydrogen isotope chironomid studies alongside those incorporating bulk aquatic moss macrofossils, and aquatic moss-derived cellulose amongst others (Puleo et al., 2022; McFarlin et al., 2023; Raposeiro et al., 2024). There is still work to be done on fully understanding the factors affecting the fractionation of oxygen and hydrogen isotopes preserved in chironomids and the impact of pre-treatment methods, particularly on hydrogen isotopes.

3.2. Relating invertebrate $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values to feeding ecology and food webs

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of invertebrate remains preserved in sediment records, can provide evidence of (1) change in availability of food sources with distinct stable isotope composition, (2) change in the stable isotope composition of the food sources, or most likely (3) a combination of the two. Unpicking this is the key to utilising the techniques outlined above for palaeoenvironmental reconstructions.

3.2.1. Recording changes in productivity

Several examples are now available of studies that record changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of invertebrate remains on timescales ranging from recent sediments to Late-glacial records (e.g., Wooller et al., 2008; Schilder et al., 2017). These studies indicate changes in $\delta^{13}\text{C}$ values of phytoplankton in response to eutrophication, or baseline shifts in $\delta^{15}\text{N}$ values in response to increasing animal waste (Cabana and Rasmussen, 1996). In these cases, invertebrates and SOM usually have very similar trends in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, something that has also been observed in studies comparing stable isotopes in modern surface sediments and invertebrate remains in many lakes (e.g., van Hardenbroek et al., 2018b; Kivilä et al., 2019).

As primary consumers, Cladocera are very sensitive to changes in primary productivity and the stable isotope composition of their remains can be used to trace the impact of anthropogenic nutrient enrichment (and recovery) of lakes in the last 100–200 years, which often leads to increasing $\delta^{13}\text{C}$ values in phytoplankton and invertebrate remains through sediment cores as productivity increases (Perga et al., 2010; Frossard et al., 2014; Kattel et al., 2020; Rantala et al., 2021).

When sediment records are longer and exceed the period of major cultural eutrophication in the recent past, the $\delta^{13}\text{C}$ values of Cladocera remains can also be used to reconstruct changes in primary producers in response to environmental change. Rantala et al. (2016) found lower $\delta^{13}\text{C}$ values of SOM and Chydoridae during the Little Ice Age in response to a reduced supply of autochthonous (algal) energy sources in Lake Námajávri, Finland. Similar trends linking changes in $\delta^{13}\text{C}$ in *Daphnia* ephippia to changes in algal pigment composition were found in sediment cores from boreal lakes Diktar Erik and Stor-Bjorsjon in Sweden (Belle et al., 2019, 2021). Shifts in algal community do not always result in changes in stable isotope composition of zooplankton as shown by Rantala et al. (2015): large changes in diatom assemblage observed in a 11,000-year record from Lake Várdoajávri in Finland, were not

reflected in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of Cladocera remains, which were stable throughout the record. These studies emphasise that the ecosystem response of invertebrate remains may not always mirror those of other taxa and it is crucial to have an understanding of their response in each case.

Long-term stable isotope records of chironomid head capsules can also indicate distinct changes in $\delta^{13}\text{C}$ values of available food sources. The $\delta^{13}\text{C}$ values of *Sergentia coracina*-type head capsules increased with eutrophication in two large, clear water lakes in Vosges Mountains (France) (Belle et al., 2017a). Changes in photosynthetic carbon also drove $\delta^{13}\text{C}$ values of *Chironomus plumosus*-type in Lake Peipsi, Latvia (Belle et al., 2018a) over much longer timescales of $\sim 10\text{ka}$, and a combination of photosynthesis- and methane-derived carbon is the most likely explanation of variations in the $\delta^{13}\text{C}$ values of *Chironomus anthracinus*-type in Lake Tollari, Estonia (Belle et al., 2017b, 2018a).

A sediment record from Lake Pyramid Inferior (Nepal) at an extreme 5067 m a.s.l. (Belle et al., 2018b) contained head capsules of *Pseudodiamesa nivosus* with unusually high $\delta^{13}\text{C}$ values of -16.9 to -8.1‰ . These values can likely be explained by the low atmospheric pressure at 5067 m a.s.l., leading to very low DIC concentrations and a drawdown of atmospheric CO_2 for the photosynthesis of phytoplankton. In these conditions, carbon available for photosynthesis is limited and fractionation reduced (there can be less preferential uptake of ^{12}C by phytoplankton), resulting in higher $\delta^{13}\text{C}$ values of algae and chironomids.

3.2.2. Understanding changes in the sources of carbon and nitrogen

Drivers of change in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of aquatic invertebrates are not always related to productivity and associated primary producers but can also be related to changes in the (type of) food sources available for invertebrates, or to changes in the carbon and nitrogen sources available to microorganisms and algae in lakes consumed by different aquatic invertebrate groups. Griffiths et al. (2010) investigated the effect of marine-derived nutrients from sea-bird colonies on arctic lakes and showed that marine inputs led to an increase in $\delta^{15}\text{N}$ values of SOM and chironomid head capsules, whereas *Daphnia* remains did not show any change in $\delta^{15}\text{N}$ at all, indicating that the two organism groups can assimilate different sources of nitrogen.

