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NOTE

Bias of ostracod stable isotope data caused by drying of sieve residues from water

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Abstract Stable isotope analysis of ostracod shells is used routinely for palaeoenvironmental studies of ostracod-bearing records. Sample treatment usually involves the disaggregation of sediments and sieving; before the sieving residues were washed with water onto petri dishes and oven-dried. In our study, we compared $\delta^{18}O$ and $\delta^{13}C$ values of shells that were oven-dried from water and from ethanol alternatively. Large isotopic differences of up to 3‰ were determined for $\delta^{18}O$ values, whereas differences in $\delta^{13}C$ values were less pronounced with differences of up to 1.6‰. Stable isotope values of shells dried from water were lower for both oxygen and carbon as a result of calcite crystals precipitated on the shell surfaces during the drying process. Therefore, ostracod shells for stable isotope analysis should not be prepared by drying from water. Instead, shells should be dried from ethanol to obtain reliable stable isotope data; likewise freeze-drying is expected to provide trustworthy results.

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Introduction

Stable isotope analysis is a standard technique in palaeoenvironmental and palaeoclimatic studies. $\delta^{18}O$ and $\delta^{13}C$ data provide important information with respect to the hydrological, biological and climatic conditions of non-marine systems (see reviews in Ito 2003; Leng and Marshall 2004). Preferentially, $\delta^{18}O$ and $\delta^{13}C$ analyses are performed on a single carbonate species to avoid signals from an unknown mixture of different carbonate types that were formed at different places and at different times in the water column and on or within the sediment. Bulk carbonate may include biogenically produced ostracod, gastropod and bivalve shells, charophyte gyrogonites and encrustations precipitated on charophyte stems, phytoplankton-induced microscopic carbonate grains, inorganically precipitated carbonate and detrital carbonate. Ostracod shells are frequently used for stable isotope analysis because the shells are usually abundant, they can be easily separated from the sediment, and information on the timing and place of shell formation is often available (DeDeckker and Forester 1988; Griffiths and Holmes 2000; Schwalb 2002; Ito et al. 2003). The cleaning of shells is crucial before stable isotope analysis can be applied to ostracod shells, and concerns about the

effects of different cleaning methods have been expressed (Holmes 1996; Keatings et al. 1999). However, studies by Holmes (1992), Sperling (2002), Barker et al. (2003) and Jin et al. (2006) focussed on the effects of cleaning methods on trace-element analysis of ostracod shells or on the effects of cleaning methods on geochemical analysis of calcareous remains of other marine organisms such as foraminifers. Most of these studies had the aim to explore the geochemical effects of different reagents used to disaggregate sediment samples and to remove adhering particles from ostracod shells. However, following the application of reagents such as sodium hypochlorite (NaOCl) or hydrogen peroxide (H_2O_2), sediment samples were usually washed through sieves with tap or deionized water and air dried (e.g. Bridgwater et al. 1999; Hammarlund et al. 1999; Holmes et al. 1997, 1999; Schwalb et al. 1999; Keatings et al. 2002; Mischke et al. 2005; Bahr et al. 2006; Mischke and Wünnemann 2006; Ortiz et al. 2006). Freeze drying of sieve residues is usually only applied if samples are rich in organic matter (Schwalb et al. 1999). The sample sieving and residue-drying procedure may be altered as by Frogley et al. (2001) who disaggregated the samples by immersion in water, used additional freezing and thawing cycles where necessary, dried the samples first and sieved the material afterwards. In general, sieving with water (tap or deionized) is followed by air-drying of residues at room temperature or by oven-drying at rather low temperatures around 40–50°C. Only a few examples exist where sieved residues were washed with ethanol in a final step following the concentration of ostracods in the sediment samples through sieving (Von Grafenstein et al. 1994; Schwalb et al. 1995; Xia et al. 1997a; Curtis et al. 1998; Rosenmeier et al. 2002, Anadón et al. 2006).

The aim of our study is to assess the possible bias of stable isotope data resulting from drying of sieving residues which where washed onto a petri dish by water in comparison to samples which were washed to dishes by ethanol.

