

**Supplementary material for**

Trophic state changes can affect the importance of methane-derived carbon in aquatic food webs

**by**

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## **METHODS**

### **Sampling of organisms currently living in Lake De Waay**

Living *Daphnia* were collected by a plankton net haul from the center of the lake to the shore at ~0.5 m depth. The contents were sieved at 300  $\mu\text{m}$  and rinsed with deionized water, after which 50 *Daphnia* were placed in pre-weighed tin cups (Elemental Microanalysis 6x4 mm, D1006) for isotope analysis. *Daphnia* ephippia were collected from the water surface with a hand net on the leeward side of the lake. Samples were rinsed with deionized water and 50 ephippia were cleaned of soft organic matter (if needed) and placed in pre-weighed tin cups. Living chironomids were collected from the littoral zone with a kick net and by collecting pieces of wood and rope from the lake bottom at a water depth of 1 to 2 m. These chironomids were placed in tap water in petri dishes to allow for gut clearance during ~24 h before rinsing with deionized water and then frozen. Later, the head capsules were separated and mounted on microscope slides for identification [1]. The body tissue was freeze dried and placed in tin cups for isotope analysis. 5 l of surface water was sampled on each occasion and sieved (250  $\mu\text{m}$ ), after which half of the water was filtered on a glass microfiber filter (GF/C, Whatman, UK). The other half was sieved again (60  $\mu\text{m}$ ) and likewise filtered. Filters were freeze dried and loaded into tin cups for  $\delta^{13}\text{C}$  analysis of POM (both size fractions).

### **Dissolved gases**

Sampling procedures for dissolved gasses in lake De Waay (as well as some of the results of concentrations and isotopic signatures of DIC and  $\text{CH}_4$ ) are presented in detail in [2] and are only briefly summarized here.  $\text{CH}_4$  for  $\delta^{13}\text{C}$  analysis was collected from anoxic sediments (at 10 cm sediment depth) retrieved at 2.5, 8 and 14.5 m water depth. In advance, 45 ml of lake surface water was equilibrated with 15 ml air in a 60 ml syringe by shaking for one minute. This equilibration was performed three times, each time with a new sample of 15 ml air. After coring, anoxic sediment from 10 cm below the sediment-water interface was transferred rapidly from the corer into an air-tight bottle (Duran, Germany) equipped with a stopcock. Next, the 45 ml prepared lake water was added and gases in the sediment sample were equilibrated with the headspace by shaking the bottle. Finally, a 45 ml headspace sample was taken and stored in a glass vial with a 10 mm thick massive butyl rubber stopper (Apodan, Denmark) filled with a saturated brine solution, using a second needle to allow some of the brine to escape.  $\text{CH}_4$  dissolved in lake water was collected at 0.5, 8 and 13 m water depth by injecting 60 ml of lake water sampled into a glass vial with a 10 mm thick massive butyl rubber stopper (Apodan, Denmark). These vials were prepared with 0.2 ml phosphoric acid to convert DIC to  $\text{CO}_2$ , and were filled with  $\text{N}_2$  gas (Carbagas, Switzerland). The vials were stored in the dark at room temperature and later the headspace was sampled and the  $\delta^{13}\text{C}$  of  $\text{CH}_4$  was measured at the Division of Climate and Environmental Physics at the Physics Institute of Bern University as described in [2].

### **Diatom-inferred total phosphorus concentrations (DI-TP)**

For diatom analysis, 17 sediment samples were treated with  $\text{H}_2\text{O}_2$  (30 %) and a couple of drops of  $\text{HNO}_3$  (30 %) to remove organic matter and then washed several times with distilled water. The suspensions were transferred to cover slips using the sedimentation tray method [3]. Slides were mounted using Naphrax<sup>®</sup> and diatoms were analyzed at a magnification of x1000 on a microscope with interference contrast optics. On each slide a minimum of 300 diatom valves was identified following [4–7]. DI-TP values were reconstructed using a transfer function developed from combined calibration datasets from Mecklenburg-Vorpommern (North-eastern Germany; [8]) and from the European diatom data base (EDDI) that consist of 429 samples and cover a range of 2 – 1189  $\mu\text{g TP l}^{-1}$  as described in [9,10]. The DI-TP reconstruction is based on weighted averaging partial least squares (WA-PLS) regression and calibration [11] of square root-transformed diatom percentage data and log-transformed TP and was calculated using