In the boreal zone and catchments with extensive peatlands, lakes can receive large amounts of allochthonous carbon (often as dissolved organic carbon - DOC), which can lead to higher heterotrophic respiration, resulting in the supply of source carbon with relatively low $\delta^{13}\text{C}$ values, which subsequently result in lower $\delta^{13}\text{C}$ values of aquatic invertebrates (Rantala et al., 2021). The situation can be more complex, however, as invertebrates can selectively feed on particular food sources when these are available. For example, when little autochthonous (algal) organic matter was available in Lake Kylmänpampi (Finland), the $\delta^{13}\text{C}$ values of *Sergentia coracina*-type head capsules became lower, and also lower than the $\delta^{13}\text{C}$ values of SOM, suggesting uptake of respired carbon with relatively low $\delta^{13}\text{C}$ values (microbially processed DOC leaching from soils) when the preferred food source (algae) was unavailable (Belle et al., 2017c). The process of incorporating respired carbon was also suggested to drive the remarkably similar directions in the trends in $\delta^{13}\text{C}$ values of SOM and remains of *Bosmina*, *Daphnia*, *Alona*, and chironomids in sediment cores from five lakes in northwest Saskatchewan going back ~ 120 years (Anas et al., 2019). The authors, however, also indicate the trend could also be (in part) explained by the release of carbon from fossil fuels with relatively low $\delta^{13}\text{C}$ values as the Suess effect. The study by Anas et al. (2019) also observed decreasing $\delta^{15}\text{N}$ values in the same remains, which can be related to increased influx of terrestrial nitrogen with relatively low $\delta^{15}\text{N}$ values in dissolved organic matter. Similar changes in the balance between allochthonous and autochthonous carbon sources have been observed in a 2000-year record from Loazžjávri, Finland, by Kivilä et al. (2020). Here, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in chironomid head capsules and SOM had similar trends; $\delta^{13}\text{C}$ values increased after $\sim \text{AD } 1400$, as allochthonous organic matter sources became less prominent during the Little Ice Age (reduced

organic matter supply from less productive soils in this colder period). The change in dominant organic matter source was also evidenced by a shift towards collector-filterers amongst chironomid functional feeding groups, so it is also possible that the change in isotope composition of chironomid head capsules is caused by a change in taxa (with different preferred feeding modes) in the samples. The ability to be able to analyse single head capsules may allow wider investigations of this nature to proceed in the future.

Measuring stable isotopes on specific, pre-selected taxa is a useful approach, as it allows interpretation of stable isotope values in-light-of habitat and dietary preferences. An earlier review (van Hardenbroek et al., 2018a) provides an overview of stable isotope studies of the remains of Chironomidae, Cladocera, and Bryozoa, and how the taxon-specific $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of these organisms are linked to their feeding ecology, hence we only highlight a few examples of studies on chironomid larvae here. Generally, remains of Orthocladinae usually have high $\delta^{13}\text{C}$ values similar to the $\delta^{13}\text{C}$ values of SOM, as many species in this subfamily are littoral dwellers feeding on available plant detritus and periphyton (Mihuc and Toetz, 1994; Merritt, Cummins and Berg, 2008; Fig. 4), especially in mesotrophic and eutrophic lakes. Chironomini head capsules have a very large range of $\delta^{13}\text{C}$ values given the wider range of feeding strategies in this tribe, including the ability to burrow, thrive in hypoxic environments and filter bacterial biomass with relatively low $\delta^{13}\text{C}$ values (van Hardenbroek et al., 2013, 2014; see also section 3.3 and 3.4).

The carbon isotope composition of organisms is derived from a mixture of different food sources. By measuring or estimating the stable isotope values of the different dietary components (end members), it is possible to provide quantitative estimates of the relative contribution of different dietary sources using isotope mixing models. Examples of this can be seen in Wooller et al. (2012) and Schilder et al. (2017), estimating contributions of methane-derived carbon to the diet of the investigated invertebrates. Similarly, Su et al. (2022) use $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ of Cladocera remains in surface sediments to estimate dietary contributions of POC, DOC, DIC/algae and terrestrial plants to cladoceran diet. With the exception of Su et al. (2022), most isotope mixing models applied to invertebrate remains have so far focused on stable isotopes of carbon, but it is possible to use other isotope systems (H, O, N, S), radiogenic isotope applications (^{14}C) as well dual isotope approaches (e.g. $\delta^{13}\text{C}/\delta^{15}\text{N}$, $\delta\text{D}/\delta^{13}\text{C}$, $\delta\text{D}/\delta^{18}\text{O}$) as applied in modern ecological studies (e.g., Soto et al., 2013; Frossard et al., 2023).

An interesting direction of research is to simultaneously analyse $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the remains from various invertebrate groups to understand changes in the structure of paleo food webs. Van Hardenbroek et al. (2018b) present a $\delta^{13}\text{C}/\delta^{15}\text{N}$ biplot of invertebrate remains in surface sediments of Lake De Waay showing how primary consumers have the lowest $\delta^{15}\text{N}$ values and predatory invertebrates have highest $\delta^{15}\text{N}$ values, as expected based on modern food web studies (e.g., Jones and Waldron, 2003). Anas et al. (2019) also measured $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values simultaneously on remains of different aquatic invertebrates (*Alona*, *Bosmina*, *Daphnia*, chironomids) in sediment cores from five lakes in Saskatchewan. In their study, they did not provide $\delta^{13}\text{C}/\delta^{15}\text{N}$ biplots, making it harder to see changes in food web structure over time.

