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# Testate Amoeba and Chironomid assemblages from Balma Lake (Piedmont, Italy): a multi-proxy record to identifying recent climate and environmental changes in alpine areas

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ABSTRACT

Alpine lakes are well known as “environmental sentinels”, which are sensitive to anthropogenic impacts, and paleoecological studies provide useful information regarding the effects of local and global changes. In the present study we investigate, for the first time, the temporal variations in testate amoeba assemblages in relation to environmental factors and anthropogenic impacts in a high-altitude lake located in the Italian Alps (Balma Lake, Piedmont, Italy). The testate amoebae are well-suited as bioindicators and have been employed to reconstruct past climate and environmental changes from lake sediments. However, these organisms have not been thoroughly studied in alpine lakes, especially in Europe. We detected significant changes in testate amoeba assemblages along an alpine lake sedimentary profile over the last 2000 years. These changes were mainly associated with variations of trace elements, nutrients, and grain size. Moreover, five different time periods were identified and related to pluvial/drought events of the last 2000 years and to the “Late Antique Little Ice Age” (LALIA). Alpine lakes are originally fishless and fish introductions for recreational purposes could cause important changes in these ecosystems. However, the introduction of brook trout in Balma Lake during the 1970s seems to have had little influence on testate amoeba assemblages. The present work provides new insights for the evaluation of temporal changes in testate amoebae through paleoecological investigations. Similarly, the comparison of testate amoeba and chironomid proxies offered an important tool for analyzing recent climatic and environmental changes in alpine lakes (in lacustrine environments), especially for the European Alps.

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## 1. Introduction

Alpine lakes or high-altitude lakes are known to be “early warning systems” for the whole mountain environment (MOLARWater Chemistry Group, 1999; Perilli et al., 2020; Cantonati et al., 2021). Due to their harsh environmental conditions, these systems allow for colonization by a few well-adapted species, resulting in low diversity communities with relatively simple structures and trophic webs (e.g., Füreder et al., 2006; Cantonati et al., 2020). Despite their remote location, small size and the high turnover of surface waters, these environments are extremely sensitive to natural and anthropogenic impact (Fjellheim et al., 2009), both on a local (water diversion, tourism, grazing, the introduction of non-native species) and global scale (long-range pollutant transport, radioactive-nuclides transport, acid rain, global climatic changes) (Psenner and Schmidt, 1992; Psenner and Catalan, 1994; Camarero et al., 1995a, 1995b; Battarbee et al., 2002; Magnea et al., 2013). In this context, paleoecological investigations in alpine lakes are of great scientific interest, as these aquatic systems can be considered good indicators for global changes (Rogora et al., 2008) and can aid in the evaluation of the large-scale effects of anthropogenic activities (e.g., Battarbee et al.,

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2009). Moreover, paleoecological reconstructions based on subfossil organism assemblage datasets are commonly used to reconstruct past environmental changes over long timescales (from hundreds of years to millennia) (Willis and MacDonald, 2011; Roberts, 2013). Assuming that species ecological requirements are stable over time, the main premise in paleoecology is that changes in species assemblages similarly reflect past environmental changes within a study site or region (Birks et al., 2016). Among biological proxies employed for paleolimnological investigations, several organisms have been used such as chironomids, diatoms, ostracods, cladocerans, pollens and testate amoebae (Douglas, 2013).

The testate amoebae (Amoebozoa: Arcellinida; Rhizaria: Euglyphida; Stramenopiles, Labyrinthulomycetes) are single-celled organisms characterized by the presence of a "shell" that protects the cytoplasm (Ogden and Hedley, 1980; Adl et al., 2019; Todorov and Bankov, 2019). These organisms are globally distributed and are abundant in freshwater environments (Patterson et al., 1985; Asioli et al., 1996; Roe and Patterson, 2006; Yang et al., 2010; Burdíková et al., 2012; Davidova and Vasilev, 2013; Ju et al., 2014). Among lotic systems, testate amoebae can be found in all types of lakes, from the littoral to the profundal zone (Sigala et al., 2016) and play a crucial role in organic matter cycling, energy flow and water purification (Han et al., 2011a). Moreover, testate amoebae are highly sensitive to environmental conditions (Neville et al., 2010; Han et al., 2011a; Ndayishimiye et al., 2021): the populations can change with natural climate variability (Yang et al., 2006; Han et al., 2011b; Burdíková et al., 2012; Ren et al., 2018) and species that can survive in the new modified environment are those with the greatest adaptation abilities (Han et al., 2011a). Due to these characteristics, testate amoebae are well-suited as bioindicators and have been employed from lake sediments to reconstruct past climate and environmental changes (Schönborn, 1973; Ruzicka, 1982; Patterson et al., 1985; Asioli et al., 1996; Charman, 2001; Mitchell et al., 2008; Qin et al., 2009; Prentice et al., 2018). Testing the response of lacustrine testate amoebae to climate and environmental changes for the Late glacial—Holocene transition in the Lake Lautrey (Jura Mountains, eastern France), Wall et al. (2010a) strengthened the potential of the lacustrine testate amoebae in the paleoclimatic and paleoecological reconstructions.