the software package C2 (version 1.5; [12]). The model features a root mean square error of prediction of 0.28 log TP units and an  $r^2$  of 0.73 as evaluated by bootstrapping cross-validation, and has been used extensively for reconstructing TP in Dutch lakes (see [9,10] for details). An earlier DI-TP reconstruction at coarser temporal resolution based on this approach was published for Lake De Waay by [10]. Errors plotted in Figure 3 in the main article represent the bootstrapped sample-specific estimated standard error of prediction.

## Microbiology

### *DNA extraction from surface sediments*

DNA was extracted using a modification of the method described in [13] based on the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA). Freeze dried sediment (0.1-0.2 g) and 780  $\mu$ l lysis buffer (200 mM NaPO<sub>4</sub> pH 7.0; 1% CTAB; 1.5 M NaCl; 2% Polyvinylpyrrolidone K30; 5 mg ml<sup>-1</sup> lysozyme (added right before use)) was added into a multimix FastPrep tube and incubated at 37°C for 30 min. MT buffer (122  $\mu$ l) was added and tubes were shaken in the FastPrep instrument (MP, Biomedicals, LLC, Solon, OH, USA) for 30 s at 5.5 m s<sup>-1</sup>. Subsequently, samples were centrifuged for 15 min at 10000 rpm and 700  $\mu$ l supernatant were collected. The pellet was re-extracted by adding lysis buffer (500  $\mu$ l) and 50  $\mu$ l MT buffer to the FastPrep tubes, shaken in the FastPrep instrument for 30 seconds at 5.5 m s<sup>-1</sup> again followed by the transfer of the second 700  $\mu$ l of supernatant into separate Eppendorf tubes. At this step, 2  $\times$  700  $\mu$ l supernatant was obtained from each sample. 5  $\mu$ l of 10 mg ml<sup>-1</sup> freshly made proteinase K was added to each tube. Tubes were incubated at 65°C for 30 min. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by a chloroform-isoamyl alcohol (24:1) extraction. 125  $\mu$ l of 7.5 M potassium acetate was added, samples were incubated on ice for 5 min and then centrifuged at 10000 rpm for 10 min. Supernatants (2  $\times$  700  $\mu$ l per sediment sample) were transferred to new tubes, 700  $\mu$ l Binding Matrix was added and tubes were mixed for 5 min on a rotator. Binding Matrix, with bound DNA, was pelleted by 1 min centrifugation at 10000 rpm. The supernatant was discarded and the pellet was resuspended in 500  $\mu$ l wash buffer. The resulting suspension was added into a Spinfilter, and centrifuged for 1 min at 10000 rpm. The eluate was discarded and the pellet was washed again in 500  $\mu$ l wash buffer. After discarding the second eluate, the Spinfilter was centrifuged for another 10 s to dry the pellet. The filter was taken into a new tube and 50  $\mu$ l of TE pH 8.0 was added. The filter was incubated at room temperature for 1 min and centrifuged for 1 min. The filter was re-eluted in the same way with 50  $\mu$ l of TE pH 8.0.