Another example of a study on changes in food web structure can be seen in Fig. 5 (van Hardenbroek and Robson, unpublished data), where $\delta^{13}\text{C}/\delta^{15}\text{N}$ biplots for different aquatic invertebrate remains in a sediment core from Loch Nam Fear (UK) show how the range of $\delta^{15}\text{N}$ values of aquatic invertebrates has decreased from 6.4‰ in the period before 1940, to 2.5‰ in the period after 1990. No significant change in $\delta^{15}\text{N}$ of SOM was observed in the same period, indicating no change in $\delta^{15}\text{N}$ baseline of organic matter derived from the catchment or produced in the lake itself. This suggests that, instead, the smaller range in $\delta^{15}\text{N}$ values of aquatic invertebrates reflects a reduction of trophic levels. In the case of Loch Nam Fear this change in food web structure may be linked with the establishment of a pine plantation in the immediate surroundings of the loch in the late 1980s, changing nutrient cycling (for

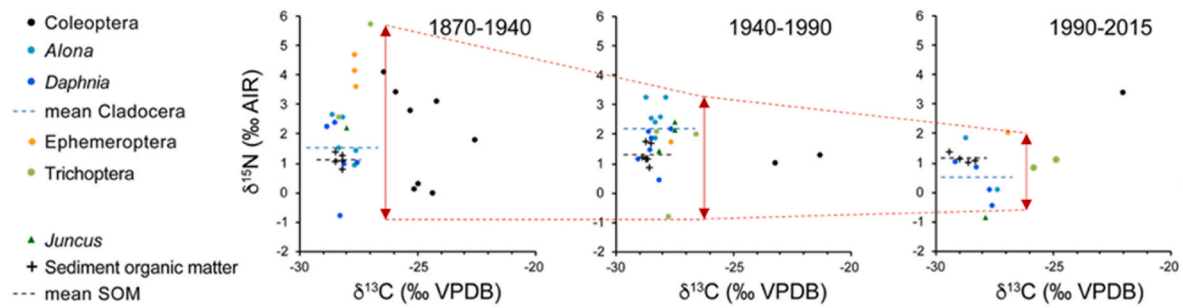


Fig. 5. Taxon-specific $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values invertebrates remains, *Juncus* and SOM in a sediment record from Loch Nam Fear, UK (van Hardenbroek and Robson, unpublished data). Samples were analysed at the Alaska Stable Isotope Facility, UAF. Replicate measurements on standard materials indicate an analytical error smaller than 0.1‰ for $\delta^{13}\text{C}$ and 0.2‰ for $\delta^{15}\text{N}$ values.

example, via the addition of nitrogen-based fertilizer during planting) and hydrology (for example, enhanced evapotranspiration resulting in less run-off) in the catchment. Although these catchment-scale processes may not affect the stable isotopes of the aquatic invertebrates directly, the change in nutrient cycling or hydrology can negatively impact the aquatic invertebrate community, leading to reduced complexity of the food web with the loss of invertebrates taking up intermediate or top-level positions, ultimately leading to a smaller range in $\delta^{15}\text{N}$ values.

3.3. Invertebrate $\delta^{13}\text{C}$ values reflect methane cycling in lakes

Several studies have used the link between methane availability and (very) low $\delta^{13}\text{C}$ values of the remains of certain chironomid and cladoceran taxa to study changes in hypoxia and methane availability over long time periods using sedimentary records. In some cases, increased methane-derived carbon in invertebrate remains is related to anthropogenic nutrient addition in lakes (Frossard et al., 2015; Belle et al., 2016a, 2016b; Schilder et al., 2017; Morlock et al., 2017). In other cases, increased primary production leading to anoxia and increased availability of methane-derived carbon is triggered by warmer/wetter climatic conditions (Wooller et al., 2012; van Hardenbroek et al., 2013). Belle et al. (2017c) recorded evidence for important contributions of methane-derived carbon to the lacustrine food web during the Holocene Thermal Maximum in Lake Tollari (Estonia) based on significantly lower $\delta^{13}\text{C}$ values in chironomid head capsules. In a study that combined multiple isotope systems, Hu et al. (2023) observed lower $\delta^{13}\text{C}$ values in chironomids and lower δD in Cladocera remains in response to wetter conditions since 1993 in a lake in the Great Plains that also experience warmer climate and increased nutrient loading.