Materials and methods

Ostracods for our study were obtained from a short core drilled in Gahai Lake, China (37.04° N, 100.58° E, 3205 m above sea level), which was known to host very abundant ostracods of the single species *Eucypris mareotica* only (unpublished data Sun Zhencheng). This species has a smooth shell surface lacking any ornamentation and therefore was regarded as appropriate for the cleanness assessment by image analysis discussed below. The core of 60 cm length was recovered at 7 m water depth in September 2005 using a UWITEC short coring device. Gahai Lake is a surficially closed-basin lake at the northeastern shore of the largest Chinese lake, Qinghai Lake (Fig. 1). The electrical conductivity measured in the lake immediately before the core had been obtained was 41.3 mS/cm, equal to a salinity of about 30 g l^{-1} applying a conversion factor of 0.725 (Hölting 1992). The pH of the lake water was 9.23. Shells of *Eucypris mareotica* (better known by the younger synonym *E. inflata*) were separated from 1 cm sediment slices of the core at 4 cm intervals.

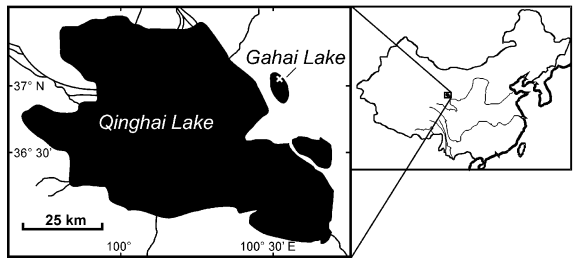

Fig. 1 Origin of studied monospecific ostracod material from Gahai Lake near the largest Chinese lake, Qinghai Lake. Small image shows the general location

Two parallel sets of ostracod stable isotope samples from the 16 sampled horizons were prepared through the following procedure. For the first set, about 7 g of fresh sediment was dispersed in tap water, sieved with a 500 µm mesh and washed to a petri dish with tap water before drying at 50°C. The sieving residues typically dried from about 25 ml of water within less than 30 h. For the second set of stable isotope samples, about 4 g of sediment was dispersed in tap water, sieved with a 500 µm mesh and washed with ethanol to a petri dish for subsequent drying at 50°C. Sieving residues dried from typically 12 ml of ethanol within less than 4 h.

Parallel to each stable isotope sample prepared in the described way, one adult *Eucypris mareotica* shell visually assessed as typical for the 15 shells selected for stable isotope analysis (see below) was

chosen for accompanying image analysis using a scanning electron microscope (SEM). SEM images were prepared from the 32 ostracod shells, each representing a stable isotope sample using similar contrast, brightness and magnification settings of the SEM.

The 'cleanness' of the water-dried shells selected for SEM analysis was visually assessed by sorting the shells into a succession starting with the most clean shell. The obtained ranking numbers were normalised to values between 0 for the most clean shell and 1 for the shell with the most contaminants on the surface.

Precipitated crystals or adhering particles on the surface of the shells used for SEM analysis were additionally quantified using image analysis techniques. The evaluation approach is based on the distinct smoothness of the shells of *Eucypris mareotica* and the idea that clean shells appear with large smooth areas in the imagery; contamination of the shells corresponds to a certain roughness. The smoothness of an image segment can be described with parameters well known in classical image processing, e.g. standard deviation of grey values in a local pixel surrounding. A number of methods was implemented and validated for cleanness investigations.

A first group of cleanness parameters for shells dried from water was retrieved by applying different filters. Low-pass filters smooth images by calculating a local weighted mean and by substitution of the current grey value by that mean following this equation:

$$g'(i,j) = \sum_{m=-I}^{I} \sum_{n=-J}^{J} w(m,n) g(i+m, j+n) \quad (1)$$

where $g'(i, j)$ is the new grey value at image row i and image column j, $w(i, j)$ is a pixel-specific weight factor with

$$\sum_{m=-I}^{I} \sum_{n=-J}^{J} w(m,n) = 1 \quad (2)$$

and $g'(i + m, j + n)$ the old grey value in a certain surrounding defined by (I, J).

The size of the area taking into account this calculation (window) is one essential parameter for filtering. Small filter windows blur or remove small objects; large filters do the same for large objects. Differences of images filtered with two different window sizes mark objects of a certain size. These remaining objects have a smaller or greater grey value than their surrounding. By setting thresholds the images can be transformed into a binary data set. The number of conspicuous pixels can be counted and gives a measure for the number of objects with a certain size and, consequently, a measure for cleanness.