Several studies have shown strong relationships between lacustrine testate amoeba assemblages composition and environmental parameters such as salinity, conductivity, pH, pollution and anthropogenic impact (Schönborn, 1965, 1966, 1973; Schönborn et al., 1965; Schönborn et al., 1984; Ruzicka, 1982; Patterson, 1996; Roe et al., 2010; Patterson et al., 2012a). Indeed, testate amoebae are sensitive to industrial contamination (Asioli et al., 1996; Patterson, 1996; Reinhardt et al., 1998) and to mine-derived contaminants including potentially toxic trace elements (Kauppila et al., 2006; Kihlman and Kauppila, 2009, 2010, 2012; Nasser et al., 2016; Gavel et al., 2018; Misailidis et al., 2017; Cockburn et al., 2020; Nasser et al., 2020a, b). The reconstruction of past climates and environments using testate amoebae proxies has focused on the water table in peatlands, pH, nutrients and recently on water temperature (Ndayishimiye et al., 2020). Recently, Charqueño-Celis et al. (2019) found that dissolved oxygen concentration and water depth are important factors affecting the composition of testate amoeba assemblages in some lakes in southern Mexico. Ndayishimiye et al. (2020) observed that changes in testate amoeba assemblages in a Chinese alpine lake could be due to soil erosion and nutrient inputs due to anthropogenic activities. Currently, there is a paucity of studies regarding testate amoebae in alpine lakes and no information is available for high-altitude lakes in the Alps. In this context, it was deemed of interest to investigate temporal changes in testate amoeba assemblages in a high-altitude lake located in the Italian Alps (Balma Lake, Piedmont, Italy). The main aims of the present research were (i) to characterize the testate amoebae paleo-assemblages of an alpine lake, located in an area where these organisms have not previously been investigated; as the main testate amoebae in lacustrine environments are Arcellinida (Marcisz et al., 2020), we focused only on this group; (ii) to assess changes in testate amoeba assemblages in relation to environmental factors and to anthropogenic impact; (iii) to compare the results obtained for testate amoeba assemblages with the effects observed in other paleoecological proxies such as Diptera Chironomidae and diatom assemblages. This research provides new insights for the evaluation of temporal changes in testate amoebae through paleoecological investigations in alpine lakes.

## 2. Materials and methods

### 2.1. Study area

Balma Lake is an alpine lake located above the tree line in the Cottian Alps (Municipality of Coazze, Piedmont, northwestern Italy), at 2116 m a.s.l. (Fig. 1a and b). It is included in the Special Area of Conservation (SAC) and Special Protection Area (SPA) IT1110006, called "Orsiera Rocciavrè" and in the Orsiera Rocciavrè Nature Park. The lake is of glacial origin with a circular perimeter equal to 414 m; the surface area is 1.21 ha, and the maximum depth is 6.42 m. The catchment is composed of ophiolite metamorphic bedrock and the landscape is dominated by rocky outcrops, ridges, and mountain walls. The ice cover generally lasts from late October to late May/early June. A small inlet is located at the western shore dividing into three small branches before entering the lake. Although a true outlet is not evident, Balma Creek originates from water filtration through the sediments at the eastern side of the basin (Fig. 1b and c) (Perilli et al., 2020; Cantonati et al., 2021). Values of the main physico-chemical parameters recorded during the sampling period are reported in Table 1.

The most relevant anthropogenic impacts in Balma Lake area over the last four decades of the 20th century are represented by the long-distance airborne transport of pollutants from the urban areas in the plain, grazing activities, and fishing (Pastorino et al., 2020; Perilli et al., 2020; Cantonati et al., 2021). Although Balma Lake was originally without fish, the brook trout *Salvelinus fontinalis* Mitchill, 1814 was introduced for recreational fishing in the 1970s (Balma et al., 1992; Pastorino et al., 2020; Perilli et al., 2020; Cantonati et al., 2021). The brook trout population is well structured, with individuals in age classes from 0+ to 4+ and an average fish biomass equal to 17.9 g $m^{-2}$ (Pastorino et al., 2020).