Eutrophication and climate are not always the drivers of changes in lake CH_4 cycling recorded by invertebrate $\delta^{13}\text{C}$ values. Belle et al. (2018c) recorded extremely low $\delta^{13}\text{C}$ values (−49.4‰) of *Chironomus anthracinus*-type and *Sergentia coracina*-type head capsules between 3.5 and 2.7 cal ka BP in a core from Lake Lielais Svētiņū, Latvia. These low $\delta^{13}\text{C}$ values were likely related to a shallowing of the lake, possibly related to increased human activity, but clearly not related to changes in nutrients as highest values of organic matter content and pigments were recorded much earlier at this site, around 6–5 ka cal BP. In one very specific case, evidence for increased methane-derived carbon uptake in *Daphnia* was related to changes in stratification stability caused by wind-induced mixing due to changes in surrounding vegetation (Rinta et al., 2016). This case is evidenced in a sediment core collected from Mekkojärvi, a small boreal lake in Finland. The lake is presently surrounded by trees, providing shelter from wind-induced mixing of the water column, leading to strong stratification, anoxia, and $\delta^{13}\text{C}$ values of *Daphnia* ephippia as low as −49‰. During two periods in the past trees were apparently cleared, likely leading to increased wind fetch, mixing and the break-up of anoxia, leading to $\delta^{13}\text{C}$ values of *Daphnia* ephippia 10‰ higher in periods without trees surrounding the lake.

In some locations, such as in regions with thawing permafrost, the

methane being emitted from a lake can have a relatively old radiocarbon age as well as a relatively low $\delta^{13}\text{C}$ value (as described above) (Brosius et al., 2023; Walter Anthony et al., 2008, 2012, 2016, 2021; Elder et al., 2019). In these instances, modern living aquatic insects, or even the consumers feeding on them, can have both relatively low $\delta^{13}\text{C}$ values and relatively old radiocarbon ages (e.g., Wooller et al., 2012; Schell, 1983; Grey, 2016). These data can be used to estimate the proportional contribution of methane-derived carbon to invertebrates using relatively simple mixing models using the $\delta^{13}\text{C}$ values of the two end member food sources, methane oxidizing bacteria and algae (e.g., Wooller et al., 2012; Belle et al., 2014, 2015b, 2016; Schilder et al., 2017) and in turn, if the flux of invertebrates is measured, the flux of methane derived carbon in the form of emergent insect carbon can be determined. In such cases, POM or SOM has been used as approximation for algae and $\delta^{13}\text{C}$ values of methane oxidizing bacteria have been estimated using $\delta^{13}\text{C}$ offsets from literature, which means this approach is associated with high levels of uncertainty due to variations in $\delta^{13}\text{C}$ values of methane and algal material within and between lakes and over the annual cycle (as discussed in section 2.4)

As an example, Big Trail Lake (Fig. 6a–c), which is set in an area of rapidly thawing permafrost in interior Alaska, has been the centre of considerable research focused on studying the production and fate of methane. Research at the site has included measurements of the $\delta^{13}\text{C}$ values of methane as well as its radiocarbon age (e.g., Walter et al., 2008 and unpublished data). In 2021, Barst and Wooller set up floating emergent insect traps (0.58 m × 0.58 m) on Big Trail Lake during a 24-day period of the growing season to capture adult emergent insects from the lake (Fig. 6d). The aim was to determine the biomass of emergent insects from Big Trail Lake and couple the data with $\delta^{13}\text{C}$ values in dietary mixing models to calculate the flux of methane-derived carbon from the lake via adult emergent insects. Since the trap areas were known and they were set out for known periods of time the weight of emergent insects from them could be used to calculate a flux of emergent insects ($\text{g}/\text{m}^2/\text{day}$) from the lake, similar to previous efforts at lower latitudes (e.g., Chumchal and Drenner, 2020). Although other taxa were present, Diptera (primarily chironomids) and Odonata (dragonflies) were most prevalent in the traps, thus the effort focused on these taxa moving forward. In this case, the flux of Diptera and Odonata only from Big Trail Lake was $0.0878 \text{ g}/\text{m}^2/\text{day}$, equating to about $0.0439 \text{ g}/\text{m}^2/\text{day}$ of carbon (given that elemental analyses of the insects determined they were composed of ~50% carbon). Sub-samples of the Diptera and Odonata were measured for their $\delta^{13}\text{C}$ values and their radiocarbon ages (Tables 3 and 4).

The insect $\delta^{13}\text{C}$ data (Table 3) were coupled with previously generated $\delta^{13}\text{C}$ values of diffusive methane (Walter Anthony and Elder unpublished data – Table 3) and plants (Wooller et al., 2007) to calculate the proportional contribution of methane-derived carbon using a two-endmember mixing model (e.g., Wooller et al., 2012). The average of three mixing models (one using the average diffusive methane $\delta^{13}\text{C}$ value for Big Trail Lake minus 1 SD of 12‰, one using the average

