



Variability in $\delta^{13}\text{C}$ values between individual *Daphnia* ephippia: Implications for palaeo-studies

Jos Schilder^{a, b, *}, Linda van Roij^c, Gert-Jan Reichart^{c, d}, Appy Sluijs^c, Oliver Heiri^a

^a Institute of Plant Sciences and Oeschger Centre for Climate Change Research, University of Bern, Altenbergrain 21, 3013 Bern, Switzerland

^b Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland

^c Department of Earth Sciences, Faculty of Geosciences, Utrecht University, Princetonlaan 8a, 3584 CB Utrecht, The Netherlands

^d Royal Netherlands Institute for Sea Research (NIOZ), Landsdiep 4, 1797 SZ 't Horntje (Texel), The Netherlands

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ABSTRACT

The stable carbon isotope ratio ($\delta^{13}\text{C}$ value) of *Daphnia* spp. resting egg shells (ephippia) provides information on past changes in *Daphnia* diet. Measurements are typically performed on samples of ≥ 20 ephippia, which obscures the range of values associated with individual ephippia. Using a recently developed laser ablation-based technique, we perform multiple $\delta^{13}\text{C}$ analyses on individual ephippia, which show a high degree of reproducibility (standard deviations 0.1–0.5‰). We further measured $\delta^{13}\text{C}$ values of 13 ephippia from surface sediments of three Swiss lakes. In the well-oxygenated lake with low methane concentrations, $\delta^{13}\text{C}$ values are close to values typical for algae (−31.4‰) and the range in values is relatively small (5.8‰). This variability is likely driven by seasonal (or inter-annual) variability in algae $\delta^{13}\text{C}$ values. In two seasonally anoxic lakes with higher methane concentrations, average values were lower (−41.4 and −43.9‰, respectively) and the ranges much larger (10.7 and 20.0‰). We attribute this variability to seasonal variation in incorporation of methane-derived carbon. In one lake we identify two statistically distinct isotopic populations, which may reflect separate production peaks. The potentially large within-sample variability should be considered when interpreting small-amplitude, short-lived isotope excursions based on samples consisting of few ephippia. We show that measurements on single ephippia can be performed using laser ablation, which allows for refined assessments of past *Daphnia* diet and carbon cycling in lake food webs. Furthermore, our study provides a basis for similar measurements on other chitinous remains (e.g. from chironomids, bryozoans).

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1. Introduction

The use of the stable isotopic composition of organic remains in lake sediments has been emerging as a means to reconstruct changes in past ecological and climatological conditions in and around lakes (Leng and Henderson, 2013). Because they are often abundantly found in sediments and are resistant to degradation (Verbruggen et al., 2010), the chitinous remains of aquatic invertebrates have a strong potential as a proxy for such reconstructions (Heiri et al., 2012). For example, the stable carbon isotopic composition (expressed as $\delta^{13}\text{C}$ values) of the shells (ephippia) of the resting eggs of water fleas (*Daphnia* spp.) has been

shown to reflect that of the maternal *Daphnia* and its diet (Schilder et al., 2015b). This allows for the use of $\delta^{13}\text{C}$ values of ephippia to investigate the $\delta^{13}\text{C}$ value of *Daphnia* diet in the past (see e.g. Morlock et al., 2016; Rinta et al., 2016; Schilder et al., 2017). Chitinous remains of other organisms, most notably chironomid head capsules, bryozoan statoblasts and *Ceriodaphnia* ephippia have also been used to investigate past changes in the stable carbon isotopic composition of aquatic invertebrates in lake food webs, often in combination with *Daphnia* ephippia (Belle et al., 2014; Frossard et al., 2013; Morlock et al., 2016; Rinta et al., 2016; van Hardenbroek et al., 2013; Wooller et al., 2012).

Daphnia feed on suspended organic particles, predominantly algae, in the water column of lakes (Lampert, 2011). In addition, *Daphnia* may ingest methanotrophic bacteria (MOB) or other microorganisms feeding on MOB. This leads to $\delta^{13}\text{C}$ values in *Daphnia* that are much lower (Kankaala et al., 2006) than typical for freshwater algae (−35 to −25‰ (Vuorio et al., 2006)), because the $\delta^{13}\text{C}$

* Corresponding author. Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland.