### 2.2. Sediment core sampling and testate amoebae identification

Coring was done during the ice-free period in October 2018 using a 50 mm Kajak-type gravity sediment corer (Renberg, 1991) and a 30-cm long sediment core was extracted from the deepest point of the lake (6.42 m depth) (Fig. 1c). The sediment core was sealed in a coring tube and brought to the laboratory where it was stored at 4 °C until subsampling. Subsamples were obtained by cutting the core into 2-cm thick interval transverse sections, in agreement with previous analyses performed by Perilli et al. (2020) and Cantonati et al. (2021) for the same site. Each level was named using the letter "L" for "level" followed by the middle depth of the section (from L1 for the top level to L29 for the bottom level). The age-depth model used for the present study was developed for Balma Lake by Perilli et al. (2020) using the RStudio Package Clam 2.3.2 (Blaauw, 2010) through smooth interpolation function, analyzing $^{14}C$ AMS data (two points) and total Pb concentrations

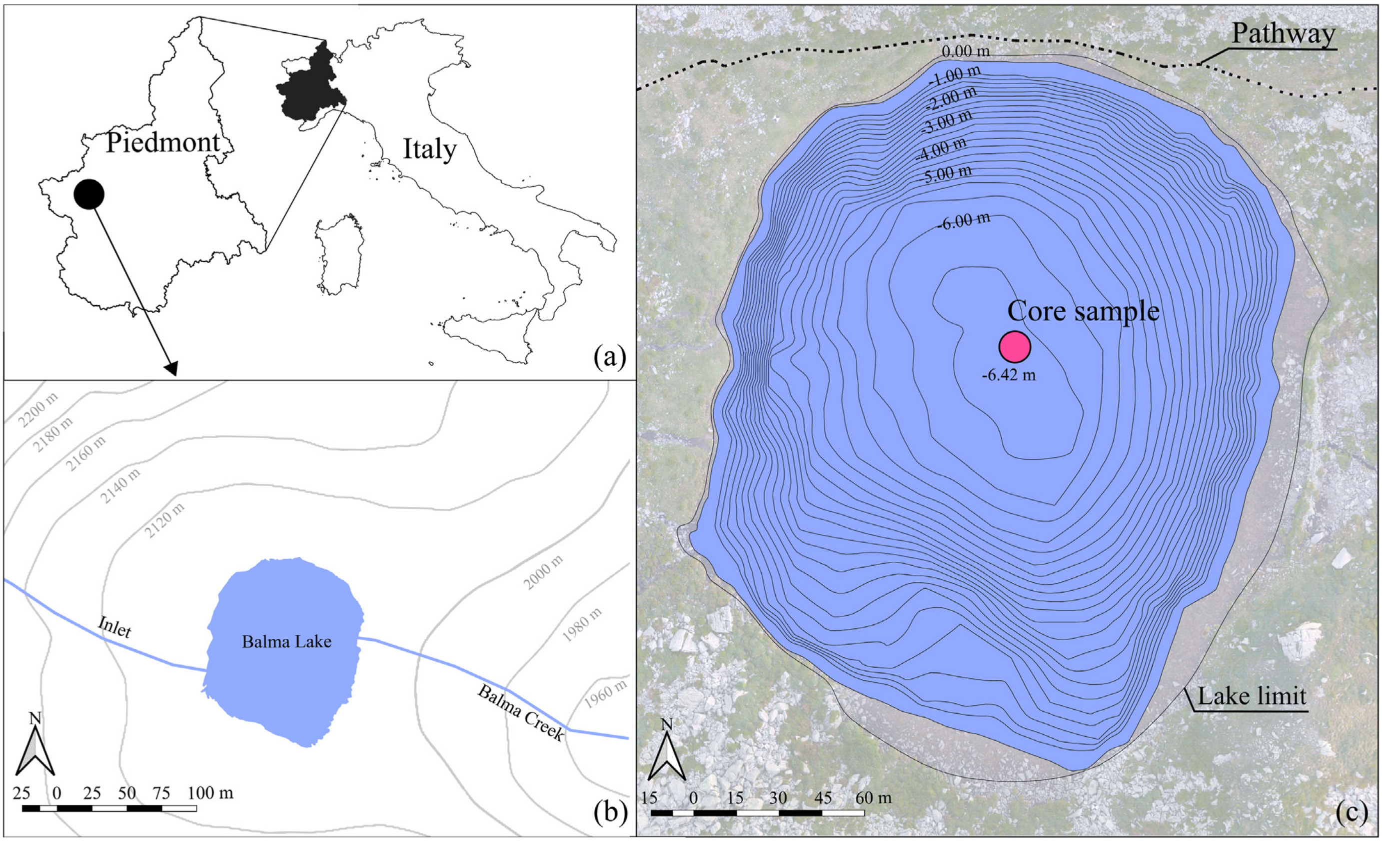

**Fig. 1.** Study area (a, b) and location of the sediment core sampling station in Balma Lake (c).

**Table 1**
Mean values and standard deviations of physico-chemical parameters measured at Lake Balma during the sampling period in autumn 2018 (from Cantonati et al., 2021). Mean values were calculated from different samplings taken both in littoral areas (5 samplings) and in the profundal zone (3 sampling points, mean depth of the water column).

| | Mean ± SD | | |
|---|---|---|---|
| Temperature (°C) | 8.44 | ± | 0.37 |
| Dissolved oxygen conc. (mg $L^{-1}$) | 5.91 | ± | 0.32 |
| Oxygen saturation (%) | 65 | ± | 4 |
| pH | 7.61 | ± | 0.10 |
| Conductivity (μS $cm^{-1}$) | 19 | ± | 1 |
| $NH_4^+$ (μg $L^{-1}$) | 70 | ± | 40 |
| $NO_3^-$ (mg $L^{-1}$) | 7.34 | ± | 2.06 |
| TP (μg $L^{-1}$) | 10 | ± | 10 |

(two points) determined by means of Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). These data were obtained from another sediment core collected in 2017 near the core sampling site considered in the present study. Average sediment accumulation rate for Balma Lake was 0.018 cm $year^{-1}$ (Perilli et al., 2020; Cantonati et al., 2021).

### 2.3. Core chemistry and sediment characteristics

In order to analyze total carbon content ($C_{tot}$), total organic carbon (TOC, %) and total nitrogen (TN, %), 15 mg of each sediment sample were manually powdered using an agata mill, and oven-dried at 105 °C for 24 h. Samples were acidified using HCl, increasing concentration up to 18%, and processed by frontal chromatography using a CHN Analyzer (model ECS 4010 CHNSO, manufactured by Costech Analytical Technologies Inc, Valencia, California, U.S.A) (Hedges and Stern, 1984). C/N ratio was then calculated.