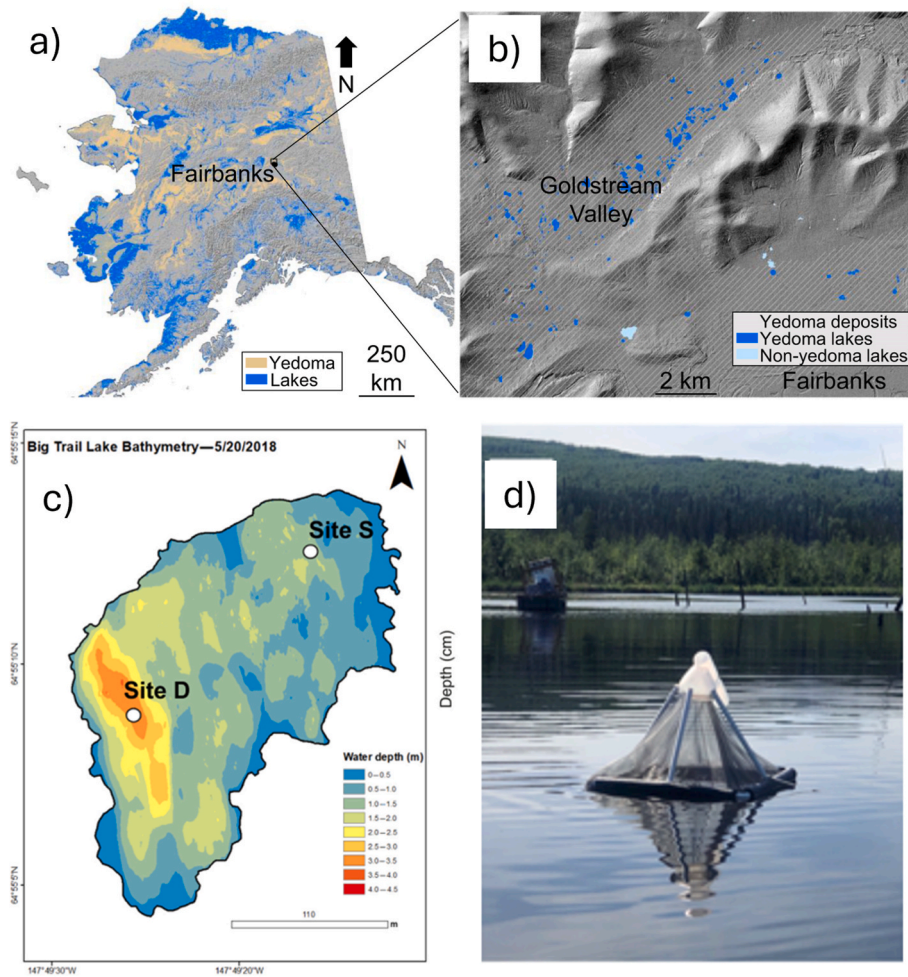


Fig. 6. The location and bathymetry of Big Trail Lake in the Goldstream valley, Alaska (a–c) (a & b reproduced from [Walter Anthony et al., 2021](#) and c from [Pellerin et al., 2022](#)) and emergent insect traps at the site in the summer of 2021(d) (photo credit B. Barst).

diffusive methane $\delta^{13}\text{C}$ value methane plus 1 SD of 12‰ and one using the average diffusive methane $\delta^{13}\text{C}$ value methane) was 68% methane-derived carbon to the insects emerging from Big Trail Lake. Radiocarbon dates were also generated from methane sampled from Big Trail Lake ([Walter Anthony et al., 2021](#)), which demonstrate that there is ‘old’ methane in the system. The radiocarbon results from analyses of certain emergent insects from Big Trail Lake ([Table 4](#)) demonstrate that some of this ‘old’ methane is entering the modern food web. We were subsequently able to use the known area of the lake ($\sim 37,053 \text{ m}^2$), our mixing model results and flux data to scale-up and estimate the flux of methane-derived insect-carbon from the whole lake, which equates to 1106 g/day. The daily summer surface to atmosphere flux of methane from Big Trail Lake, as recorded on the eddy covariance tower at the site, is $\sim 300 \text{ mg CH}_4/\text{m}^2/\text{d}$ (equivalent to $240 \text{ mg C-CH}_4/\text{m}^2/\text{d}$ when multiplied by 12/16 to convert to methane’s carbon mass). This means that the insect sink represents about 20% of the total summer emissions of methane.

These data can also be used to scale-up further using the estimated area of all Yedoma lakes ($\sim 833,000 \text{ m}^2$ from [Walter Anthony et al., 2021](#)) in the Goldstream Valley of Alaska, where Big Trail Lake is located. In this case, an estimated flux of methane-derived carbon to emerging adult Diptera and Odonata, using the data from Big Trail Lake as a basis, would equate to 25 kg/day or 3000 kg/120 day growing season, across all Yedoma lakes of the region. Although this estimate is based on many assumptions and unknowns, it serves as a useful estimation of a branch of the Arctic carbon cycle that has not previously

been determined or quantified. For example, a portion of the carbon composing emergent insects will be respired as carbon dioxide (CO_2). Although this is also a greenhouse gas, it is around 30 times less potent as a greenhouse gas on a 100-yr timescale compared with methane. Since, in the case of Big Trail Lake, a portion of this respired CO_2 from emergent insects was originally derived from lake methane, methanotrophs and emergent insects are in essence mitigating the full greenhouse gas potential of the lake from entering the atmosphere. Although this may be trivial at the scale of one lake in the sub-arctic, any scaling-up of this scenario to the large number of methane emitting lakes in the Arctic ([Walter Anthony et al., 2021](#)) could represent a substantial portion of the Arctic’s carbon budget. This thought experiment also reveals gaping holes in our knowledge about quantified estimates of emergent insects and the portion of methane-derived carbon composing them. For instance, the carbon composing emergent insects (some of which in the case of Big Trail Lake is derived from methane) can be handed on to secondary consumers (e.g., [Schell, 1983](#); [Grey, 2016](#)) and in turn a portion is also respired as CO_2 ([Fig. 7](#)). In essence, a portion of the energy budget fuelling the seasonal migration of the millions of migratory waterfowl that visit Arctic lakes is being supplied by carbon derived from ancient methane. Although chironomids and mosquitoes are notoriously ubiquitous there is a lack of information regarding estimates of their biomass and flux from lakes in the Arctic and sub-Arctic.