E-mail address: j.c.schilder@gmail.com (J. Schilder).

values of biogenic methane in lake ecosystems (-85 to -50% (Jedrysek, 2005; Rinta et al., 2015; Whiticar et al., 1986)) and MOB metabolizing this methane (Templeton et al., 2006) are exceptionally low. Schilder et al. (2015a) found that $\delta^{13}\text{C}$ values of ephippia from surface sediments were lower in small temperate lakes with high methane concentrations in the water column than in those with low methane concentrations in the water column. This suggests that the amount of methane-derived carbon in these ephippia (and consequently their $\delta^{13}\text{C}$ values) are systematically related to in-lake methane concentrations. The $\delta^{13}\text{C}$ values of ephippia deposited in lake sediments may thus provide an indication of past availability of methane in lakes. Given the importance of lakes (and other freshwater bodies) in the global carbon cycle as producers and emitters of methane (Bastviken et al., 2011; Battin et al., 2009), this method can provide valuable insight into the past response of lakes to environmental and climatic change in terms of methane productivity and carbon cycling in general (e.g. Perga et al., 2016; van Hardenbroek et al., 2014, 2013; Wooller et al., 2012). This information, in turn, can be used to better predict future responses of the lacustrine carbon cycle, and particularly of lacustrine methane production and emission, to changes in the environment and in the climate.

Seasonal fluctuations in $\delta^{13}\text{C}$ values of *Daphnia* exceed 10% in some lakes (Morlock et al., 2016; Perga and Gerdeaux, 2006; Smyntek et al., 2012) and are partly driven by changes in the $\delta^{13}\text{C}$ value of phytoplankton. For example, the seasonal succession of dominant algae species with different carbon fractionation rates can drive seasonal shifts in algal $\delta^{13}\text{C}$ values (e.g. Hollander and McKenzie, 1991) and therefore the stable carbon isotopic composition of food available to *Daphnia*. Additionally, changes in CO_2 concentrations (due to e.g. uptake of CO_2 by algae, CO_2 produced as a product of decomposition of organic matter or changes in pH that shift the equilibrium between the various dissolved carbonate species) can also influence both the baseline $\delta^{13}\text{C}$ value of CO_2 and algal carbon fractionation rates (Laws et al., 1995; Smyntek et al., 2012), forming another source of seasonal variability in *Daphnia* $\delta^{13}\text{C}$ values. As a consequence, higher $\delta^{13}\text{C}$ values of algae are typically observed during (late) spring and summer and lower values during fall and winter (Hollander and McKenzie, 1991; Morlock et al., 2016; Schilder et al., 2017; Smyntek et al., 2012). However, incorporation of MOB by *Daphnia* also likely occurs mostly in fall when algal abundance declines and hypolimnetic methane is exposed to oxygen upon lake mixing (Morlock et al., 2016; Taipale et al., 2007). This can promote large seasonal fluctuations in *Daphnia* $\delta^{13}\text{C}$ values in dimictic lakes with high methane production, leading to *Daphnia* $\delta^{13}\text{C}$ values much lower than reported for algae (-40% and lower, see e.g. Kankaala et al. (2010) and Schilder et al. (2017)). In addition, there is a seasonality in the production of ephippia themselves: Ephippia may be produced throughout the year, but there typically are distinct production peaks in spring and in fall (Cáceres, 1998; Cáceres and Tessier, 2004).

The extent to which these sources of (potential) seasonal variability affect down-core variations in $\delta^{13}\text{C}$ values of *Daphnia* ephippia is poorly constrained. In part, this is because $\delta^{13}\text{C}$ analysis of single (sub)fossil ephippia shells has to date been impossible. $\delta^{13}\text{C}$ measurements on a large number of shells from a sample (hereafter called bulk measurements) represent a weighted average of $\delta^{13}\text{C}$ values of individual ephippia which may originate from populations of very uniform or highly variable $\delta^{13}\text{C}$ values. Measurements on individual ephippia would provide insights into this within-sample variation. Furthermore, they could provide information on whether ephippia in a sample represent one or more

isotopically distinct ephippia production peaks or *Daphnia* populations (e.g. spring and autumn production peaks of the same or different *Daphnia* species), and how food sources different from algae (e.g. MOB) contributed to the diet of *Daphnia* producing these ephippia.