A similar approach can be used by applying median filters as a core algorithm. Median filters are rank filters which are able to suppress noise in a very effective way. Applying two median filters with different window sizes and subtracting the resulting images give an image with remaining structures of a certain size again. The number of conspicuous pixels can be counted and gives a measure for the number of objects with a certain size.

The last parameter implemented is based on local stochastic measurements and is called information entropy. This value is a measurement of the information content of an image. The frequency of grey values in a local surrounding is counted. High entropy values correspond to rough image regions (high contamination); low entropy values are correlated with smooth image regions (high cleanness). The following equation describes the calculation of this parameter:

$$e = -\sum_{m=0}^{255} p(g_m)^* \log p(g_m) \quad (3)$$

e is the entropy, m is grey value, and $p(g_m)$ is the probability that the value g_m will occur in a certain surrounding of the current pixel.

The obtained three parameters were normalised to values between 0 for the smoothest (most clean) shell and 1 for the most rough shell (with the most contaminants on the surface). The mean of the normalised parameters was used as the cleanness measure derived from image analysis.

Some bias of the quantification of crystals or particles on the shell surface by image analysis may arise from the varying number of preserved setae on the shell surface, although its influence is regarded as clearly subordinate in comparison to those of the undesirable contaminant precipitates on shell surfaces.

For stable isotope analysis, 15 adult shells of *Eucypris mareotica* were randomly picked from the dried sieving residues. The stable isotope analysis

followed the standard procedure of the Leibniz Laboratory for Stable Isotope Research, Kiel. The results are reported as per mil deviation with respect to the international standard V-PDB, with a reproducibility of less than 0.05‰.

The statistical significance of the difference in the stable isotope data for water-dried and ethanol-dried ostracod shells was tested using paired t tests for the stable isotope data with sample-depth related pairs of stable isotope values for water- and ethanol-dried shells. Individual tests were conducted for the $\delta^{18}O$ and $\delta^{13}C$ values of water- and ethanol-dried shells, respectively.

The mineralogy of the cored sediments and the precipitated crystals on ostracod shells was assessed through X-ray diffraction analysis of a bulk sediment sample from 10 cm core depth and of 100 ostracod shells from the core level, which was identified to provide the most heavily overgrown ostracod shells (i.e. at 9 cm core depth, see results and discussion sections and Fig. 5, shell numbers 7 and 8) using a Philips PW 1050 X-ray spectrometer. The powdered samples were scanned from 10° to 60° using Cu-Kα radiation.

Results

The stable oxygen isotope values of shells dried from water ($\delta^{18}O_w$) range from –0.2 to 1.6‰, whereas shells dried from ethanol ($\delta^{18}O_e$) display values between 1.1 and 2.9‰ (Fig. 2). The $\delta^{18}O$ values of shells dried from water are lower than those of shells dried from ethanol (Fig. 2). The differences between the stable oxygen data for shells from the same sampled level dried in ethanol and in water ($\Delta\delta^{18}O_{e-w}$) range from about 0 to a maximum of 3‰ with an average of about 1‰ (Fig. 3).

The stable carbon isotope values of shells dried from water ($\delta^{13}C_w$) range from –1.0 to 1.2‰, whereas the corresponding values for ethanol-dried shells ($\delta^{13}C_e$) from the same sampled horizons are higher with the exception of one sample (Fig. 2). The $\delta^{13}C_e$ values range from 0.3 to 1.3‰. The differences between $\delta^{13}C_e$ and $\delta^{13}C_w$ values for the same sampled level ($\Delta\delta^{13}C_{e-w}$) range from about –0.3 to 1.6‰ (Fig. 3). The average $\delta^{13}C$ deviation arising from the different ways of sample treatment is about 0.5‰.