Table 3

$\delta^{13}\text{C}$ values (‰) from a sample of adult emergent insects from Big Trail Lake – this study. (*diffusive site methane data from Big Trail Lake are from Walter Anthony and Elder unpublished data and plant $\delta^{13}\text{C}$ values are from Wooller et al., 2007). These compiled data were used to estimate the proportional contribution of methane-derived carbon to the emergent insects using a two end-member mixing model (as in Wooller et al., 2012). $\delta^{13}\text{C}$ values of insects were measured at the Alaska Stable Isotope Facility following previously published protocols and analytical uncertainties were 0.2 ‰ (Wooller et al., 2012).

Sample name	Insect $\delta^{13}\text{C}$ values (V-PDB ‰)	Average site summer dissolved methane $\delta^{13}\text{C}$ value* (V-PDB ‰)	Plant $\delta^{13}\text{C}$ value* (V-PDB ‰)
Diptera pupa	-31	-43	-28
Diptera pupa	-32	-43	-28
Diptera pupa	-31	-43	-28
Diptera pupa	-32	-43	-28
Diptera pupa	-31	-43	-28
Diptera pupa	-31	-43	-28
Diptera pupa	-33	-43	-28
Diptera pupa	-35	-43	-28
Diptera pupa	-31	-43	-28
Diptera pupa	-32	-43	-28
Dragonfly	-29	-43	-28
Mean	-32 (+1)		

Table 4

Radiocarbon dating results from analyses of emergent insects from Big Trail Lake – this study. Radiocarbon analyses of insects were measured at the KECK radiocarbon dating facility and were calibrated using Calib 8.2 following previously published protocols (Wooller et al., 2012). Calibrated radiocarbon date for diffusive methane from the site was 1122 years before present (Walter Anthony and Elder previously unpublished data).

UCIAMS #	Sample name	^{14}C age (BP)	±	Insect median calibrated age (BP)	1 Sigma range
258851	Diptera pupa	820	20	712	690–731
258852	Diptera pupa	895	15	782	742–897
258853	Diptera pupa	930	20	852	791–903
258854	Diptera pupa	830	20	724	689–736
Mean				768	

4. Summary and outlook

There has been a significant range of new studies over the last few years addressing improvements to the existing methodology of stable isotope analysis on aquatic invertebrates as detailed above. Moving forwards, these improvements open a range of research opportunities and have the potential to be applied on a large scale. Below we summarise the key advancements in the field and offer some thoughts on what we consider to be important next steps and opportunities.

4.1. Analytical improvements

The studies exploring the impact of degradation and chemical pretreatments indicate that preservation and taphonomic processes do not seem to impact the stable isotope values of chitinous remains substantially, with the potential exception of thin-walled remains.

Pretreatments using a range of different approaches also seem to have limited impact on the stable isotope signals preserved in fossil remains, with the exception of $\delta^{18}\text{O}$ values which show variations depending on the chosen laboratory protocol. Current pretreatment assessments have mainly focused on $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ ratios in chironomid or cladoceran remains, and further tests on a wider range of elements, laboratory procedures on invertebrate remains can further our understanding of the impacts of taphonomic processes and laboratory pretreatments as these applications grow in number. Available studies with *Daphnia* indicate that offsets between soft tissue and the fossilizing, sclerotized structures are relatively small and constant, a finding that should be verified also for other aquatic invertebrate groups with fossilizing exoskeleton structures. Studies have shown that hydrogen isotopes preserved in chironomid tissues also largely originate from the water and the food ingested by larvae (Belle et al., 2015b), although little is known about the δD values different body parts, apart from one study of beetle fragments (Gröcke et al., 2010). A focus for further work could be to expand this to other, more commonly used, invertebrate fossils.

A major step-change has come about through the next generation (TC)EA-IRMS systems and experimental work undertaken by the authors here has demonstrated that, for $\delta^{18}\text{O}_{\text{chitin}}$ measurements at least, a sample size of ca. 25–30 μg means that taxon-specific isotopic measurements are now within reach, preventing the complexity of interpreting $\delta^{18}\text{O}_{\text{chitin}}$ measurements on samples composed of a mix of taxa. Improvements in (TC)EA-IRMS (section 2.2) and laser ablation nano combustion gas chromatography isotope ratio mass spectrometry (LA/nC/GC/IRMS) as undertaken by Schilder et al. (2018) open the possibility of new areas of research that have been too time-consuming to be viable in the past as it can be challenging to produce long-term records that consistently measure isotope values on the same taxon.