For trace element (Pb, Zn, As and Cd) determination, freeze-dried (CoolSafe 55-4 SCANVAC) sediment samples were manually powdered using an agata mill and aliquots of 0.300 g were acid-digested in PTFE vessels in a closed microwave system (Multiwave PRO Anton Paar, Graz, Austria). The acid-digestion was performed using a mixture of 5 mL of nitric acid ($HNO_3$, 65–69% v/v, VWR) and 1 mL of hydrogen peroxide ($H_2O_2$, 30% m/v, Supelco), following the modified EPA Method 3052. The obtained solutions were then centrifuged to remove solid residue particles and diluted up to a volume of 25 mL by adding MilliQ water. Samples were diluted 1:20 and analyzed by ICP-MS using a NexION 350X Spectrometer equipped with an ESI SC Autosampler (PerkinElmer, Waltham, Massachusetts, U.S.A.). The instrument was calibrated using five standard solutions (ranging between 0.5 and 100 μg $L^{-1}$) prepared by diluting a multistandard solution for ICP analysis (10 mg $L^{-1}$, Periodic Table MIX 1, Sigma Aldrich). Analysis was performed using the KED (Kinetic Energy Discrimination) mode in order to avoid and reduce cell-formed polyatomic ion interference. The precision of the analysis expressed as RSD % was <3%.

For grain size analysis, 2 g of each sediment sample was treated with hydrogen peroxide ($H_2O_2$, 3%) in order to eliminate the majority of the organic matter. After 24 h samples were washed with distilled water and filtered with a sieve (1000 μm mesh) to eliminate the coarse component which was then considered separately for analysis. Samples were then processed with a Malvern Mastersizer 2000 laser diffraction particle size analyzer. As indices of high transport capacity, one percentile Cμ and median diameter Mμ were considered.

### 2.4. *Testate amoebae extraction and identification*

In order to facilitate sediment break-up, freeze-dried samples were washed with distilled water and subsequently screened with a 500 μm sieve to remove coarse organic particles and then with a 45 μm mesh filter to remove fine organic and mineral detritus and in order to avoid the loss of small specimens (Medioli et al., 1994; Asioli et al., 1996; Boudreau et al., 2005; Roe and Patterson, 2006; Wall et al., 2010b). The residues on the 45 μm sieve were retained for testate amoebae analysis.