### *Quantitative PCR (qPCR)*

#### *pmoA*

Three methanotrophic sub-groups (Ia, Ib and II) were quantified by *pmoA*-(particulate methanemmonoxigenase) based quantitative PCR based on the assays described by [14]. The type Ia and II assays were carried out as described by [15]. For the type Ib assay, DNA standards were prepared by dilution of a known amount of PCR product amplified from a reference clone by using the 189-Mc468 primer set [14]. PCR reactions of 25  $\mu$ l in volume, contained 12.5  $\mu$ l 2  $\times$  SYBR green mix (AB gene, Epsom, UK), 2.5  $\mu$ l of diluted DNA template and 0.8 mM each of primers. The samples were diluted accurately to 1 ng /  $\mu$ l. The thermal cycle started with an initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 20 sec, annealing at 64 °C for 20 sec, and extension at 72 °C for 45 sec. Fluorescence was recorded at 84 °C and DNA melting curve analysis was performed at temperatures ranging from 70 °C to 99 °C. All of these three assays were performed with a Rotor Gene 6000 thermal cycling system (Corbett Research, Eight Mile Plains, Qld, Australia), where samples were added to aliquots of the master mixture using a CAS-1200 (Corbett Robotics Eight Mile Plains, Qld, Australia) liquid handling system. Every sample was performed in duplicate. Quantification analysis was performed by the

RotorGene software. Cell numbers were calculated assuming a PCR product length of 412 bp, 279 bp and 423 bp for type Ia, type Ib and type II methanotrophs, respectively.

### mcrA

The number of methanogens were assessed by qPCR of the gene coding for methyl coenzyme M reductase (mcrA) using the primers mcrA forward and mcrArev as described by [16]. Assay conditions and reaction volumes were as described for pmoA except for an annealing temperature of 64 °C for mcrA. Calibration curves were constructed using a clone generated from genomic DNA from *Methanosaeta concilli*.

### **Mass spectrometry (solids)**

Isotope analyses of chitinous material for both the field survey and the down-core analyses were carried out at the Utrecht University Geochemistry Laboratory, on a Euro Vector Elemental Analyzer coupled to a Thermo Electron Delta V Advantage Isotope Ratio-Mass Spectrometer (IRMS). Samples were calibrated against an internal standard of known relation to international standards (see [17] for details). The analytical error based on replicate standard measurements was  $\pm 0.05$  ‰ (n = 15). Bulk sediment organic matter  $\delta^{13}\text{C}$  and C:N was analyzed at the Netherlands Institute for Ecology on a Thermo Scientific TRACE GC Ultra element analyzer coupled Thermo Electron Delta V Advantage IRMS. The analytical error based on replicate measurements on a casein standard was  $\pm 0.02$  ‰ (n = 18). The analysis of  $\delta^{13}\text{C}$  of the filtered POM samples was carried out at UC Davis Stable Isotope Facility (California, US), on a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20-20 IRMS. Analytical error based on replicate standard (bovine liver, glutamic acid and nylon) measurements was  $\pm 0.05$  ‰ (n = 54).

### *Isotope mixing model*

To provide an estimate of how much carbon in the diet of Chironomini and *Daphnia* could derive from  $\text{CH}_4$ , and how strongly this contribution may have varied in the past ca. 100 years, we applied a two-source mixing model [18] to the down-core invertebrate data following [19,20]. When selecting  $\delta^{13}\text{C}$  values of potential carbon sources for the aquatic invertebrates we aimed to provide conservative estimates of the potential importance of  $\text{CH}_4$  in the diet.

Perga [21] has shown for a large oligo- to mesotrophic prealpine lake  $\delta^{13}\text{C}$  values of *Daphnia* ephippia resemble those of the whole body, but become on average 1.3 ‰ higher after 126 days of incubation in the water column in a sediment trap [22]. In culturing experiments, Schilder et al. [23] have shown that *Daphnia* ephippia have on average 0.7 ‰ higher  $\delta^{13}\text{C}$  values than the food source of the *Daphnia* and on average 0.2 ‰ higher  $\delta^{13}\text{C}$  values than the whole organism. Whether ephippia degradation in the water column plays a role in Lake De Waay is unclear. By using the fossil ephippia  $\delta^{13}\text{C}$  values as a proxy for those of *Daphnia* we potentially slightly (by 1.3 ‰ or less) overestimate *Daphnia*  $\delta^{13}\text{C}$  values, and therefore provide conservative estimates of  $\text{CH}_4$ -derived carbon in the diet. The qPCR analysis of sediments from the lake (Table 1) revealed that methanotrophs of the type 1A dominated the MOB community. Available literature indicates that type 1A methanotrophs have ca. 6 ‰ lower  $\delta^{13}\text{C}$  values than the  $\text{CH}_4$  they oxidize [24]. Therefore, for the MOB end member we used the lowest (i.e. not or least oxidized)  $\text{CH}_4$   $\delta^{13}\text{C}$  value measured in the sediments of Lake De Waay (-69 ‰), representing the  $\delta^{13}\text{C}$  value of  $\text{CH}_4$  entering the lake ecosystem, modified by -6 ‰ [24]. The algae endmember of  $\delta^{13}\text{C}$  was estimated by the average  $\delta^{13}\text{C}$  value of POM for the field survey (-32.9 ‰). We assumed no change in baseline  $\delta^{13}\text{C}$  of  $\text{CH}_4$  and DIC and did not account for a change in algal  $\delta^{13}\text{C}$  over time because the bulk organic matter  $\delta^{13}\text{C}$  is very stable within our record (-30.5  $\pm$  0.5 ‰, Figure 3 in main article). This approach assumes negligible contribution by MOB to POM which seems reasonable for a hypertrophic lake with a very high algal production. The results give an estimate of