Many of the studies discussed here have illustrated why single taxon studies could be transformative. For example, variations in methane flux can differentially impact the $\delta^{13}\text{C}$ values of the head capsules of littoral-dwelling chironomid groups compared to profundal-dwelling ones. Isotopic changes between species in the same lake, between the same species in different part of the lake and especially across different seasons are also evident (see sections 2.5.1 and 2.5.2). For example, we note in section 2.5.1 that $\delta^{13}\text{C}$ values for living larvae of *C. plumosus* in the deepest part of Lake Holzsee had values of -56.0 to 60.0 ‰, compared to remains of *C. plumosus*-type, which showed higher values of -38.9 ‰, suggesting seasonal changes in $\delta^{13}\text{C}$ values of *C. plumosus* at the sampling site throughout the year that are not represented by the collections of live larvae during the summer stratification period. This contrast is masked in a bulk measurement of remains containing up to 40 individual head capsules, resulting in long-term averaging across seasons, years and across different micro habitats within a lake. The ability to be able to isolate and analyse single taxon groups can be a large step forward in such situations. For example, the study by Belle et al. (2018b) of *Pseudodiamesa nivosa* chironomid head capsules extracted from a sediment record from Lake Pyramid Inferior (Nepal) at high altitude had unusually high $\delta^{13}\text{C}$ values of -16.9 to -8.1 ‰, related to an altitude-induced drawdown of atmospheric CO_2 . Records of $\delta^{13}\text{C}$ values of *Micropsectra contracta*-type and *Sergentia coracina*-type from Lake Annecy (Frossard et al., 2014) demonstrated contrasting $\delta^{13}\text{C}$ values between deep-water and shallower locations. These two studies clearly show the potential insights that could be gained from more detailed studies and the improvements in mass-spectrometry will inevitably open this approach more widely by impacting the time and cost implications.

However, there needs to be consideration of the natural variability of isotopic change evident compared to background noise and the extra analytical error that may be introduced through a higher sensitivity method (Table 2). There will be circumstances when a bulk measurement of several invertebrate types is preferred to give a robust estimate of average values for a taxonomic group. Lowering the sample size can result in a different set of concerns surrounding the risk of bias and linearity effects. The experiment outlined in this paper (Fig. 2) focused

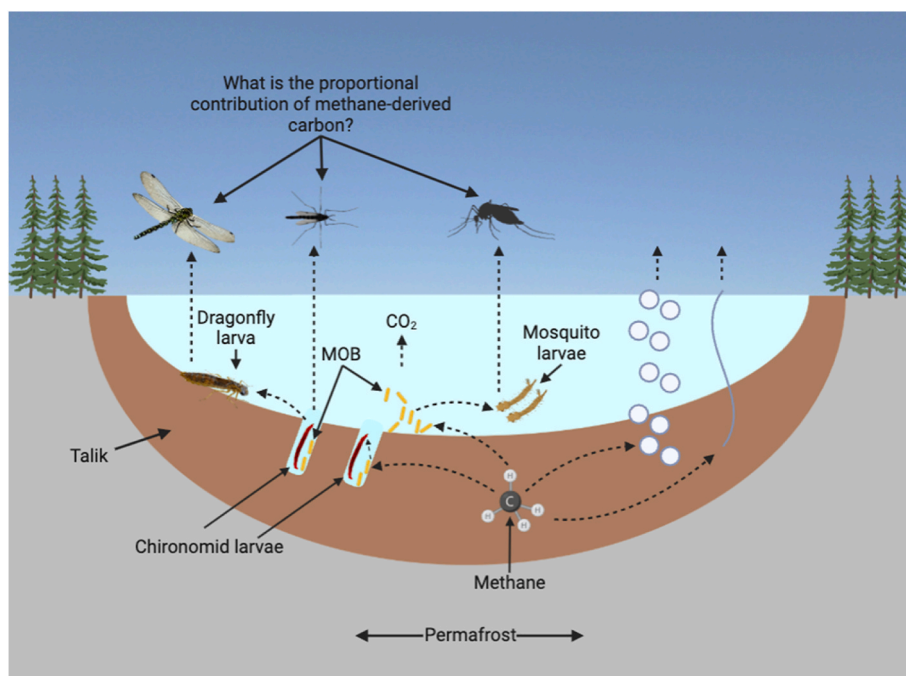


Fig. 7. The transfer of methane derived carbon in an aquatic food web. Solid arrows indicate labels and dashed arrows indicate processes. Figure created using Biorender.

on $\delta^{18}\text{O}$ values of chironomids, but similar issues also apply to all isotope studies on invertebrate remains and more comparable tests are needed to determine "how low you can go" using the latest instrument advances.

4.2. Understanding the importance of lake type and heterogeneity

In terms of the spatial and temporal variation in the stable isotope composition of invertebrate remains, the processes are less well understood and within-lake variability of stable isotopes of invertebrate remains has only been investigated in a few studies to date. What is apparent is that stable isotope values are highly variable depending on lake conditions (section 2.5.1). This is evident for studies focusing on carbon isotopes but is less studied in other isotope systems and there is further work to be done to fully understand within-lake processes for all isotope systems.