Testate amoebae were then extracted, mounted on slides, and identified and counted using a light microscope at 200–400 × magnification. Identification and classification were performed according to Mazei & Tsyganov (2006), Mazei and Warren (2012, 2014, 2015), Siemensma (2021) and Todorov and Bankov (2019). 300 individuals per sample were identified; generally, an amount of 150 individuals is considered representative for paleolimnological investigation, sufficient for community data statistical analysis, and for a correct estimation of rare species abundances (Patterson and Fishbein, 1989; Payne and Mitchell, 2009; Wall et al., 2010b). Three subsamples were considered for each section of Balma Lake sediment core and testate amoeba densities (ind $g^{-1}$) were calculated for each subsample.

### 2.5. *Statistical analysis*

A principal component analysis (PCA) was used to investigate the testate amoeba assemblages, using the Q-mode and the VARIMAX rotation (Malmgren and Haq, 1982; Mackensen et al., 1990; Majewski et al., 2018). PC loadings exceeding a value of 0.4 are regarded as statistically significant after Malmgren and Haq (1982). Three principal components explain 97.0% of the total variance. Most PCA components are characterized by a single species with the highest positive PCA scores contribute to the definition of the assemblages. Taxa that accounted for at least 2% of the total association in at least two samples were included. Based on the PCA results, five core section groups, corresponding to different core zones, were identified (named Groups 1, 2, 3, 4 and 5, from the bottom to the top of the core).

A resemblance matrix was obtained from testate amoebae density data using the Bray–Curtis measure and one-way PERMANOVA (Anderson, 2001; McArdle and Anderson, 2001) was performed to check significant differences among assemblages of the core section groups, which were pairwise compared. Prior to analyses, data were transformed (log $[x+1]$) to reduce the influence of very abundant taxa (Clarke and Gorley, 2006) and the multivariate homogeneity of group dispersions was checked using PERMDISP2 (Anderson, 2006). A SIMPER analysis (Clarke, 1993) was applied to the data matrix to identify the main taxa which most highly contributed to observed significant differences highlighted by the PERMANOVA.

Redundancy Analysis (RDA) (ter Braak and Smilauer, 1998; Legendre and Legendre, 1998) was used to investigate the relationship between testate amoebae and environmental variables through the core section. RDA was chosen after applying the Detrended Correspondence Analysis (DCA), as the gradient lengths were <4 standard deviations (ter Braak & Smilauer, 2012). To avoid multicollinearity, a subset of environmental variables was chosen after correlation analyses. The Pearson product-moment correlation coefficient ($r$) was used for this purpose. If two or more variables were strongly correlated (threshold value $r < [|0.7|$ and $p < 0.001$), one of them was excluded from the analysis (Dormann et al., 2013). In addition, the presence of fish was also considered a variable. The total explained variance within the testate amoebae species data was partitioned among four groups of variables associated with nutrients, trace elements, sediment characteristics and the presence of fish using variation partitioning analysis (VPA) (Borcard et al., 1992). The contribution of each variable group was then plotted as a Venn diagram. The significance of each variable group and relative interactions were checked using the Monte Carlo permutation test with 999 permutations.

The analyses described above (PERMANOVA, SIMPER, RDA and VPA), were also applied to the dataset regarding Chironomidae assemblages reported for Balma Lake by Perilli et al. (2020), to check for common ecological trends between testate amoeba and chironomid assemblages. Chironomid assemblages were determined via the analysis of another 30 cm-long sediment core, previously collected in Balma Lake in 2017, near the core sampling site considered in the present study. Analyses were based on core section groups identified by the PCA performed on the testate amoebae and described in this research.

All analyses were performed using RStudio version 2021.9.0.351 (R Core Team, 2021). Figures were produced with RStudio and with Origin (Pro), (Version 2021b; OriginLab Corporation, Northampton, MA, USA) and processed with software Inkscape version 0.92.

## 3. Results

### 3.1. *Core chemistry and sediment characteristics*

The grain size analysis highlighted the presence of sandy silt in all core samples, representing the deep zone of the lake. The highest values of Mμ (61.2) and Cμ (789) were recorded at the top of the core (Fig. 2). Sedimentological changes were observed along the core sections with poorly sorted sediments in the top and bottom core sections interspersed by poorly sorted sandy pelite sediments.

