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# The Ostracod Clumped-Isotope Thermometer: A Novel Tool to Accurately Quantify Continental Climate Changes

Marta Marchegiano[1,2], Marion Peral[2], Jeroen Venderickx[3], Koen Martens[3,4], Antonio García-Alix[1], Christophe Snoeck[2], Steven Goderis[2], and Philippe Claeys[2]

[1]Departamento de Estratigrafía y Paleontología, Universidad de Granada, Granada, España, [2]Archaeology, Environmental Changes and Geo-Chemistry, Vrije Universiteit Brussel, Brussel, Belgium, [3]Royal Belgian Institute of Natural Sciences, Natural Environments, Freshwater Biology, Brussels, Belgium, [4]Department of Biology, Ghent University, Ghent, Belgium

**Key Points:**

- $\Delta_{47}$—ostracod signal accurately records the shell calcification temperature
- $\Delta_{47}$—ostracod signal is not affected by the so called "vital effect"
- The unified calibration of Anderson et al. (2021) can be used to convert the $\Delta_{47}$—ostracod signal into accurate temperatures

**Supporting Information:**

Supporting Information may be found in the online version of this article.

**Correspondence to:**

M. Marchegiano,
martamarchegiano@ugr.es

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**Author Contributions:**

**Conceptualization:** Marta Marchegiano
**Formal analysis:** Marta Marchegiano
**Funding acquisition:** Marta Marchegiano, Christophe Snoeck, Steven Goderis, Philippe Claeys
**Investigation:** Marta Marchegiano
**Methodology:** Marta Marchegiano, Jeroen Venderickx, Koen Martens
**Resources:** Marta Marchegiano, Koen Martens, Antonio García-Alix, Christophe Snoeck, Steven Goderis, Philippe Claeys
**Validation:** Marion Peral, Jeroen Venderickx, Koen Martens, Antonio García-Alix
**Visualization:** Marta Marchegiano
**Writing – original draft:** Marta Marchegiano



**Abstract** This study presents a methodological advancement in the field of clumped-isotope ($\Delta_{47}$) thermometry, specifically tailored for application to freshwater ostracods. The novel ostracod clumped isotope approach enables quantitative temperature and hydrological reconstruction in lacustrine records. The relationship between $\Delta_{47}$ and the temperature at which ostracod shell mineralized is determined by measuring $\Delta_{47}$ on different species grown under controlled temperatures, ranging from $4 \pm 0.8$ to $23 \pm 0.5$°C. The excellent agreement between the presented $\Delta_{47}$ ostracod data and the monitored temperatures confirms that $\Delta_{47}$ can be applied to ostracod shells and that a vital effect is absent outside the uncertainty of measurements. Results are consistent with the carbonate clumped-isotope unified calibration (Anderson et al., 2021, https://doi.org/10.1029/2020gl092069), therefore, an ostracod-specific calibration is not needed. The ostracod clumped-isotope thermometer represents a powerful tool for terrestrial paleoclimate studies all around the world, as lakes and ostracods are found in all climatic belts.

**Plain Language Summary** In the framework of global warming, the reconstruction of past climatic conditions is important to understand the future evolution of climate and its impact. Lake sediments can be used as archives to quantify these effects. This study presents a novel paleo-thermometer based on the application of clumped-isotope technique (i.e., measurement of the number of $^{13}C–^{18}O$ bonds in carbonate minerals that depends on the temperature of carbonate precipitation) on carbonatic microcrustacea, named ostracods that commonly live in lakes. By using ostracods that formed their shells at known temperatures, we demonstrate that they can be easily used to reconstruct water temperature and hydrological conditions (precipitation/evaporation). The ostracod clumped-isotope thermometer represents a powerful tool for terrestrial paleoclimate studies around the world, as lakes and ostracods are located in all climatic belts.

## 1. Introduction

The projected future global temperature increase (IPCC, 2023; Stocker et al., 2013) will affect continental areas differently depending on their geographic position. Lakes are distributed worldwide, on different climate belts, and from boreal to tropical regions. Lacustrine sedimentary archives constitute one of the most useful recorders of past climate changes (Cohen, 2003). Because of their smaller size compared to oceans, lakes are more responsive to environmental variations and thus record even rapid climatic events. In paleoclimatology, the reconstruction of past temperatures and hydrology in lake-waters that directly reflects atmospheric changes is of particular interest.

Carbonate clumped-isotope ($\Delta_{47}$) paleothermometry (Eiler, 2007, 2011) opens the way to accurate determination of lacustrine paleotemperatures and hydrology. So far, the $\Delta_{47}$ paleothermometer was mainly applied to reconstruct past climate variations on marine environments (e.g., Cummins et al., 2014; Henkes et al., 2018; Marchegiano & John, 2022; Modestou et al., 2020; Peral et al., 2020; Tagliavento et al., 2019; Van der Ploeg et al., 2023; Wichern et al., 2022).