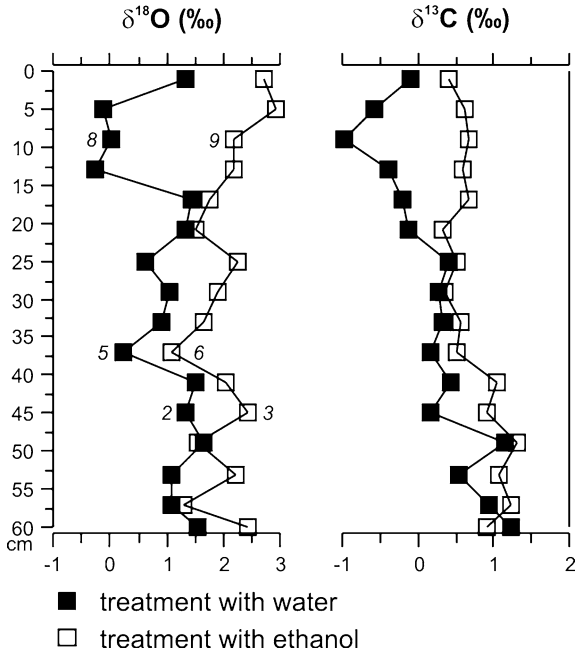

Fig. 2 $\delta^{18}O$ and $\delta^{13}C$ data of two parallel sets of ostracod shell samples dried from water or ethanol after sieving. Numbers in italics refer to Fig. 5, see below

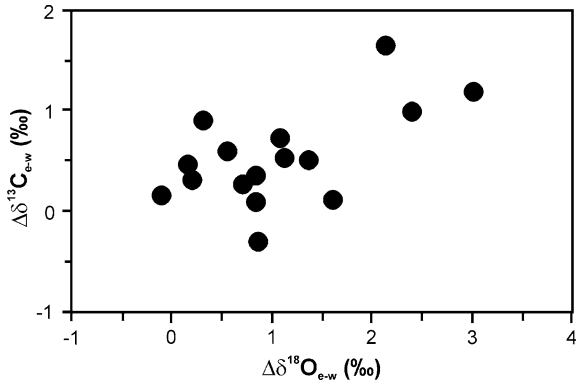

Fig. 3 Crossplots showing the differences between $\delta^{18}O$ and $\delta^{13}C$ data for samples from the same stratigraphic levels dried from ethanol and from water

Image analysis of water-dried shells which were visually selected as representative of the shells used for stable isotope analysis revealed highest values (heavily contaminated shells) in the upper and middle part of the core (Fig. 4). Lowest values, i.e. most clean shells, were identified from the basal part. The visual ordering of the shell cleanness resulted in a similar pattern with highest values (heavily contaminated shells) near the core top and lowest values in the basal part (Fig. 4).

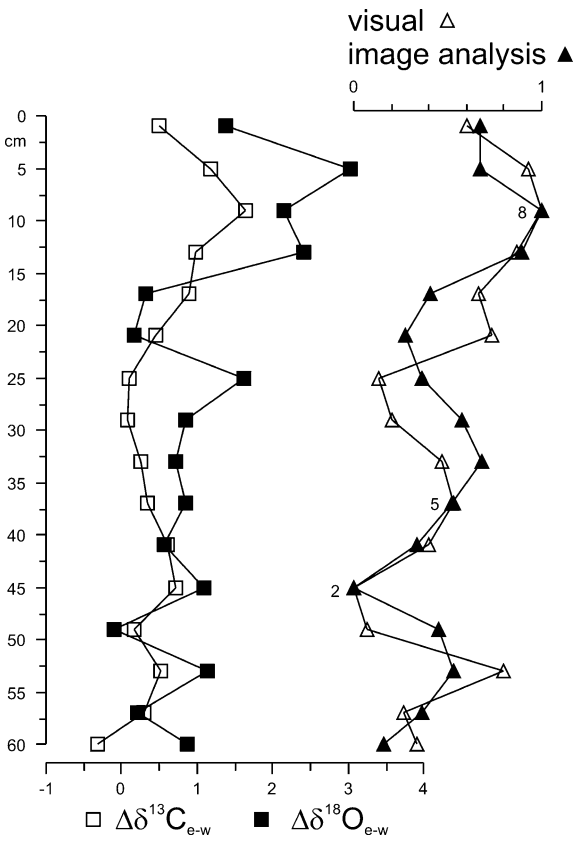

Fig. 4 Stratigraphic presentation of differences between $\delta^{18}O$ and $\delta^{13}C$ data for samples from the same stratigraphic levels dried from ethanol and from water on the left. Cleanness assessment resulting from image analysis of shells and from visual inspection on the right. Cleanness assessment values were normalised to values between 0 and 1. Numbers in italics refer to Fig. 5, see below

Correlation coefficients for the cleanness assessment values revealed by image analysis (IA) and the stable isotope deviation for ethanol- and water-dried shells between the cleanness assessment values revealed by IA and visual inspection and between the $\delta^{18}O$ and $\delta^{13}C$ deviation for ethanol- and water-dried shells are given in Table 1.