The amount of *Daphnia* ephippia needed for down-core ephippia $\delta^{13}\text{C}$ analysis constrains the resolution of presently available records, often resulting in records with a relatively low level of taxonomic and temporal resolution (e.g. Morlock et al., 2016; Schilder et al., 2017). The number of ephippia needed is in turn strongly constrained by the size (mass) of the ephippia found in a sediment record and the lower limit of sample mass that can be measured with a given analytical setup. For standard total combustion isotope ratio mass spectrometry (TC/IRMS), 20 or more individual ephippia are typically measured (Morlock et al., 2016; Rinta et al., 2016; Schilder et al., 2017). The $\delta^{13}\text{C}$ analysis on invertebrate remains is at times performed on samples as small as $20\ \mu\text{g}$ (see e.g. Belle et al., 2017) and given the potentially large weight of *Daphnia* ephippia (0.5 – $5\ \mu\text{g}$ per ephippium, with very large ephippia even exceeding $5\ \mu\text{g}$; Van Hardenbroek et al., 2018) measurements could be performed on samples containing only 5 to 10 individual ephippia (or even less). However, if the variability in $\delta^{13}\text{C}$ values is high in a sediment sample, measurements on bulk samples containing a low number of ephippia may also lead to high apparent variability in down-core records, which can complicate their interpretation. Here we apply a novel analytical setup capable of analysing $\delta^{13}\text{C}$ values of individual ephippia which we use to investigate within-bulk sample variability.

Recent advances in analytical procedures (e.g. approaches based on laser-ablation or Spooling Wire Microcombustion) now allow the measurement of the isotopic composition of samples considerably smaller than those conventionally measured in isotope studies of lacustrine invertebrate remains (see e.g. Eek et al., 2007; Moran et al., 2011; Pearson et al., 2016), potentially allowing single or even multiple measurements on individual microfossils (e.g. Nelson et al., 2007; van Roij et al., 2017; Zhao et al., 2017). We use a laser-ablation based setup for measuring the $\delta^{13}\text{C}$ values of individual ephippia. The setup has been developed for $\delta^{13}\text{C}$ analysis of small organic particles, such as organic microfossils, and has been shown to produce high accuracy and precision data for an international standard, as well as for single grains of pollen and dinoflagellate cysts (Sluijs et al., 2018; van Roij et al., 2017). We analyse individual *Daphnia* ephippia from surface sediments from three Central European lakes that vary in their geographical and elevational setting (subalpine versus lowland), occurrence of anoxia in deeper water layers, and the extent to which methane enters the open water column. We aim to explore the potential of this approach to investigate the variability behind $\delta^{13}\text{C}$ values based on bulk *Daphnia* ephippia samples such as presented by Schilder et al. (2015a). For each lake we present multiple $\delta^{13}\text{C}$ measurements on the same ephippium to constrain the reproducibility of the approach and assess whether individual ephippia were homogeneous in their stable carbon isotopic composition. Furthermore, for each lake we analysed another 13 individual ephippia from the same surface sediment sample once to investigate the variability in *Daphnia* ephippia $\delta^{13}\text{C}$ values within a fossil ephippia assemblage. We expected to find larger variability in ephippia $\delta^{13}\text{C}$ values in the stratified lakes with high methane concentrations than in the well-mixed lake with low methane concentrations, since it can be expected that in the lakes with high methane concentrations *Daphnia* diet may be supplemented by strongly ^{13}C -depleted, methane-derived carbon during part of the seasonal cycle (e.g. Morlock et al., 2016; Rinta et al., 2016; Schilder et al., 2017; Taipale et al., 2007).

2. Methods

2.1. Description of sites

For this study the surface sediments of three dimictic Swiss lakes, Burgäschisee (BUR), Hinterburgsee (HIN) and Seealpsee (SEE) were investigated. HIN and SEE are mountain lakes (1516 and 1141 m above sea level (asl), respectively), whereas BUR is situated in the lowlands of the Swiss plateau (434 m asl). At the time of sampling (late summer 2011), all three lakes were thermally stratified. BUR and HIN were characterized by anoxic bottom waters, whereas the water column of SEE was fully oxygenated. Surface water methane concentrations were 1.15 (BUR), 2.80 (HIN) and 1.04 (SEE) $\mu\text{mol l}^{-1}$, and the bottom water methane concentrations were 226.63 (BUR), 13.40 (HIN) and 1.26 (SEE) $\mu\text{mol l}^{-1}$ (see Table 1 for further details on the lakes and Rinta et al. (2015) for full details on the trace gas measurements and information on the lakes).