The $\Delta_{47}$ technique is based on the temperature-dependent abundance of $^{13}C–^{18}O$ bonds in carbonate minerals (Eiler, 2007, 2011). The increased abundance of these bonds in carbonate, expressed using the $\Delta_{47}$ notation, which corresponds to the small excessive abundance of $^{13}C–^{18}O$ bonds relative to a stochastic isotopic distribution, is associated with decreasing water temperatures (Eiler, 2011; Ghosh et al., 2006; Passey & Henkes, 2012; Stolper & Eiler, 2016). It provides a direct insight in carbonate temperature precipitation. Furthermore, combining $\Delta_{47}$ with $\delta^{18}O$ of carbonate, makes it possible to reconstruct the isotopic composition of the water ($\delta^{18}O_w$) from

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which the carbonate precipitated. Previous studies showed that $\Delta_{47}$ is not influenced by the water isotopic composition (Schauble et al., 2006), salinity (Grauel et al., 2013; Peral et al., 2018) or pH (Peral et al., 2022; Tripati et al., 2015). Whether $\Delta_{47}$ values are affected or not by the so-called "vital effect" (i.e., offset between the isotopic signal of biogenic carbonate and inorganic calcite precipitated under same conditions) remains an ongoing area of investigation. Organisms such as foraminifera and marine molluscs are not affected by it (de Winter et al., 2022; Grauel et al., 2013; Huyghe et al., 2022; Meinicke et al., 2020; Peral et al., 2018; Tripati et al., 2010). However, certain corals, echinoderms, brachiopods and terrestrial snails are (Bajnai et al., 2018; Davies & John, 2019; Dong et al., 2021; Spooner et al., 2016; Zhai et al., 2019).

Bernasconi et al. (2021) describe the efforts of the clumped-isotope community to establish an international standardization (ETH 1–4, IAEA-C1&2 and MERCK; Meckler et al., 2014; Bernasconi et al., 2018, 2021), and a uniform method for measuring and processing data, aimed at a robust and accurate comparison between $\Delta_{47}$ measurements carried out in different laboratories. This approach resolved the large discrepancy between previously published calibrations and allowed the establishment of a unified clumped-isotope calibration (Anderson et al., 2021). The unified $\Delta_{47}$ calibration recently published by Anderson et al. (2021) may be applied across a wide range of natural carbonate material precipitated at temperatures between 0.5 and 1,100°C. This calibration includes exclusively authigenic continental carbonates (i.e., tufa, travertines, speleothems) and synthetic carbonates. However, the good correspondence with previous published calibrations on foraminifera (e.g., Daëron & Gray, 2023; Meinicke et al., 2020; Peral et al., 2018) and marine bivalves data (Huyghe et al., 2022) confirms its validity in biogenic carbonate that do not present any isotopic disequilibrium. So far, no biogenic continental carbonates were included in the calibrations.

In terrestrial environments, the $\Delta_{47}$ paleothermometer has mainly been applied on paleosol (e.g., Kelson et al., 2020; Passey et al., 2010; Quade et al., 2013) and speleothems (e.g., Affek et al., 2008; Daëron et al., 2011; Fernandez et al., 2023; Kluge & Affek, 2012; Nehme et al., 2023). In lacustrine records, the $\Delta_{47}$ technique was used on inorganic carbonates (e.g., Hudson et al., 2017; Huntington et al., 2010; Katz et al., 2023) and molluscs (e.g., Dong et al., 2021; Grauel et al., 2016; Tobin et al., 2014; Wang et al., 2016; Zaarur et al., 2011) and was only recently applied to ostracods to reconstruct paleoaltitude and paleoclimate (Marchegiano et al., 2024; Song et al., 2022; Yue et al., 2022).

Ostracods are small aquatic crustaceans (mostly 0.3–5 mm) that occur in almost all aquatic environments in all climatic belts (Ghaouaci et al., 2017; Jiang et al., 2022; Meisch, 2000; Mischke et al., 2012; Pugh et al., 2002; Wojtasik and Kczyńska−Wiśnik, 2012) and have an excellent and abundant fossil record since the Ordovician (Siveter et al., 2010). They are sensitive to the variation of several ecological variables (i.e., pH, salinity, temperature, water level, and oxygen saturation) (Meisch, 2000). For these reasons ostracods are useful in biostratigraphy and are valuable archive to reconstruct past environmental and climatic changes (Holmes & De Deckker, 2012; Horne et al., 2012; Marchegiano et al., 2018, 2019, 2020; Mischke et al., 2010; Pérez et al., 2013; Ruiz et al., 2013; Schwalb et al., 2002; Von Grafenstein et al., 1999). The main advantage of applying $\Delta_{47}$ technique on ostracods relies on their stable low-Mg calcite shell mineralogy, which usually are well preserved in the fossil record (e.g., Bajpai et al., 2013; Forel et al., 2021; Janz, 2000), compared to aragonitic mollusks. Also, through the analyses of ostracod assemblage, it is possible to establish if shells were autochthonous or allochthonous (Zacarías et al., 2019). Therefore, they reliably record temperature and hydrological conditions of the waterbody in which they were found. This aspect constitutes a major advantage for the ostracod clumped-isotope paleothermometer over inorganic carbonate archives that can be composed of a mix of allogenic and authigenic carbonates.