Calcite was observed as the only mineral species by X-ray diffraction analysis of shells heavily overgrown with crystals. The analysis of a bulk sediment sample revealed quartz, calcite, halite and gypsum as the major constituents of the sediment, plagioclase feldspar was detected in smaller amounts.

Table 1 Pearson's correlation coefficient (r) for the cleanness assessment of water-dried shells by image analysis (IA) and the $\delta^{18}O$ difference between ethanol and water-dried shells, for the cleanness assessment by IA and the $\delta^{13}C$ difference between ethanol and water-dried shells, for the cleanness assessment by IA and by visual inspection, and for the $\delta^{13}C$ and $\delta^{18}O$ differences between ethanol and water-dried shells

Correlation between	r
IA and $\Delta\delta^{18}O_{e-w}$	0.57
IA and $\Delta\delta^{13}C_{e-w}$	0.56
IA and visual cleanness assessment	0.67
$\Delta\delta^{13}C_{e-w}$ and $\Delta\delta^{18}O_{e-w}$	0.58

Discussion

Crystals growing on the surface of ostracod shells consist of calcite, as shown by X-ray diffraction analysis. One hundred ostracod shells with a similar appearance as shells 7 and 8 in Fig. 5 were used for X-ray diffraction analysis, and we assume that the volume of the crystals covering these shells is well above the detection limit. Although we investigated material from one lake only, the overgrowth of ostracod shells with calcite crystals during the drying of sieving residues from water is regarded as a general problem, since calcium carbonate is present in various sedimentary samples from nonmarine subaquatic environments either as biogenic or biogenically induced carbonate in the form of ostracod shells, gastropod and bivalve carbonate, charophyte gyrogonites or stem encrustations, or as chemically precipitated carbonate or detrital material. Therefore, the possible influencing of the original stable isotope signal is a widespread phenomenon in palaeolimnological analysis if samples were dried from water after sieving.

According to SEM analysis of shells dried from water and ethanol, inorganic crystals grew on the surface of water-dried shells to a maximum size of 30 μm (Fig. 5), big enough to be easily recognized even under a low-power stereo microscope. More often, crystals reached a size of about 10–15 μm and are more difficult to detect then. In contrast, ostracod shells dried from ethanol display a relatively smooth and clean surface (Fig. 5).

The cleanness assessment by image analysis and by visual inspection revealed relatively similar results indicated by the correlation between the obtained