Total carbon ($C_{tot}$) concentrations ranged between 1.2% and 8.1%, showing a fluctuating trend with increasing depth, with three lower values at sections L25, L21 and L9 (1.9%, 1.9% and 1.2%, respectively) and the maximum values reached at sections L19 and L29 (6.8% and 8.1%, respectively). Total organic carbon (TOC) and TN values showed similar parallel trends with maximum values (0.6% and 7.4%, respectively) in section L29 (bottom of the core) and alternating increasing/decreasing phases, with lower values at three levels along the core (in sections L25, L15 and L5). The C/N ratio increased with increasing depth reaching the maximum value (11.2) at the bottom, with a drop-down point at sections L13 and L15 (values equal to 9.1 and 9.2, respectively).

Trace element concentrations showed a constant decreasing trend from the top to the bottom of the core. Pb, Zn and As maximum values (73.1, 78.1 and 2.3 μg $g^{-1}$, respectively) were recorded at section L3, whereas maximum value for Cd was recorded in section L5 (0.25 μg $g^{-1}$). Levels of Pb, Zn and to a lesser extent As, showed higher concentrations in the upper sections of the core, decreasing from section L7 towards the bottom. Cadmium reached generally low concentrations, especially at the top of the sedimentary sequence decreasing with increasing depth from section L9 to the bottom.

There was a strong significant correlation among trace element concentrations ($r^2 > 0.79$, $n = 45$, $p < 0.001$), therefore, only Pb and As were chosen for further analyses. Total organic carbon (TOC) was strongly correlated with total nitrogen (TN) ($r^2 = 0.98$, $n = 45$, $p < 0.001$), therefore, TOC was excluded from the analyses reported below. TN was chosen as a significant factor affecting testate amoeba assemblages (Gilbert et al., 1998). Other correlations showed values of the Pearson coefficient under the threshold level indicated by Dormann et al. (2013) ($r < [|0.7|$ and $p < 0.001$). The final dataset used for RDA and VPA analyses included the following