Ostracods have a bivalve dorsally articulated shell. They grow by moulting and undergo eight juvenile stages (designated in descending order from A-8 being the first instar to A-1 adult stage) in their development before reaching adulthood (A). They secrete their shell relatively rapidly, few days or even hours (Chivas et al., 1983), starting from ions present in the host water at the time of valve calcification (Turpen & Angell, 1971). Ostracods record temperatures that represent a snapshot of the conditions existing when they precipitate their shells instead of mean annual average temperature, such as mollusks and inorganic carbonate. Marchegiano et al. (2024) demonstrated that by combining paleontological (i.e., species identification), biological (i.e., calcification season), and geochemical (i.e., $\Delta_{47}$ technique and $\delta^{18}O$) information, it is possible to reconstruct past seasonal temperature and hydrological conditions. Because mean annual temperature and rainfall amount can be significantly biased by seasonality, it is of primary importance to disentangle these parameters. The ostracod-$\Delta_{47}$ methodology presented in this study represents a novel tool able to achieve this goal.

The main limitation of the ostracod-$\Delta_{47}$ paleothermometer lies in the relatively large amount of carbonate required for $\Delta_{47}$ analyses that, depending on the size and thickness of the shells, can be highly variable (e.g., from 40 to 60 valves in Marchegiano et al., 2024 and this study). To overcome this, Yue et al. (2022) perform $\Delta_{47}$ analyses on different ostracod shell size (i.e., adult, and different juvenile stages). The study showed no difference in $\Delta_{47}$ results suggesting the possibility of combining distinct life stages and thus increase the amount of carbonate availability in sediments. Also, considering the instrumental advances made since the initial development of $\Delta_{47}$ technique carbonate amount is becoming less of a limiting factor.

This article improves the current understanding of the application of $\Delta_{47}$ to lacustrine environments with a particular focus on ostracod shells by: (a) investigating for the first time, the relationship between $\Delta_{47}$ and precipitated temperatures for ostracod shells and, whether it is affected by the so-called "vital-effects". This is essential to properly apply the ostracod clumped-isotope technique to reconstruct past temperatures; (b) establishing if clumped-isotope variations in ostracods are consistent with the unified calibration of Anderson et al. (2021). To achieve these objectives $\Delta_{47}$ analyses were carried out on ostracods grown under known temperatures (from lab cultures and natural environments). The selected ostracod species are among the most widespread and easily recovered in both recent and fossil records.

## 2. Materials and Methods

### 2.1. Ostracod Shells

#### 2.1.1. Lab Grown *Heterocypris incongruens*

The cosmopolitan ostracod species *Heterocypris incongruens*, common in lakes, shallow seasonal ponds and small permanent water bodies (Meisch, 2000), was cultured at the Royal Belgian Institute of Natural Science (Brussel, Belgium) from September to December 2021 under controlled temperature at 23°C (±0.5°C) (Figure S1 in Supporting Information S1). Other two cultured experiments were installed at 10 and 15°C (±0.5°C), but the mortality rate was high, and the eggs did not hatch. These two cultures did not produce enough shells for clumped-isotope analyses and thus could not be included in the data set.

To set up the lab experiment, living specimens of *H. incongruens* were sampled using a hand-net (120 µm mesh size) in a temporary pond in Bertem (Belgium, 50.86898°N, 4.61908°E) in August 2021. The main population was kept alive in an aquarium filled with fine sand sediments (ca. 2 cm thickness at the bottom) and water coming from their natural environment. Every 3 days ostracods were fed with *Spirulina* algae. For this study, juvenile specimens were selected from the last three stages (A-1, A-2, A-3 valves). These specimens were isolated to make satellite populations in a (self-made) incubator system under controlled temperature at 23°C (±0.5°C) and 50–50 Light-Dark diurnal circle. The juvenile valves were discarded and only the deceased adult *H. incongruens* specimens, which precipitated their valves in the isolated temperature-controlled aquarium, were used for $\Delta_{47}$ analysis.

#### 2.1.2. Ostracod Collected in Natural Environments

Living ostracods were collected in their natural environments using a hand-net (120 µm mesh size) during two field campaigns in January ("Drongengoed" nature area, Ursel, Belgium—51.151 N, 3.474 E) and February 2022 (Padul, Granada, Spain 37.016° N, 3.607° W) (Figure S1 in Supporting Information S1). Water temperatures were measured periodically during December 2021 and January 2022 in Ursel using a HANNA thermometer (Figure S2 in Supporting Information S1) and compared with mean daily air temperature (average of 4.6 ± 0.5ºC, from Weather Spark) (Figure S2 in Supporting Information S1). Mean daily water temperatures were measured in Padul between the 1 of November 2021 to the 28 of February 2022 by means of an ONSET HOBO TidbiT MX 400 temperature data logger (Figure S3 in Supporting Information S1).