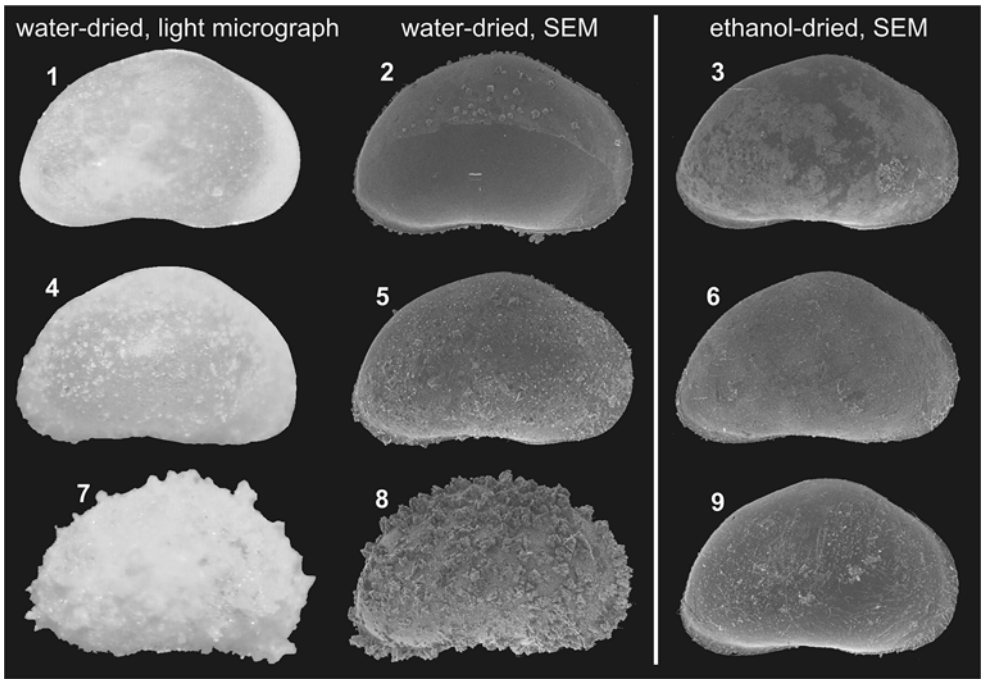

Fig. 5 Three water-dried shells in the middle column which were selected by image analysis (IA) as representing the most clean shell (2, having a normalised cleanness assessment value by IA of 0), the shell with a medium contamination (5, normalised cleanness assessment value by IA of about 0.5), and the shell with the heaviest contamination (8, normalised cleanness assessment value by IA = 1). Ethanol-dried shells from the corresponding levels are shown on the right for comparison. The three samples with the lowest, medium and largest contamination revealed by IA are marked with the corresponding image numbers in italics in Figs. 2 and 4 too. Shells in the left column represent light micrographs to illustrate the appearance of the calcite crystals precipitated on the surface of the shells under a low power binocular stereomicroscope. Shells 1 and 2, 4 and 5, and 7 and 8 are not identical, but their contamination by calcite crystals is regarded as comparable. (All images show right shells of *Eucypris mareotica*. All shells about 1 mm in length)

data of both analyses (Fig. 5, Table 1). However, the cleanness assessment by image analysis is regarded as the less subjective method and its results will be discussed together with the stable isotope data below.

The $\delta^{18}O$ values of ethanol-dried shells are generally higher in comparison to those of water-dried shells with an observed deviation of up to 3‰. The observed difference between $\delta^{18}O_e$ and $\delta^{18}O_w$ is statistically significant as revealed by a paired t test. The deviation is attributed to the microscopic crystals or adhering particles on the surface of water-treated shells. The correlation coefficient for the cleanness assessment by image analysis and the deviation of the stable oxygen isotope data ($\Delta\delta^{18}O_{e-w}$) is relatively low (0.57) and shows that a reliable correction of already existing $\delta^{18}O_w$ data based on a later cleanness assessment of reference material by IA cannot be established. Furthermore, in consideration of the low correlation between IA-derived cleanness parameters and the observed $\delta^{18}O$ deviation, it is not sufficient to check ostracod shells dried in water visually or by IA before shells were selected for stable isotope analysis. Even shells with a relatively clean appearance may yield stable isotope values which include a large bias from microscopic contaminations. Thus, the common visual inspection of shells prior to stable isotope analysis might not be appropriate to avoid the observed bias in stable isotope data resulting from water-drying of sieve residues.

The $\delta^{13}C$ values of ethanol-dried shells are higher than those of the water-dried shells, although the deviation is less pronounced in comparison to the $\delta^{18}O$ data. However, the bias of the $\delta^{13}C_w$ data from the $\delta^{13}C_e$ data is statistically significant according to a paired t test; the bias is still 1.6‰ at maximum or 0.5‰ on average and cannot be ignored during most stable isotope applications. The cleanness assessment by IA does not mirror the observed $\Delta\delta^{13}C_{e-w}$ data convincingly according to the relatively low correlation coefficient of 0.56 (Table 1). In general, a large

deviation of stable oxygen isotope data for ethanol- and water-dried shells corresponds to a large deviation of stable carbon isotope data ($r = 0.58$, Table 1).

The use of tap water or deionized water for washing the sieving residues to petri dishes before drying is probably not advisable because tap water may be a source of additional carbonate, whereas deionized water is very mildly acidic and may cause carbonate dissolution and later precipitation during the drying of the sieving residues.