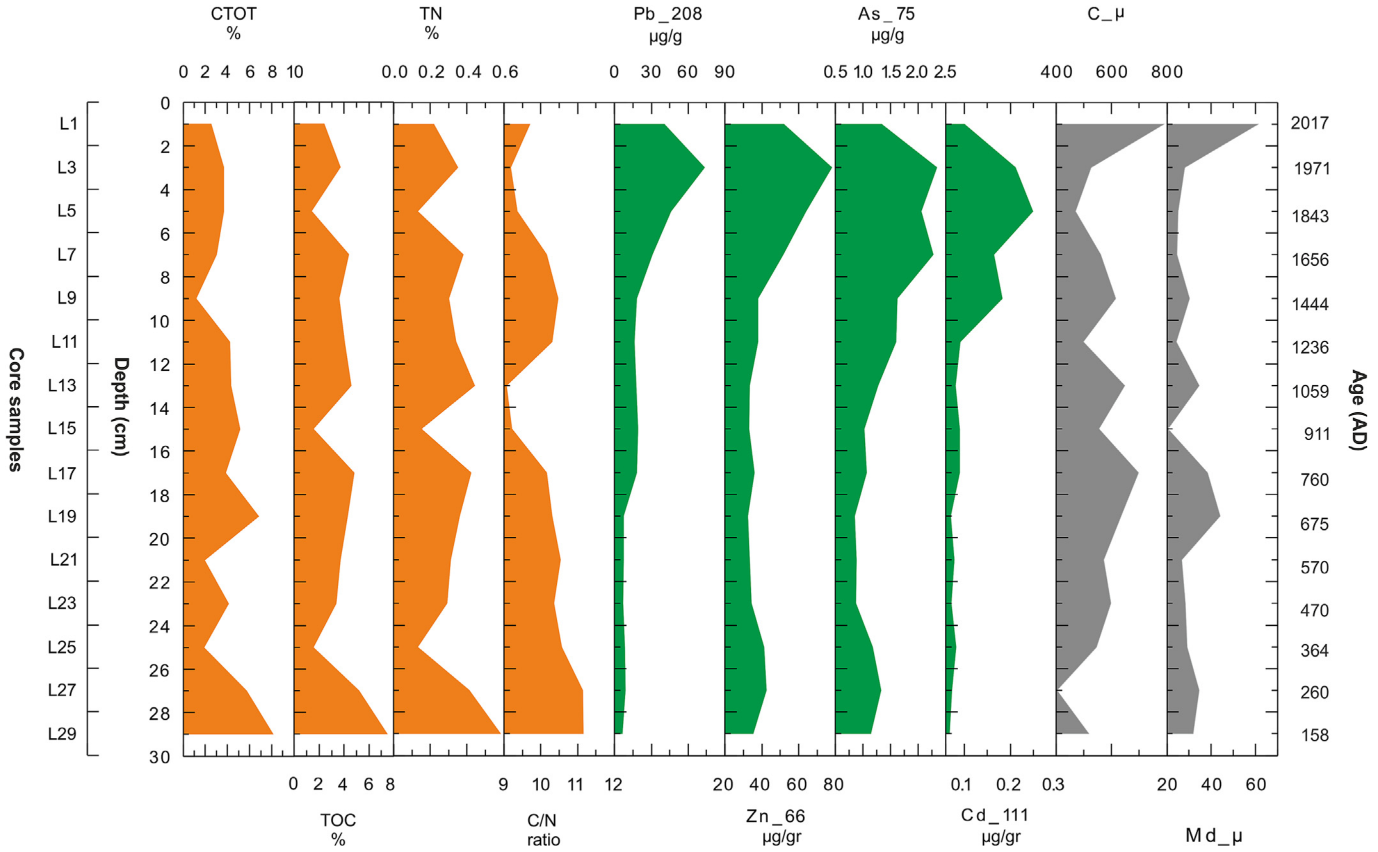

**Fig. 2.** Values of total carbon ($C_{tot}$%), total organic carbon (TOC%), total nitrogen (TN%), C/N ratio, trace element concentrations (Pb, Zn, As, Cd, µg $g^{-1}$) and descriptors of sediment characteristics (first percentile Cµ and median diameter Mµ) observed along the sediment core samples of Balma Lake. Samples are indicated by the letter L, followed by the average depth of the section (L1-L29).

variables: $C_{tot}$, TN and C/N for nutrients, Pb and As for trace elements, one percentile Cµ and median diameter Mµ for sediment characteristics and the presence of fish.

### 3.2. *Testate amoeba assemblages*

Analyses allowed for the identification of 6946 testate amoebae individuals (4 genera and 11 species) belonging to Order Arcellinida Kent, 1880, with four variants (Fig. 3a, Fig. 4, Table 2). The average density of individuals observed along the core was 644 ind $g^{-1}$. The maximum and minimum number of individuals were observed in sections L7 (1746.7 ind $g^{-1}$) and L25 (95.8 ind $g^{-1}$), respectively. The only exception was section L21 where the testate amoebae almost disappeared and only a single species was recorded with very low density (*D. viscidula*, 2.3 ind $g^{-1}$). Except for section L21, the number of taxa ranged between 9 and 13 (Fig. 5a). Values of the Shannon-Wiener index ranged between 1.46 and 2.02 (sections L13-L7) and was generally higher downcore than in the top levels (Fig. 5b). Dominance ranged between 0.16 and 0.35 (sections L15 and L25) (Fig. 5c).

Genus *Difflugia* was the most abundant along the core, with 8 species (4 variants) (Fig. 3a, Table 2) recorded. Among these species, *Difflugia viscidula* was observed in all the core levels and was the most abundant, ranging from 2.3 to 429.6 ind $g^{-1}$ (mean density 229 ind $g^{-1}$). *Difflugia rotunda* and *Difflugia acuminata* var. *Brevicaulis*, also showed high densities along the core (ranges 12.1–407.6 ind $g^{-1}$ and 10.2–372.6 ind $g^{-1}$, respectively). *D. rotunda*, *D. acuminata* var. *Brevicaulis*, *Difflugia oblonga* and *Difflugia pyriformis* were found in all the core levels with the only exception being section L21. Other *Difflugia* species were observed discontinuously through the core. Three species did not belong to the genus *Difflugia*: *Lesquereusia modesta*, *Centropyxis cassis* and *Pontigulasia bigibbosa*. The latter species was present in all the core levels except L21, whereas *L. modesta*, and *C. cassis* were mainly found at the top of the sedimentary sequence (Fig. 3a, Table 2).