*Eucypris virens* and *Bradleystrandesia fuscata* comes from a monitored temporary pond (Ghent-Belgium) of 10 cm depth. The collected adult specimens precipitated their shells between December and beginning of January 2022 under temperature of 4°C (±0.8°C) (Figure S2 in Supporting Information S1). *Eucypris virens* is a winter-early spring species (December/April) widely distributed (Europe, Greenland, Azores, North Africa, Central

**Table 1**
*Ostracod Clumped Isotope Results*

| Samples name | Species | Environmental $T$ (°C) | $N$ | Δ47 (‰) I-CDES | 1SE | Δ47−$T$(C°) (Anderson et al., 2021) | Δ47−$T$(C°) (Meinicke et al., 2020) | δ13C‰ (VPDB) | δ18O‰ (VPDB) | δ18Ow/o‰ (Kim & O'Neil, 1997) | 1SE ‰ | δ18Ow‰ (measured) | Offset ‰ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ETH4 | 0.4505 (Bernasconi et al., 2021) | | 79 | 0.4483 | 0.0055 | | | | | | | | |
| OSTRA-1Ghe | *E. virens* | 4.0 (±0.8) | 11 | 0.6636 | 0.0103 | 3.8 (±1.7) | 5.4 (±0.7) | −13.7 | −4.0 | −6.4 | 0.2 | −9.0[b] | −2.6 |
| OSTRA-2Gra | *H. brevicaudata* | 10.1 (±2) | 9 | 0.6374 | 0.0110 | 11.3 (±2) | 12.8 (±0.5) | −9.0 | −6.6 | −7.3 | 0.2 | −8.4 | −1.1 |
| OSTRA-3Ghe | *B. fuscata* | 4.0 (±0.8) | 10 | 0.6658 | 0.0108 | 3.3 (±1.8) | 4.8 (±0.54) | −13.3 | −3.9 | −6.4 | 0.2 | −9.0[b] | −2.6 |
| OSTRA-4Lab | *H. incongruens* | 23.0 (±0.5) | 5 | 0.6021 | 0.0145 | 22.2 (±3.4) | 23.8 (±0.5) | −10.3 | −3.7 | −2.1 | 0.3 | −2.7[b] | −0.6 |
| TRAR1Tras | *C.angulata* | 8.2 (±2)[a] | 6 | 0.6485 | 0.0144 | 8.0 (±2.9) | 9.7 (±0.5) | −0.5 | 3.13 | 1.6 | 0.3 | −0[c] | −2.2[d] |
| TRAR2Tras | *C.torosa* | 21.2 (±4)[a] | 10 | 0.6012 | 0.0111 | 22.5 (±2.3) | 24.1 (±0.6) | −3.2 | 0.42 | 2 | 0.2 | 1.7[c] | −0.8[d] |

*Note.* $N$ = number of replicates per sample. Offset between $\delta^{18}O_{water/ostr}$, calculated with Kim and O'Neil (1997), and the measured $\delta^{18}O_w$. [a]environmental $T$ values are from Worldlake database 2013. [b]δ18Ow‰ values are from Bowen & Wilkinson (2002). [c]δ18Ow‰ values are from Frondini et al. (2019). [d]vital effect values are from Von Grafenstein et al. (1999) for *C. angulata* and from Keatings et al. (2007) for *C. torosa*.

Asia, China, North America and Middle East) that prefers to live in temporary habitats, mostly ponds, which dry up before summer, as well as in lakes, streams, rivers, and rice fields (Meisch, 2000). Adults have a life span of about 2 months and die shortly after egg production (Meisch, 2000).

*Bradleystrandesia fuscata* is a species that typically occurs in seasonal temporary ponds, mainly from autumn to spring and very rare during summer. In winter ponds larvae develop faster than in those in other seasons and they live only for a few weeks (Meisch, 2000). It has been found in Europe, the Middle East and North America (Meisch, 2000), in the Central and South America (Coviaga et al., 2018) and in Western Australia (Koenders et al., 2012, 2016).

*Herpetocypris brevicaudata* specimens were collected in a small and very shallow river (less then 20 cm deep), with continuous running water, that is fed by groundwater in the Padul lake area (Granada-Spain). The collected adults precipitated their shells between November and the beginning of February, which is a period of relatively constant water temperature (10.1 ± 2°C) (Figure S3 in Supporting Information S1). This species occurs in Europe and North Africa and prefers springs, slowly flowing streams, and the littoral zone of lakes. Little is known about the life cycle of *H. brevicaudata* but it is supposedly similar to that of its closest congener, *H. reptans*, which produces two generations per year (April/August and November/overwinter).

All the collected ostracods were euthanized and preserved in 70% ethanol up to 3 months. Knowledge of the life style of the selected ostracod species ensures that the timing of calcification is well constrained and in combination with the temperature monitoring of the calcification environment, assess the robustness of the ostracod clumped isotope paleothermometer.