Some of the observed deviation of stable isotope data between shells dried in ethanol and in water could be possibly caused by the stable isotope variability in ostracod samples from sediment slices of 1 cm thickness. However, the relatively large number of shells per sample used for stable isotope measurements (15) will have reduced the risk that the stable isotope data reflect the stable isotope variability within the analysed shells. The general oxygen isotope deviation of the water-derived samples towards lower values points to a bias caused by the precipitation of inorganic microscopic crystals on the surfaces of water-dried shells. Stable isotope studies on ostracod shells and inorganic carbonate precipitated in isotopic equilibrium with host waters under laboratory and natural conditions have shown that $\delta^{18}O$ values of ostracod shells are about 1 to 3‰ higher than $\delta^{18}O$ values of inorganic carbonate depending on the ostracod species (Xia et al. 1997b, Von Grafenstein et al. 1999). The species-specific deviation of $\delta^{13}C$ values of ostracod shells from the $\delta^{13}C$ values of dissolved inorganic carbon is generally not as large as for $\delta^{18}O$. The $\delta^{13}C$ values of ostracod shells are for some species even lower than the corresponding $\delta^{13}C$ values for the dissolved inorganic carbon (Von Grafenstein et al. 1999). The crystals on the surface of the ostracod shells may be formed from various sources: different inorganic carbonate species present in the sediments (or provided with the tap water if this is used), thin-shelled early instar ostracod shells which are probably easily affected by partial or complete dissolution, or shells of thin-shelled ostracod species and other biogenic carbonate if appropriate environments different from the studied lake sediments are considered. The portion to which each possible carbonate species is contributing to the later formation of the crystals on the surface of the ostracod shells is uncertain, and even if the stable isotope composition of the different potential carbonate sources is known, a prediction of the resulting stable isotope deviation of the overgrowth crystals will not be possible at the present state of our understanding. Based on the results of Xia et al. (1997b) and Von Grafenstein et al. (1999), a bias towards distinctly lower $\delta^{18}O$ values points to the contribution of inorganic carbonate to the formation of the covering crystals.

In general, a similar bias of stable isotope values as a result of oven-drying of sediment samples has been observed for foraminifera by Sperling et al. (2002). They compared stable isotope data of foraminifera tests obtained from sediment samples which were oven-dried before sieving with foraminifera tests which were obtained from sieving residues without drying of the sediment samples prior to sieving. The drying procedure of the sieve residues is not specified in their work. However, Sperling et al. were able to show that oven-drying of the sediment samples could cause partial dissolution of the foraminifera tests and precipitation of inorganic calcite crystals very similar to our observations on ostracod shells. $\delta^{18}O$ and $\delta^{13}C$ values of foraminifera tests from oven-dried sediment samples were generally lower than the values obtained for pristine unaltered tests. $\delta^{18}O$ values were up to 2.8‰ lower, whereas the difference between $\delta^{13}C$ values was less pronounced and rarely exceeded 0.5‰. Again, these observations are well comparable to the bias observed in the course of our study on water- and ethanol-dried ostracod shells. Sperling et al. (2002) argue that the oxidation of pyrite in organic-rich sediment samples led to the formation of H_2SO_4, a resulting decrease in pH and the dissolution of carbonate. The direction of the observed stable isotope deviation as a result of oven-drying is not discussed in Sperling et al. (2002).

It remains uncertain whether the characteristics of our core sediment samples with respect to the mineral composition, organic content, grain size, ostracod calcite to inorganic carbonate ratio and further physical and chemical properties are extraordinarily favouring the heavy overgrowth of ostracod shells during the drying of sieve residues in water. Similarly overgrown shells have not been observed in earlier stable isotope studies by the authors thus far (Mischke et al. 2005, 2006). However, the facts (1) that the precipitation of crystals on ostracod shells during sample drying from water may significantly

alter the isotopic signature of the ostracod shells and (2) that the magnitude of the stable isotope deviation is not easily assessed by a visual inspection of shells prior to stable isotope analysis show that water-drying of sieve residues should be generally avoided. Further studies are required that focus on the conditions favouring the precipitation of carbonate crystals on ostracod shells during the drying procedure.

Conclusions

Ostracod shells dried from water after sieving showed a relatively large deviation both in $\delta^{18}O$ and $\delta^{13}C$ in comparison to shells dried from ethanol in our comparative study. Stable isotope values obtained on water-dried shells were lower by 1‰ for oxygen and 0.5‰ for carbon on average. The observed isotopic depletion of the water-dried shells in comparison to ethanol-dried shells is attributed to the precipitation of inorganic calcite on the ostracod shell surfaces revealed by X-ray diffraction and scanning electron microscope analyses. The degree of contamination of the shells assessed by visual inspection and image analysis is not strongly correlated to the observed bias, showing that the selection of shells with a clean appearance for stable isotope analysis is not an appropriate measure to obviate the stable isotope bias of water-dried ostracod shells. Freeze-drying is probably an alternative procedure to ethanol-drying of sieving residues to obtain reliable stable isotope data from ostracod shells.

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