2.2. Surface sediment ephippia

In the deepest part of the lakes, surface sediment (the top 2 cm) was retrieved using a gravity corer (UWITEC, Austria). The sediment was treated chemically (2 h 10% KOH and 20 h 2M NH_4Cl buffered in 0.35 M NaOH) to deflocculate the sediment and remove carbonates (following Verbruggen et al. (2010)), sieved (100 μm) and ephippia were isolated from the sieving residue using forceps and a binocular (20–50 \times magnification) as described by Schilder et al. (2015a). For each sample, 25 ephippia were transferred to a glass vial containing demineralized water for short-term (2 months) dark storage and transport. NaCl was added to each vial to prevent biological degradation during this period. Later, these ephippia were rinsed in demineralized water to remove the salt. From each lake, the $\delta^{13}\text{C}$ value of one ephippium was determined three to four times to assess whether a single measurement is representative of the whole specimen. From 13 further ephippia the $\delta^{13}\text{C}$ value was measured once.

2.3. Mass spectrometry

Individual ephippia $\delta^{13}\text{C}$ values were measured using the laser ablation, nano combustion gas chromatography and isotope ratio mass spectrometry (LA/nC/GC/IRMS) setup described by van Roij et al. (2017). In short, 2 to 4 ephippia were placed between two nickel discs (6 mm diameter) and pressed with a hydraulic press (3.5 ton cm^{-2}) to attach the ephippia to the nickel discs. A nickel disc with ephippia was then introduced to a small ablation chamber positioned under a 193 nm ArF laser system (COMPex 102; Lambda Physik, Göttingen, Germany). Using the laser, a small section of one ephippium (100 μm diameter hole: Fig. 1) was ablated

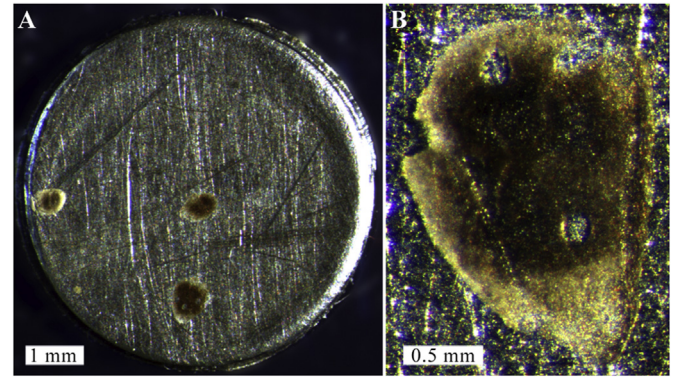


Fig. 1. Laser-ablated ephippia. (A) Photograph showing a 6 mm nickel plate with three ephippia pressed onto it. (B) An ephippium with two holes resulting from the laser ablation.

and carried to a GC combustion III interface (ThermoFinnigan, Bremen, Germany) coupled on line to a Delta V Advantage isotope ratio mass spectrometer (ThermoFinnigan) by a helium flow. A microscope equipped with a camera was used to aim the laser beam. Aside from avoiding the edges of the ephippia, no particular area of the ephippia was targeted specifically. The analytical precision (based on the results of 2–3 analyses of the laboratory standard IAEA CH-7 (polyethylene, PE) per sequence) ranged from ± 0.05 to $\pm 0.5\%$. 2 to 4 ephippium measurements were performed per sequence. A small fragment of the PE standard was placed on the same nickel disc as the ephippia and ablated at the beginning and end of each measurement sequence.

2.4. Statistical analyses

Shapiro-Wilk tests were used to test for normal distribution of the results. To compare the variance of *Daphnia* ephippia $\delta^{13}\text{C}$ values retrieved from the three lake sediment samples the Brown-Forsyth test for homogeneity of variance was applied. Hartigan's dip-test was applied to test for unimodality of the distributions of $\delta^{13}\text{C}$ values within a sediment sample. All statistics were performed using the R software package (R Core Team, 2013).

3. Results

Repeated measurements on a single ephippium from each lake show very consistent results (Fig. 2), with a standard deviation of 0.1‰ (SEE, $n = 3$) and 0.5‰ (HIN and BUR, $n = 4$ and 3, respectively). This is comparable to the standard deviation observed for the laboratory standard, implying that a *Daphnia* ephippium is as

Table 1
Location and limnological characteristics of the three study lakes.