### 2.2. Clumped-Isotope Analyses

The four samples represent three different temperatures (see Table 1) and are used for clumped-isotope ($\Delta_{47}$) analyses. To avoid contamination, each ostracod is cleaned to remove the organic matter. The two ostracod valves are gently opened to remove the body using two needles and each shell accurately cleaned with a thin brush and 70% ethanol under the microscope for few minutes. The efficacy of the cleaning is tested by checking 10 valves per species under the scanning electron microscope (SEM) (Figure S1 in Supporting Information S1). Only adult specimens are selected for the following clumped analyses to prevent a potential size effect in the sample. Moreover, it is not possible to accurately remove the body and organic matter from the smaller shells. A total of 40–60 valves per replicate is needed to assure enough material (0.5–0.6 mg per replicate), whereby the exact number depends on the species (i.e., size and thickness of the shells). Each sample is replicated 5 to 11 times, the use of a large number of valves, between 300 and 700, per sample ensures the consistency of the analyses. The $\Delta_{47}$ analyses of ostracod shells are carried out at the Archeology, Environmental changes and Geo-Chemistry (AMGC) clumped isotope lab of the Vrije Universiteit Brussel (VUB), using a Nu Instruments Perspective-IS stable isotope ratio mass spectrometer in conjunction with a NuCarb carbonate sample preparation system, as described in detail in De Vleeschouwer et al. (2022). The carbonate powder reacts for 10 min with 105% $H_3PO_4$ at 70°C. The analyses are performed between October 2021 and November 2022. The ETH standards (Meckler et al., 2014) are measured following the recommendations of Kocken et al. (2019) with a sample-to-standard ratio of 1:1. The carbonate standard ETH-4 is systematically measured and compared to InterCarb values (Bernasconi et al., 2018, 2021) to ensure the measurements quality control. Analyses and results were monitored using the Easotope software (John & Bowen, 2016). The raw measured $\Delta_{47}$ values are processed using the IUPAC Brand's isotopic parameters (Brand et al., 2010; Daëron et al., 2016; Petersen et al., 2019) and converted to the ICDES 90°C scale, using the most recent values for the ETH-1, ETH-2, and ETH-3 carbonate reference materials (Bernasconi et al., 2021) within the ClumpyCrunch software (Daëron, 2021; Daëron et al., 2016). Both analytical (SE in Table 1 calculated by ClumpyCrunch web version) and calibration uncertainties are propagated to calculate the final uncertainties on temperatures. The ANCOVA (Analysis Of COVAriance) test is used to determine the difference between ostracod $\Delta_{47}$ temperature and the unified calibration of Anderson et al. (2021) (Table S1 in Supporting Information S1).

### 2.3. Water Sample Analyses

Four water samples were collected in Padul (Granada), sealed, cold-stored at 4°C, and analyzed by means of laser spectrometry-based analyzer, using a Picarro L2140-i at the Centro de Instrumentación Científica of the University of Granada (Table 1). Analytical uncertainty (2SD) is ±0.2‰ for $\delta^{18}O_w$ and ±0.4‰ for δD. International IAEA standards, including VSMOW2 and GISP, were used in the calibration. For the other two locations, being temporary ponds, the correspective seasonal precipitation $\delta^{18}O_w$ value was used following the model from Bowen and Wilkinson (2002).

## 3. Results

The $\Delta_{47}$ values of the ostracod samples range from 0.6021 to 0.6658‰ with a standard error (1SE) from 0.0103 to 0.0145‰ (Figure 1 and Table 1). The availability of sample material directly determined the number of replicated measurements carried out per sample. The large number of replicates per specimen ensures the robustness of the results. The repeatability along the 9 sessions of the standards used to standardize the results and the ostracod samples is 31.3 and 26.6 ppm, respectively. The ETH4 standard, used to ensure and control the quality of the analyses, presents a $\Delta_{47}$ value of 0.4483‰ (in Bernasconi et al., 2021, ETH4 value is 0.4505 ± 0.0018‰) and 0.0055‰ 1SE for 79 replicates (Table 1). The homogeneity in the $\delta^{18}O$ and $\delta^{13}C$ values (EarthChem database) in the samples coming from natural environments, indicates that the non-linear mixing effect (i.e., $\Delta_{47}$ offset due to sample heterogeneity in $\delta^{18}O$ and $\delta^{13}C$ and independent of equilibrium temperatures, Defliese & Lohmann, 2015) does not affect the measurements. The compiled results for $\Delta_{47}$, $\delta^{18}O$ and $\delta^{13}C$, including all standards and sample replicates, are available in Supporting Information S1. The water sample from the locality in Padul, Granada, gave a value of $\delta^{18}O_w - 8.4 \pm 0.2$‰ (Table 1). Other $\delta^{18}O_w$ are estimated using Bowen and Wilkinson (2002) data set.

## 4. Discussion

### 4.1. Relationship Between Ostracod $\Delta_{47}$ and Temperature and Implication in Paleotemperature Reconstruction

To determine the $\Delta_{47}$—$T$ relationship for ostracod shells, clumped-isotopes were measured on four species that precipitated their shells at three different temperatures (4 ± 0.8°C, 10.1 ± 2°C and 23 ± 0.5°C). The two datapoints from Marchegiano et al. (2024), obtained from recent shells (last 1kyr) of two other different species living today at Lake Trasimeno (central Italy) (i.e., *C. torosa* $\Delta_{47}-T$ of 22.5 ± 2.3°C and *C. angulata* $\Delta_{47}-T$ is 8.0 ± 2.9°C) at 21.2 ± 4°C (average warmer season temperatures) and 8.2 ± 2°C (average cold season temperatures) (World Lake Database, 2023), were also included (Table 1 and Figure 1). All the measured ostracod samples fall within the 95% confidence interval of the regression line of Anderson et al. (2021):