the percentage of methane-derived carbon in the tissue of the invertebrates, either through direct ingestion of MOB or by consuming organisms that feed on MOB (e.g. ciliates).

### *Cross correlations*

Cross correlations were calculated between log-transformed DI-TP and Fe : Mn as well as between Fe : Mn and  $\delta^{13}\text{C}$  values of *Daphnia* and Chironomini in the De Waay record following Oliveira et al. [25] and using the ccf function in the R package stats [26]. Cross-correlation was restricted to lags of up to 1/4 of the examined sequence [27] and to positive lags between potential drivers and response variables. This resulted in comparisons of 0 to 4 sample steps (equivalent to ca. 0 to 20 years in the De Waay record) between log-transformed DI-TP (leading variable) and Fe : Mn, as well as between Fe : Mn (leading variable) and the  $\delta^{13}\text{C}$  values of *Daphnia* and Chironomini. We follow Oliveira et al. [25] in plotting the 95% confidence intervals in the correlogram to provide an indication of the strength of the observed relationships relative to those expected for unstructured, uncorrelated datasets. However, due to issues associated with assessing correlations between structured time series (autocorrelation, multiple testing; [27,28]) these intervals should not be interpreted as a direct test of statistical significance. Before analysis all records were linearly detrended using the program PAST [28].

## **RESULTS**

### *DNA extraction from sediments*

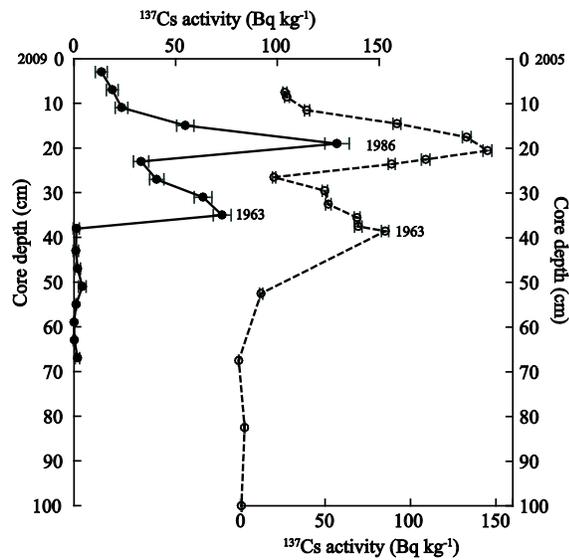
Table 1 summarizes the results from the DNA extraction and qPCR analysis.

**Supplementary Table 1:** Numbers of methanotrophs and methanogens in the top (0-2 cm) sediment layer of Lake De Waay. ND indicates numbers were below the detection limit.