	Seealpsee (SEE)	Hinterburgsee (HIN)	Burgäschisee (BUR)
Longitude (WGS 84; decimal)	9.4000	8.0675	7.6686
Latitude (WGS 84; decimal)	47.2689	46.7173	47.1690
Altitude (m asl)	1141	1516	434
Max depth (m)	14.5	11.4	30
Area (ha)	13.5	4.5	20.4
pH surface water	8.7	8.9	8.6
TN surface water ($\mu\text{g l}^{-1}$)	550	800	1200
TP surface water ($\mu\text{g l}^{-1}$)	10	11	14
[O ₂] surface water (mg l^{-1})	10.33	10.83	9.92
[O ₂] bottom water (mg l^{-1})	10.08	0.13	0.05
[CH ₄] surface water (μM)	1.04	2.8	1.15
[CH ₄] bottom water (μM)	1.26	13.40	226.63

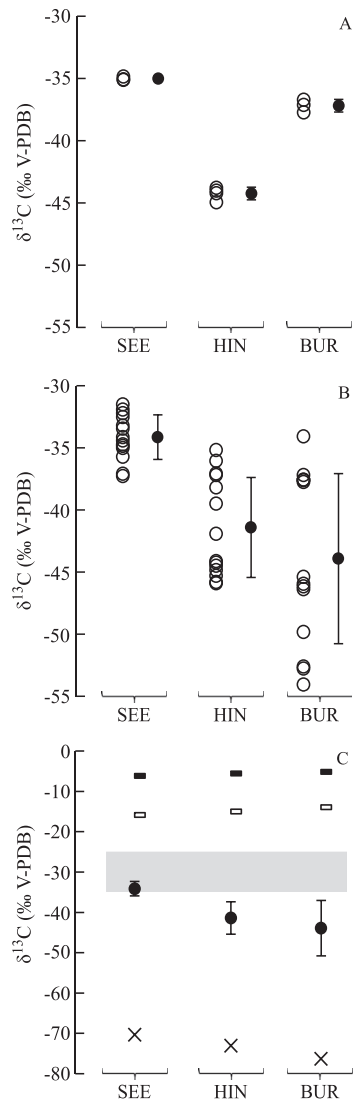


Fig. 2. Individual ephippia $\delta^{13}\text{C}$ values. A: $\delta^{13}\text{C}$ values of repeated measurement of a single ephippium from the sediments of SEE ($n = 3$), HIN ($n = 4$) and BUR ($n = 3$). Open circles represent the measurements and the closed circles represent the average, the error bars indicate the standard deviation. B: The result of measurements on 14 individual ephippia from the same sediments. Open circles indicate individual measurements and the closed circles the average value (error bars represent the standard deviation). C: $\delta^{13}\text{C}$ values of dissolved inorganic carbon (black bars), dissolved carbon dioxide (open bars) and dissolved methane (cross marks) measured in the lakes (for details see Rinta et al. (2015) and Schilder et al. (2015a)). The grey area indicates $\delta^{13}\text{C}$ values typical for algae (-35 to -25 ‰, Vuorio et al. (2006)) and the black circles with error bars indicate the average and standard deviation of the 14 individual ephippium $\delta^{13}\text{C}$ values from each lake (as in B).

homogeneous in its $\delta^{13}\text{C}$ value as the PE standard. Repeated measurements on PE and on single ephippia therefore both indicate an analytical uncertainty and repeatability of our measurements of ≤ 0.5 ‰ (expressed as 1 standard deviation).

There is a considerable amount of variation in $\delta^{13}\text{C}$ values of individual *Daphnia* ephippia within each surface sediment sample, as well as a large difference in variability between the lakes. Ephippia $\delta^{13}\text{C}$ values from SEE ranged from -37.3 to -31.5 ‰ (range 5.8 ‰, standard deviation 1.8 ‰), values from HIN ranged from -45.9 to -35.2 ‰ (range 10.8 ‰, standard deviation 4.0 ‰) and those from BUR ranged from -54.1 to -34.1 ‰ (range 20.0 ‰, standard deviation 6.8 ‰). The average values are in line with Schilder et al. (2015a) who reported much lower bulk *Daphnia*

ephippia $\delta^{13}\text{C}$ values in HIN (-45.2 and -47.4 ‰) than in SEE (-35.5 ‰) isolated from different surface sediment samples than analysed in this study. The Shapiro-Wilk tests indicate that the data from HIN are not normally distributed ($p < 0.05$), whereas those of SEE and BUR are. The Brown-Forsyth test confirms a strong dissimilarity in the variance of the $\delta^{13}\text{C}$ values between the lakes ($p < 0.0005$). Furthermore, Hartigan's dip-test suggests that the distribution of ephippia $\delta^{13}\text{C}$ values from BUR was not unimodal (i.e. at least bimodal, $p < 0.05$).