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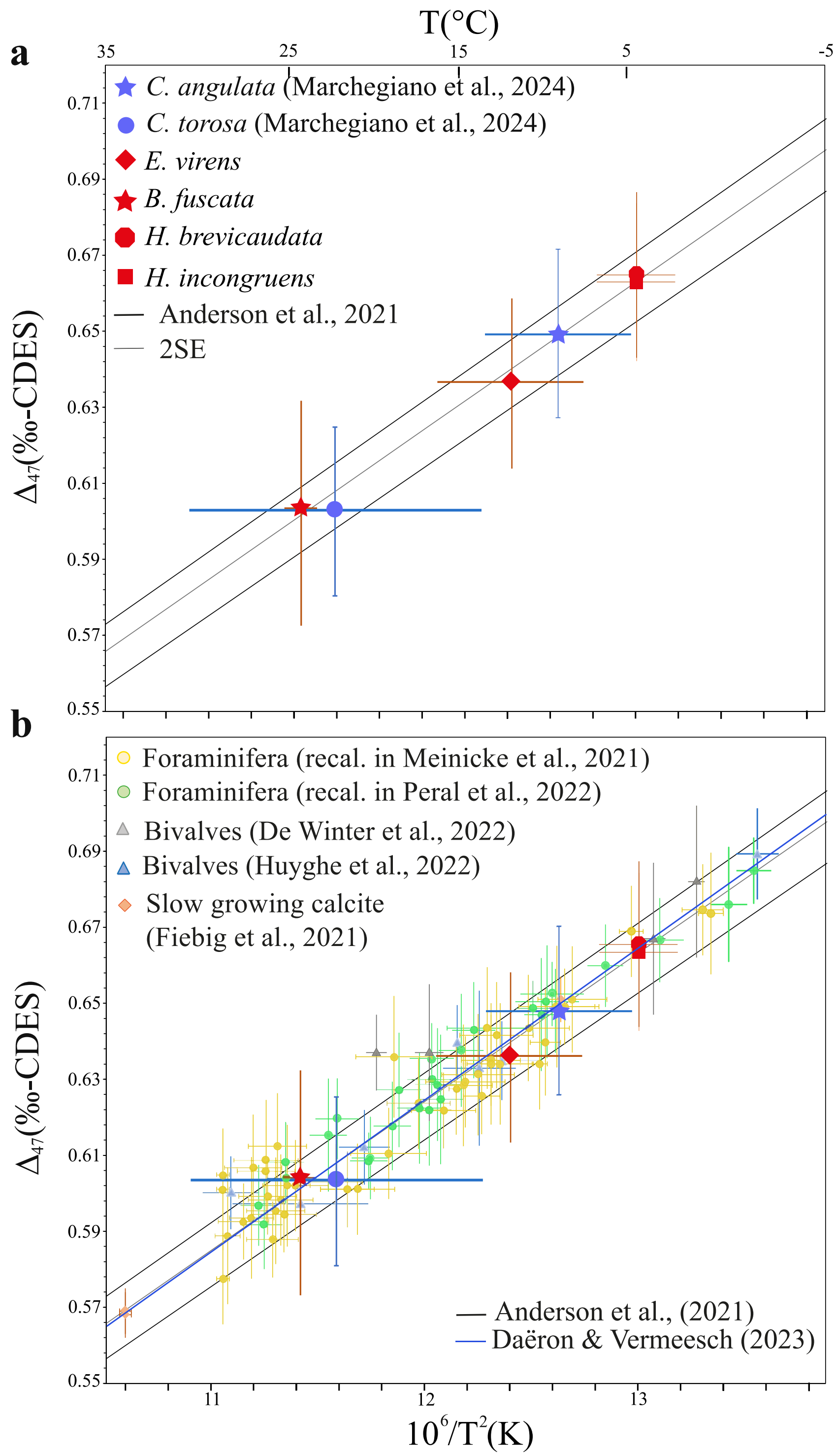


**Figure 1.** (a) Clumped-isotope ($\Delta_{47}$) measured on living and recent ostracods shells are presented against the known precipitated temperatures. The ostracod-$\Delta_{47}$ values are compared to the unified calibration of Anderson et al. (2021) (black line; gray lines indicate the 2SE confidence interval). (b) Ostracod clumped-isotope data against precipitated temperatures compared with clumped-isotope data from marine biogenic carbonate and continental slow grown calcite speleothems (De Winter et al., 2022; Fiebig et al., 2021; Huyghe et al., 2022; Meinicke et al., 2021; Peral et al., 2022) and the calibration from Daëron and Vermeesch (2023). All $\Delta_{47}$ errors are expressed as 2SE. Final $\Delta_{47}$ values are calculated using the ClumpyCrunch software (Daëron, 2021; Daëron et al., 2016). The horizontal lines indicate the variations of the temperature measured in the field per each sample.