<b>Water depth (m)</b>	<b>Type Ia (gene copies g dry sediment<sup>-1</sup>)</b>	<b>Type Ib (gene copies g dry sediment<sup>-1</sup>)</b>	<b>Type II (gene copies g dry sediment<sup>-1</sup>)</b>	<b>Methanogens (gene copies g dry sediment<sup>-1</sup>)</b>	<b>Nested PCR (presence of MOB)</b>
<b>2.5</b>	ND	ND	ND	1.85*10 <sup>5</sup>	+
<b>8</b>	7.19*10 <sup>6</sup>	ND	ND	3.20*10 <sup>7</sup>	+
<b>14.5</b>	1.53*10 <sup>7</sup>	2.12*10 <sup>6</sup>	ND	1.25*10 <sup>7</sup>	+

## Chronology

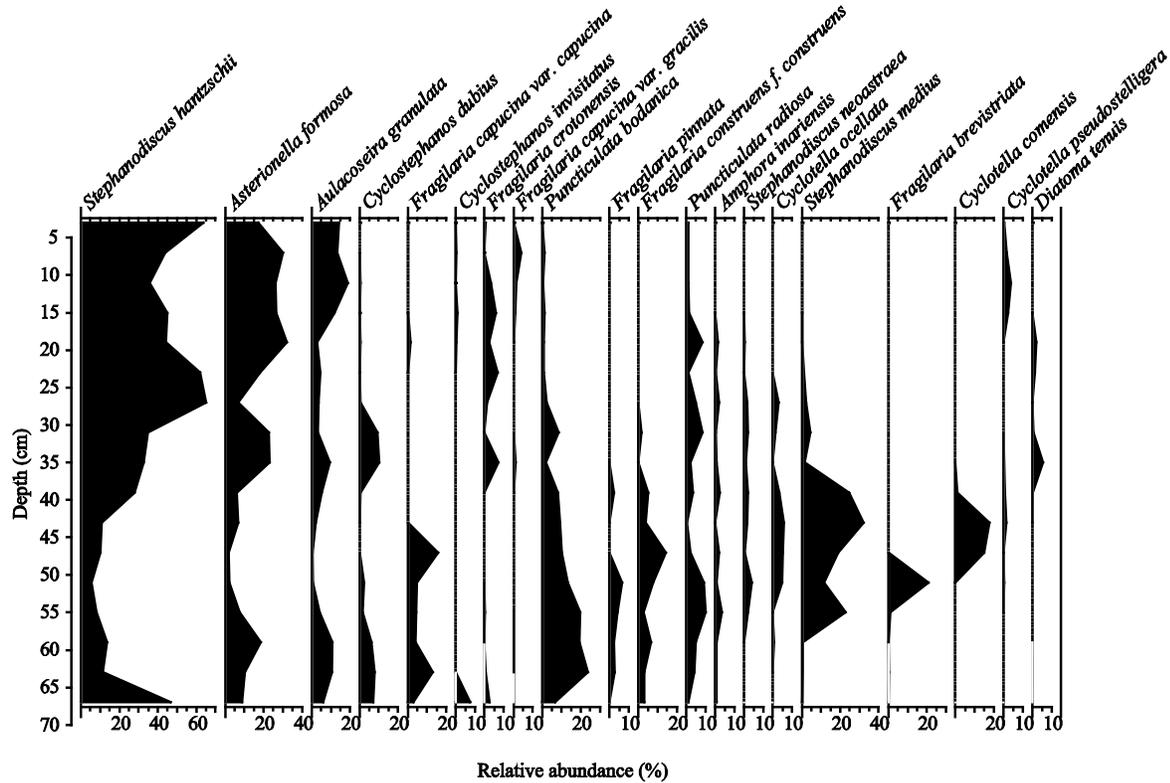
The depth-age relationship for core WAY05 is described in detail in [10]. For the 20th century it is based on  $^{137}\text{Cs}$  dating, providing a number of dated marker horizons (e.g. 1986, 1963, 1954). The age of older sediment sections is inferred based on the sedimentation rate between the oldest  $^{137}\text{Cs}$  dated layer and the lowermost sediment layer which represent the earliest sedimentation after formation of the lake after a dyke breach event in the 17<sup>th</sup> century. The chronology for WAY09 is mainly based on the  $^{137}\text{Cs}$  activity in the uppermost 35 cm (Figure 1). The 1986 Chernobyl event is clearly defined at 19 cm depth and the 1963 bomb test peak at 35 cm. The  $^{137}\text{Cs}$  data indicate a near-linear relationship between  $^{137}\text{Cs}$ -dated horizons in both WAY05 and WAY09. To provide an age estimate for sediment layers below 35 cm depth in core WAY09 we therefore extrapolated sedimentation rates for the  $^{137}\text{Cs}$ -dated sections to the bottom of the core. The  $^{137}\text{Cs}$ -profiles of both cores are very similar (Figure 1) and therefore the two cores can be correlated with confidence at least for the past ca. 50 years (i.e. 1963 to present).