4. Discussion

4.1. Within-sample variability in $\delta^{13}\text{C}$ values

The repeated measurements on single ephippia demonstrate that the setup used in our study provides measurements with a high degree of replicability for ephippia analyses. They also show very low heterogeneity in $\delta^{13}\text{C}$ values within ephippia, which means that results from different ephippia based on single measurements may be compared meaningfully.

In SEE, the variability between individual ephippia is relatively small (standard deviation 1.8 ‰) and the $\delta^{13}\text{C}$ values (-37.3 to -31.5 ‰) typically are in the range of those reported for algae (-35 to -25 ‰ (Vuorio et al., 2006)). We consider it likely that this variability represents seasonal (or inter-annual) changes in $\delta^{13}\text{C}$ values of algae that dominated in diet of *Daphnia* in this lake. Considering the low concentrations of methane in this lake it is very unlikely that MOB formed a major part of *Daphnia* diet and this is reflected in the absence of low *Daphnia* ephippia $\delta^{13}\text{C}$ values (Fig. 2).

In HIN, the standard deviation of $\delta^{13}\text{C}$ values (4.0 ‰) is more than twice as large as in SEE and 8 out of 14 ephippia have a value of -41.9 ‰ or lower, much lower than can be expected for algae (Vuorio et al., 2006). We therefore interpret these low ephippia $\delta^{13}\text{C}$ values as indicating that these remains originate from *Daphnia* that incorporated methane-derived carbon. It appears that the variability is caused by both variation in algal $\delta^{13}\text{C}$ values and variation in the importance of MOB as a dietary component. We do note that because the top 2 cm of sediments in our studied lakes likely represents multiple seasonal cycles, the observed variability may reflect both seasonal and inter-annual variations. MOB incorporation by *Daphnia* occurs predominantly in fall and winter (Schilder et al., 2017; Taipale et al., 2007). The $\delta^{13}\text{C}$ values may hint at more than one production peak (one during fall and one during e.g. spring). However, the test for multimodality was not significant so we cannot state with confidence that timing of ephippia production was a causal factor in the variation of ephippia $\delta^{13}\text{C}$ values we observed. Additional measurements may reveal evidence for multimodality of ephippia $\delta^{13}\text{C}$ values within this sample.

The distribution of ephippia $\delta^{13}\text{C}$ values from BUR was identified as multimodal and a clear separation can be seen between very low values (-54 to -45 ‰), much lower than can be expected from algae, and values more in line with those expected from algal primary productivity (-37 to -34 ‰). This pattern of $\delta^{13}\text{C}$ values can be most easily explained by two or more seasonal production peaks of *Daphnia* ephippia during periods in the seasonal cycle when *Daphnia* either preferentially feed on algal material or on a mixed diet of phytoplankton and organisms incorporating methane-derived carbon (e.g. MOB or micro-organisms feeding on MOB). In Central European lakes such periods would include, e.g., early summer and autumn overturning, periods in which at least some *Daphnia* species are known to produce ephippia (Cáceres, 1998; Cáceres and Tessier, 2004).

4.2. Implications for palaeo-studies

The sample from BUR demonstrates that the range of seasonal and/or inter-annual variability in $\delta^{13}\text{C}$ values of ephippia-producing *Daphnia* is preserved and reflected in the fossil record. Furthermore, it demonstrates that the distinct imprint of multiple production peaks remains preserved in such samples. We interpret this variability as mainly reflecting seasonal changes in the relative importance and $\delta^{13}\text{C}$ values of food sources ingested by *Daphnia*. Because of the peak-wise production of ephippia in many lakes (Cáceres, 1998) and the observation that *Daphnia* and ephippia tend to have very low $\delta^{13}\text{C}$ values indicative of incorporation of methane-derived carbon typically during autumn and winter (Schilder et al., 2017; Taipale et al., 2007), it is likely that seasonal variation in ephippium production is the main contributor to the observed inter-ephippium variability. We regard seasonal variability especially relevant as a source for inter-ephippium variability in lakes where methane-derived carbon enters the pelagic food web. However, other sources of variation, such as species-dependent differences in feeding strategy or timing of ephippium production may also contribute to this variability. We also note that in lakes where ephippia are only produced once per year during a short period, strong seasonal variability in *Daphnia* $\delta^{13}\text{C}$ values may not be faithfully recorded by the ephippia preserved in the sediment.