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$$\Delta_{47}(\mathrm{I-CDES90°C}) = 0.0391 \pm 0.0004 \times \frac{10^6}{T^2} + 0.154 \pm 0.004\left(\mathrm{r}^2 = 0.97\right)$$

The ANCOVA test indicates no significant difference in slope between the ostracod $\Delta_{47}$ temperature relationship and the unified calibration of Anderson et al. (2021) (*p-value* of 0.8552) or intercept (*p-value* of 0.8558). To convert the $\Delta_{47}$ fossil ostracod value into temperatures, Song et al. (2022) and Yue et al. (2022) relied on the dolomite calibration from Bonifacie et al. (2017) and the travertine calibration from Kele et al. (2015) recalculated in Bernasconi et al. (2018), respectively. Instead, Marchegiano et al. (2024) used the unified calibration of Anderson et al. (2021). The $\Delta_{47}$ analyses conducted on living ostracods that formed their shells under precisely known temperatures make it possible to select the most suitable calibration. In this study, ostracods show a similar T-$\Delta_{47}$ relationship to forams, bivalves and slowly precipitated inorganic carbonates, supporting a common temperature relationship between biogenic and authigenic carbonates from both marine and continental environments when vital effects are absent (Figure 1). Consequently, the unified calibration is applied to convert ostracod-$\Delta_{47}$ values in temperatures (Table 1). *E. virens* and *B. fuscata* recorded $\Delta_{47}{-}T$ of 3.8 ± 1.7°C and 3.3 ± 1.8°C, respectively, and their monitored environmental $T$ is of 4 ± 0.8°C. *H. brevicaudata* lived at $T$ of 10.1 ± 2°C and recorded $\Delta_{47}{-}T$ of 11.3 ± 2°C. The species *H. incongruens* cultivated in the lab at 23 ± 0.5°C, yields a $\Delta_{47}{-}T$ of 22.2 ± 3.4°C. The difference between the calculated temperatures and the temperature measured in situ is between 0.5 and 1.2°C, which falls within the uncertainties of the measurements. Consequently, clumped-isotope thermometry can be applied to ostracods shells, the unified calibration can be used to convert $\Delta_{47}$ value into temperature and a ostracod-specific calibration is not needed.

De Winter et al. (2022) suggested that for lower earth surface temperature, the combined foraminifera-based calibration from Meinicke et al. (2020) is more suitable as Anderson et al. (2021) leads to a bias to colder temperatures in aragonitic marine mollusks. However, our data (Table 1) agree better with the unified calibration. A comparison with the latest calibration from Daëron and Vermeesch (2023) (Figure 1b), does not reveal substantial differences within the uncertainties.

The clumped ostracod study of Song et al. (2022) did not use the InterCarb standardization method (Bernasconi et al., 2018, 2021). Therefore, it is not possible to recalculate the $\Delta_{47}$ temperature values for comparison with our study. However, we recalculate the temperatures of Yue et al. (2022) with the last ETH1-4 values from Bernasconi et al. (2021) and converted the $\Delta_{47}$ temperature with the unified calibration (Anderson et al., 2021) (see Table S2 in Supporting Information S1). A difference of 3°C is found between the recalculated temperatures (Table S2 in Supporting Information S1) and the ones presented in the original paper of Yue et al. (2022). The use of the most up-to-date and universally recognized standardization method and the most appropriate calibration are fundamental to reconstruct accurate temperatures.

### 4.2. Absence of Vital Effect in Ostracod Clumped-Isotopes Measurements

Research on potential vital effect in the $\Delta_{47}$ signal of terrestrial biogenic carbonate, conducted on modern organisms that precipitated their shells under known temperatures, is scarce and so far limited to land snails in which the presence of such effect is still under debate (Bao et al., 2023; Bricker et al., 2023; Dong et al., 2021; Guo et al., 2019; Wang et al., 2021; Zaarur et al., 2011; Zhai et al., 2019; Zhang et al., 2018). A vital effect, linked to metabolic processes (i.e., rate of incorporation of $HCO_3^-$ or of $CO_3^{2-}$ into the calcification sites during calcitic cuticle growth, Decrouy et al., 2011), exists for the $\delta^{18}O$ signal of several, but not in all ostracod species. The vital effect ranges from 0.5 to 3‰ with respect to the isotopic equilibrium (von Grafenstein et al., 1999; Holmes & Chivas, 2002) and currently limits unambiguous paleotemperature reconstructions based on $\delta^{18}O$. Yet, Marchegiano et al. (2024), demonstrate the absence of an interspecific vital effect on the $\Delta_{47}$ signal of fossil ostracods coming from the same sediment sample by analyzing different species (i.e., *Sarcypridopsis aculeata*, *Heterocypris salina* and *Eucypris mareotica*) that precipitated their shells at the same time, season and in the same temperature and environment (samples TRA2-*S. aculeata* 24.9 ± 2°C and TRA2b-*H. salina* 24.3 ± 2.2°C; samples TRAH2-1-*S. aculeata* 13.0 ± 2.7°C and TRAH2-2- *E. mareotica* 13.2 ± 3.3°C). Furthermore, recent shells (last 1 kyr) of the two species *C. torosa* and *C. angulata* both recorded temperatures that overlap with the ones measured today at Lake Trasimeno during the respective shell precipitation season (*C. torosa* $\Delta_{47}{-}T$ is 22.5 ± 2.3°C –warm season average temperature is 21.2°C today; *C. angulata* $\Delta_{47}{-}T$ is 8.0 ± 2.9°C –cold season average temperature is 8.2°C today), excluding a vital effect in their $\Delta_{47}$ signal (Marchegiano et al., 2024; Yue et al., 2022, reached a similar conclusion for *L. inopinata* at Dali Lake (Eastern Asia). *S. aculeata*, *H. salina* and *E. mareotica* do not seem to have a vital effect in their $\delta^{18}O$ signal either (Li & Liu, 2010; Marchegiano et al., 2024); however, *L. inopinata*, *C. torosa* and *C. angulata* are well known to display such effect (Decrouy et al., 2011; Keatings et al., 2007; Marco-Barba et al., 2012; Von Grafenstein et al., 1999).