**Supplementary Figure 1:** The  $^{137}\text{Cs}$  activity ( $\text{Bq kg}^{-1}$ ) profile of core WAY09 obtained from 8 m water depth (solid line) and core WAY05 obtained by [10] at 15 m water depth in the centre of the lake (dashed line). The Chernobyl (1986) and bomb test (1963) peaks as well as the coring year (2009) were used to obtain a depth-age model for WAY09.

## Diatom assemblages

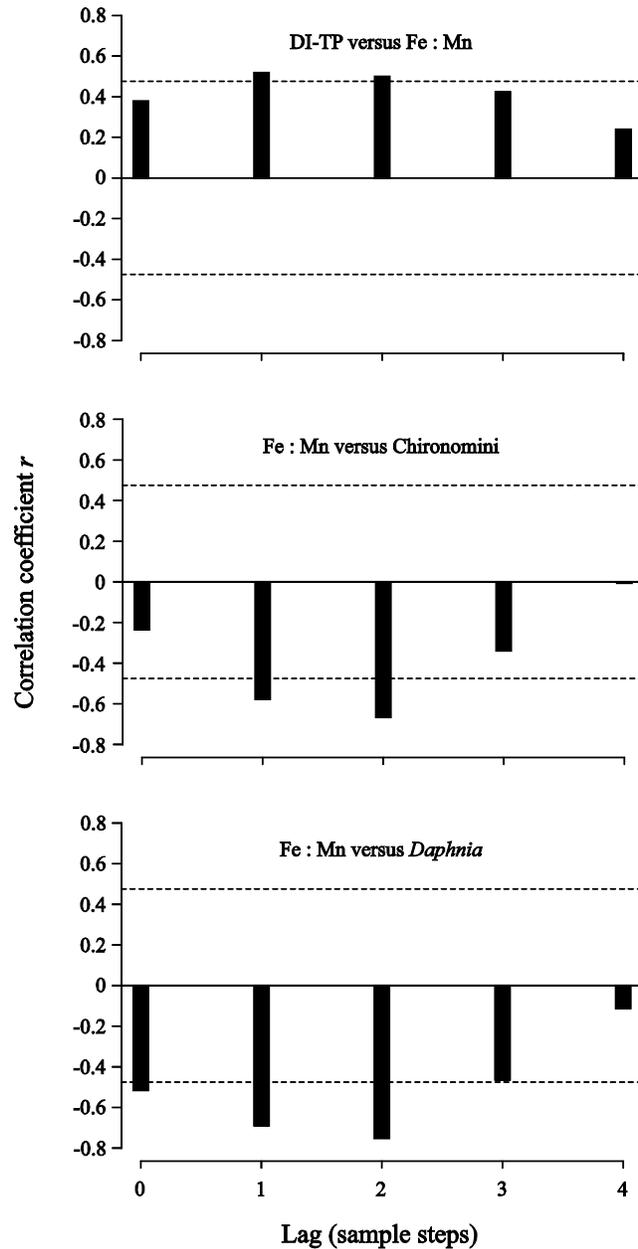
In the lower parts of the sequence, until ca. 1925, diatom assemblages were dominated by taxa typical for eutrophic and hypertrophic lakes, e.g. *Stephanodiscus hantzschii* (Figure 2). Later (ca. 1925 – 1955), diatom assemblages consisted mainly of taxa more typically found in oligotrophic and mesotrophic lakes, including e.g. *Stephanodiscus medius*, *Cyclotella ocellata*, *Cyclotella comensis*, and *Puncticulata* spp. Between ca. 1955 and 1985 we found a typical succession of taxa that are highly competitive at high P concentrations such as *Cyclostephanos dubius*, *Diatoma tenue*, and *Aulacoseira granulata*, indicating a re-eutrophication. In the youngest sediment samples the composition of the diatom assemblages again resembles the one found before ca. 1925, indicative of eutrophic to hypertrophic conditions