Several studies have measured significant differences in $\delta^{13}\text{C}$ between invertebrates from depth compared to littoral samples during part of the year due to the source of carbon at depth and/or changes in eutrophication and hypoxia including new data presented here from lakes in Germany and The Netherlands (Holzsee, Plußsee, Schöhsee, De Waay; Rinta et al., 2017), see section 2.5.1., which emphasises the need to assess the environmental setting of each study site to enable a thorough assessment of the experimental design in terms of sample number and degree of sampling intensity.

This is especially relevant in terms of seasonal changes and the need to take into account what moment in the annual cycle is captured by the isotopic composition of invertebrate remains. The studies reviewed here also raise the question whether this variability can change from year to year – and what the impact of changes in the timing of seasons can have on $\delta^{13}\text{C}$ values of invertebrates from sediment records covering hundreds or thousands of years. A focus needs to remain on producing quality calibration data sets that cover a wider remit (multi-isotope and multi-lake) in order to fully realise the potential of the technique. This is also true for the global application of the technique. The fact that aquatic invertebrates are ubiquitously present in most freshwater bodies in the world allows for these newly developed techniques to be applied across a much larger range of lakes, potentially even globally. Focus has

primarily been on temperate and high-altitude lakes to date with little application in tropical environments or the global south. Further work is needed to assess the systematics of invertebrate isotope variation in such, as yet, understudied environments.

4.3. Offsets and isotope systematics

Studies to date have generally confirmed that the stable isotope composition of invertebrate remains preserved in lake sediments reflects the living organism, which in turn reflects the stable isotope composition of the diet and/or water in their environment. The number of food web studies are growing, and these types of studies could be strengthened by analysing stable isotopes of different food sources for aquatic invertebrates, e.g. by analysing stable isotopes of plant macro-remains or compounds specific to algae and microbial biomass (Castañeda and Schouten, 2011; Taipale et al., 2015; Holtvoeth et al., 2019).

In their 2018 review of the application of organic and inorganic fossils in lake sediment records, van Hardenbroek et al. (2018a) highlighted the importance of calibration studies, noting that they were essential to better understand the relationships between stable isotope composition and ecological/environmental variables. Recently, Calibration studies continue to play an important role in developing isotope-based approaches analysing chitinous invertebrate remains and to study changes in the isotopic composition of invertebrate remains as a consequence of varying environmental conditions (e.g., in different lakes along a temperature or lake-water $\delta^{18}\text{O}/\delta\text{D}$ gradient). Employing a multi-element isotope approach may help to untie many complex processes, and ultimately (start to) differentiate effects from climate from those of nutrient enrichment. The improved sensitivity of EA-IRMS further opens this possibility. In this light, the hydrogen isotope analysis is less often applied but offers interesting unexplored options and new possibilities. For example, Hu et al. (2023) recently observed a fall in δD of Cladocera remains in response to wetter conditions in a study that combined multiple isotope systems, hinting that δD may offer a palaeohydrological proxy. In addition, there is potential to investigate a wider range of ecological conditions using sulfur isotope systematics.

Instrumentation development has particularly helped with the

analysis of low concentration sulfur organisms (Sayle et al., 2019). Sulfur isotope values can potentially offer insights to within-lake energy sources to consumers namely, the incorporation of sulfide-S into freshwater benthic food webs (Croisette et al., 2009; Karube et al., 2012; Lescord et al., 2019). Previous studies have relied on carbon isotopes to understand these processes so the reduction in sample size required for sulfur isotope studies now make its addition more feasible going forwards and hopefully will increase research opportunities to study biogeochemical sulfur cycling in lacustrine settings. A review by Raoult et al. (2024) explores these opportunities more widely.

In terms of oxygen isotopes, the temperature dependence of $\delta^{18}\text{O}$ in chironomids recently identified by Lombino et al. (2021a) opens up further questions in terms of how we utilise $\delta^{18}\text{O}$ as a palaeoclimatic proxy in invertebrates. Further work to test the implications of this relationship by Lombino et al. (2021b) has highlighted that diagenetic alteration of the head capsules may systematically bias the isotope-derived temperature estimates in some circumstances and thus there is still much to explore here before we can fully utilise this technique further.

4.4. Final thoughts

Finally, in the last few years members of the isotope community have made a concerted effort to develop and provide a centralised repository for all isotope data, following in the same vein as the role GenBank serves for the genetic and molecular community (Shipley et al., 2024). This resource, known as IsoBank, will help perpetuate comparative studies and the expansion of larger data sets to maximize the benefits to all from new research and we fully support this initiative.

CRediT authorship contribution statement

Angela L. Lamb: Project administration, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Benjamin D. Barst:** Formal analysis, Writing – original draft, Writing – review & editing. **Clayton D. Elder:** Investigation, Formal analysis, Writing – review & editing. **Stefan Engels:** Supervision, Investigation, Writing – original draft, Writing – review & editing. **Chris Francis:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Maarten van Hardenbroek:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Oliver Heiri:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Alex Lombino:** Investigation, Resources. **Hannah J. Robson:** Formal analysis. **Katey Walter Anthony:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Matthew J. Wooller:** Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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