To further investigate the potential ostracod vital effect, this study measured $\Delta_{47}$ on four ostracods species that precipitated their shells under monitored temperatures. Two of them, *E. virens* and *B. fuscata*, lived at the same time and environment and precipitated their shells at the same temperature (4 ± 0.8°C). All the four species (*H. brevicaudata*, *H. incongruens*, *E. virens*, and *B. fuscata*) coming from lab culture and different natural environments (i.e., river and temporary pond) record the corresponding environmental temperatures, using the unified calibration of Anderson et al. (2021). This observation indicates the absence of a vital effect larger than the analytical uncertainty (SE from ±1.7 to 3.4°C), in the $\Delta_{47}$ signal. This conclusion is reinforced by the absence of a consistent offset ($\Delta\Delta_{47}$ 0.002 ± 0.01‰, Table 1), within the analytical uncertainty, between the species *E. virens* and *B. fuscata*.

The presence of a vital effect in the $\delta^{18}O$ signal of the analyzed species was not investigated before. Only *Herpetocypris reptans*, which is considered to have living conditions highly similar to *H. brevicaudata* (Meisch, 2000), displays a strong $\delta^{18}O$ vital effect (+2.5‰ Keatings et al., 2002). In this study, an offset from ~2.6 to 0.6‰ is found between the reconstructed $\delta^{18}O_{water/ostr}$ (i.e., combining $\delta^{18}O_{ostr}$ and $\Delta_{47}$ value and the equation of Kim & O'Neil, 1997), and the measured $\delta^{18}O_w$ signal of the water in which ostracod lived, which suggests the presence of a vital effect in the $\delta^{18}O_{ostr}$ (see Table 1 and Figure S2 in Supporting Information S1). This observation confirms the absence of any correlation between vital effects in $\delta^{18}O$ and the $\Delta_{47}$ signal. It also implies that, although $\Delta_{47}$ paleotemperatures remain unaffected by the vital effect, it becomes imperative to correct the $\delta^{18}O_{ostr}$ with the species-specific offset when combining $\delta^{18}O_{ostr}$ and $\Delta_{47}$ to reconstruct hydrological conditions (i.e., $\delta^{18}O_w$).

Although this study does not cover the entire possible variability in lacustrine environments and/or ostracod species environmental preferences, previous studies showed that the $\Delta_{47}$ signal is not affected by parameters such as salinity, pH, and $\delta^{18}O_w$ (Grauel et al., 2013; Peral et al., 2018, 2022; Schauble et al., 2006; Tripati et al., 2015). Thus, the number of analyzed samples (six different genera and species and five different temperatures, plus three coming from fossils record) is sufficient to demonstrate that the ostracods-$\Delta_{47}$ signal records the temperature at which the organisms precipitated their shells. Previous calibration studies conducted on different organisms (i.e., corals, echinoderms, and terrestrial snails) used a comparable sample number to detect the presence (or not) of a vital effect for the entire class or phylum (Davies & John, 2019; Dong et al., 2021; Guo et al., 2019; Spooner et al., 2016).

This study confirms that clumped-isotope thermometry is applicable to ostracods to reconstruct reliable paleotemperatures and past hydrological conditions and that a vital effect does not affect the obtained ostracod-$\Delta_{47}$ values. However, to reconstruct reliable paleotemperatures, it is capital to document the life history (e.g., calcification season and habitat) of the used ostracod species.

## 5. Conclusions

Ostracod-$\Delta_{47}$ constitute a novel paleothermometer to reconstruct temperature and hydrological conditions in continental environments. The absence of a vital effect, outside the uncertainty, enables the use of the paleothermometer with different ostracod species and throughout geological time. This makes the ostracod-$\Delta_{47}$ thermometer a new powerful tool to reconstruct past continental climate change. Because of the good agreement between the ostracod data and the unified calibration (Anderson et al., 2021), the latter can be used for future applications of clumped-isotopes in ostracods and an ostracod-specific calibration is not needed. The establishment of this new lacustrine proxy opens the door to novel high-resolution continental paleoclimate and paleoenvironmental reconstructions and therefore, has the potential to become a key tool in future lacustrine research.

## Data Availability Statement

Research Data are deposited in EarthChem database. Marchegiano (2024) "The Ostracod Clumped-Isotope Thermometer: a Novel Tool to Accurately Quantify Continental Climate Changes".

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