**Supplementary Figure 2:** Relative abundance (%) of diatom remains found in core WAY09 for those taxa that had abundances higher than 3 % in the record.

Cross-correlation

Figure 3 shows the results of the cross-correlation analyses after linear de-trending of the time series.



**Supplementary Figure 3:** Cross-correlation analysis of log-transformed DI-TP versus Fe : Mn (top), Fe : Mn versus Chironomini  $\delta^{13}\text{C}$  values (middle panel) and Fe : Mn versus *Daphnia* ephippia  $\delta^{13}\text{C}$  values (bottom panel). The dashed lines indicate the 95 % confidence interval.

## DATA

Semi-colon delimited table showing  $\delta^{13}\text{C}$  values of Chironomini head capsules, *Daphnia ephippia* and bulk sediment organic matter, as well as the results of the DI-TP reconstruction.

Depth (cm);d13C Chironomini head capsules (‰ V-PDB);;Depth (cm);d13C *Daphnia ephippia* (‰ V-PDB);;Depth (cm);d13C bulk sediment organic matter (‰ V-PDB);;Depth (cm);DI-TP ( $\mu\text{g l}^{-1}$ );DI-TP - error ( $\mu\text{g l}^{-1}$ );DI-TP + error ( $\mu\text{g l}^{-1}$ )

0-4;-38.46;;0-4;-41.52;;3\_4;-31.28;;3\_4;222.6;113.9;435.0  
4\_8;-32.35;;4\_8;-39.51;;7\_8;-30.72;;7\_8;131.3;67.7;254.6  
8\_12;-32.99;;8\_12;-38.78;;11\_12;-30.51;;11\_12;133.7;69.2;258.4  
12\_16;-31.76;;12\_16;-37.31;;15\_16;-30.52;;15\_16;132.4;68.5;256.0  
16\_20;-31.56;;16\_20;-37.92;;19\_20;-30.34;;19\_20;102.8;53.3;198.3  
20\_24;-33.15;;20\_24;-36.66;;23\_24;-30.39;;23\_24;103.2;53.0;200.8  
24\_28;-31.17;;24\_28;-34.84;;27\_28;-30.42;;27\_28;113.9;58.7;220.7  
28\_32;-32.48;;28\_32;-33.7;;31\_32;-30.40;;31\_32;51.5;26.2;101.1  
32\_36;-32.65;;32\_36;-35.88;;35\_36;-30.41;;35\_36;82.3;42.6;159.2  
36\_40;-33.27;;36\_40;-38.36;;39\_40;-30.95;;39\_40;46.0;23.7;89.4  
40\_44;-33.26;;40\_44;-36.75;;43\_44;-31.22;;43\_44;27.2;13.9;53.1  
44\_48;-33.77;;44\_48;-40.6;;47\_48;-30.93;;47\_48;29.3;15.0;57.5  
48\_52;-33.15;;48\_52;-39.4;;51\_52;-30.71;;51\_52;30.0;15.2;59.1  
52\_56;-36.15;;52\_56;-38.51;;55\_56;-30.23;;55\_56;33.8;17.0;67.4  
56\_60;-37.98;;56\_60;-39.17;;59\_60;-29.82;;59\_60;45.2;23.0;88.9  
60\_64;-34.7;;60\_64;-39.1;;63\_64;-29.91;;63\_64;35.8;18.1;70.9  
64\_68;-35.69;;64\_68;-40.02;;67\_68;-29.54;;67\_68;100.3;51.3;196.0

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