Analyses of within-sample distribution patterns of *Daphnia* ephippia $\delta^{13}\text{C}$ values may provide new avenues for reconstructing past environmental and ecological changes based on geochemical analyses of *Daphnia* remains. For example, measuring the stable oxygen isotope ratios and/or Mg/Ca values of individual marine foraminifera shells (Ganssen et al., 2011; Wit et al., 2010) to reveal changes in the range of seasonal sea surface temperatures allowed to further refine reconstructions of past (average) sea surface temperatures, which normally target mean annual values only. Moreover, individual marine dinoflagellate cyst $\delta^{13}\text{C}$ analyses not only showed species-specific ecological variations but also yielded information on the ecological flexibility or fitness of populations (Sluijs et al., 2018). Similarly, down-core changes in the variability of within-sample $\delta^{13}\text{C}$ variation of ephippia and/or extreme (particularly minimum) values may provide information on changes in carbon sources for *Daphnia* ephippia in lakes. For example, is the relative abundance of ephippia containing methane-derived carbon in the sample changing, or is the amount of methane-derived carbon in these ephippia changing, thereby altering the mean $\delta^{13}\text{C}$ values of these ephippia? Measuring individual ephippia $\delta^{13}\text{C}$ values may therefore allow for more sensitive assessments as to whether methane-derived carbon contributed to the planktonic food web of lakes. In addition, such information may potentially reveal changes in the production period of *Daphnia* ephippia in the past.

Our findings also allow an assessment of the expected variability of fossil ephippia $\delta^{13}\text{C}$ values of bulk ephippia samples that originates from between-ephippium variability of $\delta^{13}\text{C}$ values. Low ephippia numbers in such bulk samples may lead to substantial down-core variability in records due to an overrepresentation of ephippia with relatively (compared to the sample's average) low or high $\delta^{13}\text{C}$ values in some samples. This is especially of concern for lake systems in which strong influences of methane-derived carbon in *Daphnia* diet may be expected (given the large variability in BUR and to a lesser degree HIN as opposed to SEE). To illustrate this, we used our 14 data points per lake (under the assumption that these properly reflect the variability within the lake's sediments, and that the ephippia all have equal mass) to randomly generate 40 bulk ephippia samples for each of 17 different sample sizes between 1 and 150 ephippia, for each lake. Fig. 3 shows the standard deviation

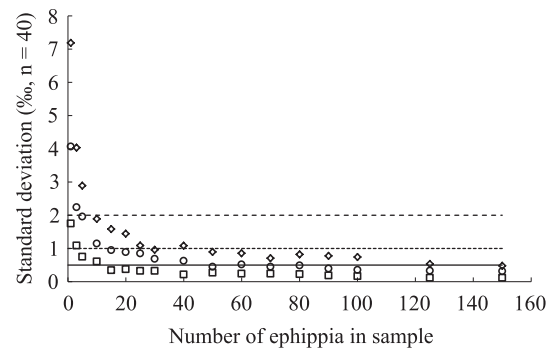


Fig. 3. Sample size and standard deviation. The standard deviation of 40 randomly generated ephippia samples (see main text for details) for various sample sizes (between 1 and 150 ephippia) based on our 14 measurements on ephippia from BUR (diamonds), HIN (circles) and SEE (squares). The lines indicate a standard deviation of 0.5, 1 and 2%.

from the average for those 40 samples for each sample size and lake. The calculations clearly show that for SEE, with relatively low between-ephippium variability, bulk samples with 20 ephippia are much more likely to return values within 1‰ of the true average than for HIN and BUR. This implies that especially in lakes with a high variability in ephippia $\delta^{13}\text{C}$ values, short (i.e. single sample), low-amplitude isotope excursions should not be over-interpreted in bulk *Daphnia* ephippia $\delta^{13}\text{C}$ records, unless they are based on a very large amount of ephippia or well-supported by corroborating evidence. Measuring one or more secondary, independent proxies can help to separate such minor variations in bulk *Daphnia* $\delta^{13}\text{C}$ values which represent true changes in aquatic food webs and past environments from those which are due to large heterogeneity of ephippia $\delta^{13}\text{C}$ values within samples. For example, earlier studies have used geochemical analyses, diatom-inferred total phosphorus (Schilder et al., 2017), $\delta^{13}\text{C}$ measurements on other invertebrate taxa (e.g. chironomids, bryozoans, *Ceriodaphnia*) or bulk sediment organic matter $\delta^{13}\text{C}$ analyses (Morlock et al., 2016; van Hardenbroek et al., 2013), or have analysed invertebrate remains from a second core from the same lake (Frossard et al., 2014) to support the interpretation of down-core invertebrate $\delta^{13}\text{C}$ records based on the analysis of bulk samples.

In sediments that contain two or more distinct groups or clusters of ephippia $\delta^{13}\text{C}$ values (such as is the case in BUR), down-core changes in bulk ephippia $\delta^{13}\text{C}$ values may reflect changes in one or two of these groups. For example, variations may be driven only by changes in fall ephippia $\delta^{13}\text{C}$ values. In such cases changes in bulk ephippia $\delta^{13}\text{C}$ values may be effectively dampened by stability of ephippia produced during other parts of the seasonal cycle, for example ephippia from spring/early summer *Daphnia* that feed predominantly on algae. Such mechanisms may need to be taken into account when interpreting down-core bulk ephippia $\delta^{13}\text{C}$ records. Further investigations of the main ephippia production peaks in our study lakes and of their stable carbon isotopic composition (e.g. with the help of sediment trap studies) would help to further constrain the effects of between-year variability in *Daphnia* ephippia $\delta^{13}\text{C}$ values and multiple production peaks on reconstructed *Daphnia* $\delta^{13}\text{C}$ values.

5. Conclusion

The data we present show that LA/nC/GC/IRMS analysis of *Daphnia* ephippia is possible and a feasible approach to investigate ephippia $\delta^{13}\text{C}$ values on individual ephippia or even investigate the variability within the same ephippium with a high degree of

repeatability. Our results imply that the within-sample variability of ephippia $\delta^{13}\text{C}$ values, related to e.g. seasonal variability in *Daphnia* $\delta^{13}\text{C}$ values and timing of ephippia production peaks, can have an impact on a fossil bulk ephippia $\delta^{13}\text{C}$ record. This has some implications for preparing samples for down-core records based on bulk ephippia $\delta^{13}\text{C}$ analysis and interpreting the results, but also opens up new avenues for future research.

Our results indicate that bulk samples with low $\delta^{13}\text{C}$ values may represent averages of ephippium assemblages with a high between-ephippium variability in $\delta^{13}\text{C}$ values. Bulk ephippia samples therefore ideally consist of sufficiently high numbers of ephippia to increase the likelihood of properly reflecting the true average $\delta^{13}\text{C}$ value of the deposited ephippia during a certain period of time. The optimal amount of ephippia depends strongly on the (expected) variability within the sample and the desired accuracy of the reconstruction (Fig. 3). Especially when dealing with low amounts of ephippia, the use of secondary, independent proxies can produce necessary constraints to interpretations of down-core records. Measuring individual ephippia for certain parts of a record, for example for sections with relatively high or low rates of incorporation of methane-derived carbon, can also provide an avenue for constraining and improving interpretations. Moreover, this approach can potentially also be used to investigate how the range of ephippia $\delta^{13}\text{C}$ values produced during different seasons and/or by different *Daphnia* populations changed over time.

Finally, our results show that $\delta^{13}\text{C}$ analyses of individual chitinous microfossils in lake sediments are possible and produce reproducible results. This opens the opportunity for applying this method to chitinous fossils of other invertebrate groups such as chironomid head capsules, cladoceran (e.g. bosminid or chydorid) head shields and carapaces or bryozoan statoblasts. Possible research questions which could be explored include, e.g. the within assemblage variability of $\delta^{13}\text{C}$ values for individual taxa and fossil structures, but also inter taxon variability for fossil groups that produce too small amounts of fossil chitinous material to measure with conventional bulk measurement techniques.

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