The Q-mode PCA allowed for the highlighting of five core section groups which were identified on the basis of the most abundant species (Fig. 3b). In Group 1 (L29-L27cm/158–260 DC), *D. viscidula* and variants of *D. acuminata* were identified as the dominant species, in particular *D. acuminata* var. *Brevicaulis*. *D. acuminata* species decreased in Group 2 (L25-L21 cm/260–675 AD), which was dominated by *D. viscidula.* Group 3 (L19-L11 cm/675–1444 AD) was mainly characterized by both *D. viscidula* and *D. acuminata*. The densities of the latter species increased in Group 3, reaching values similar to those observed at the bottom of the core. *L. modesta* appeared in this group. However, densities of the testate amoeba species showed fluctuating trends (Fig. 3; Table 2). Group 4 (L9-L7/1444–1843 AD) was dominated by *D. rotunda*, *D. acuminata* and *D. viscidula*. With the only exception being *D. acuminata* var. *Inflata*, all the observed species increased in density in this group, especially in section L7. Finally, Group 5 (L5-L1/1843–2017 AD) differed from the others as densities of *D. acuminata* and *D. viscidula* decreased at the top of the core, whereas *D. rotunda* became the dominant species.

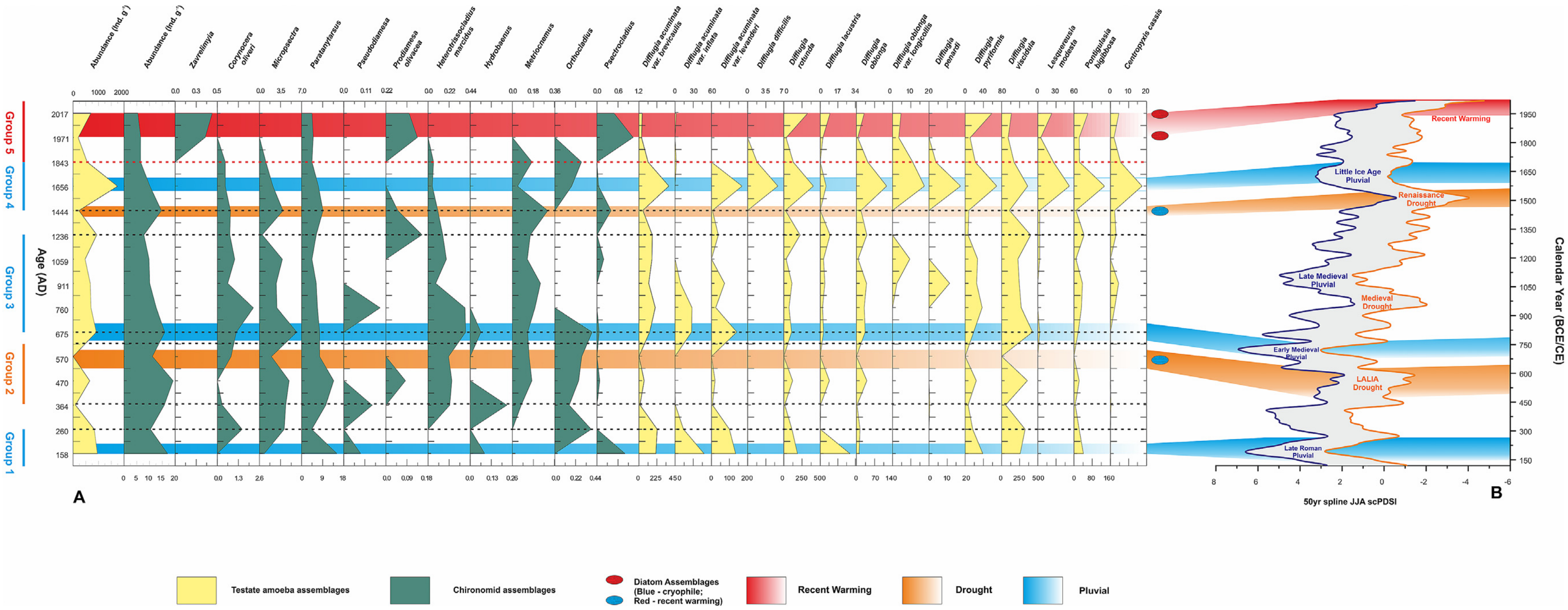

**Fig. 3.** Distribution of testate amoebae species (ind g$^{-1}$, yellow) along the sediment core of Balma Lake (a). Groups of the sediment core samples highlighted by the Q-mode PCA with VARIMAX rotation are reported on the left side between depths and ages of the sediment core samples. The age depth model was developed for Balma Lake by Perilli et al. (2020). Distribution of Chironomidae genera/species observed by Perilli et al. (2020) (ind g$^{-1}$, green) were also reported. The right side of the figure (b) shows the reconstruction of the summer temperatures (June, July, August) for Central Europe in the last 2000 years and the main climatic events which occurred in Europe during the Late Holocene (source: Büntgen et al., 2021, modified). Characteristics of diatom assemblages studied by Cantonati et al. (2021) in Balma Lake are indicated for each event (red and blue dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

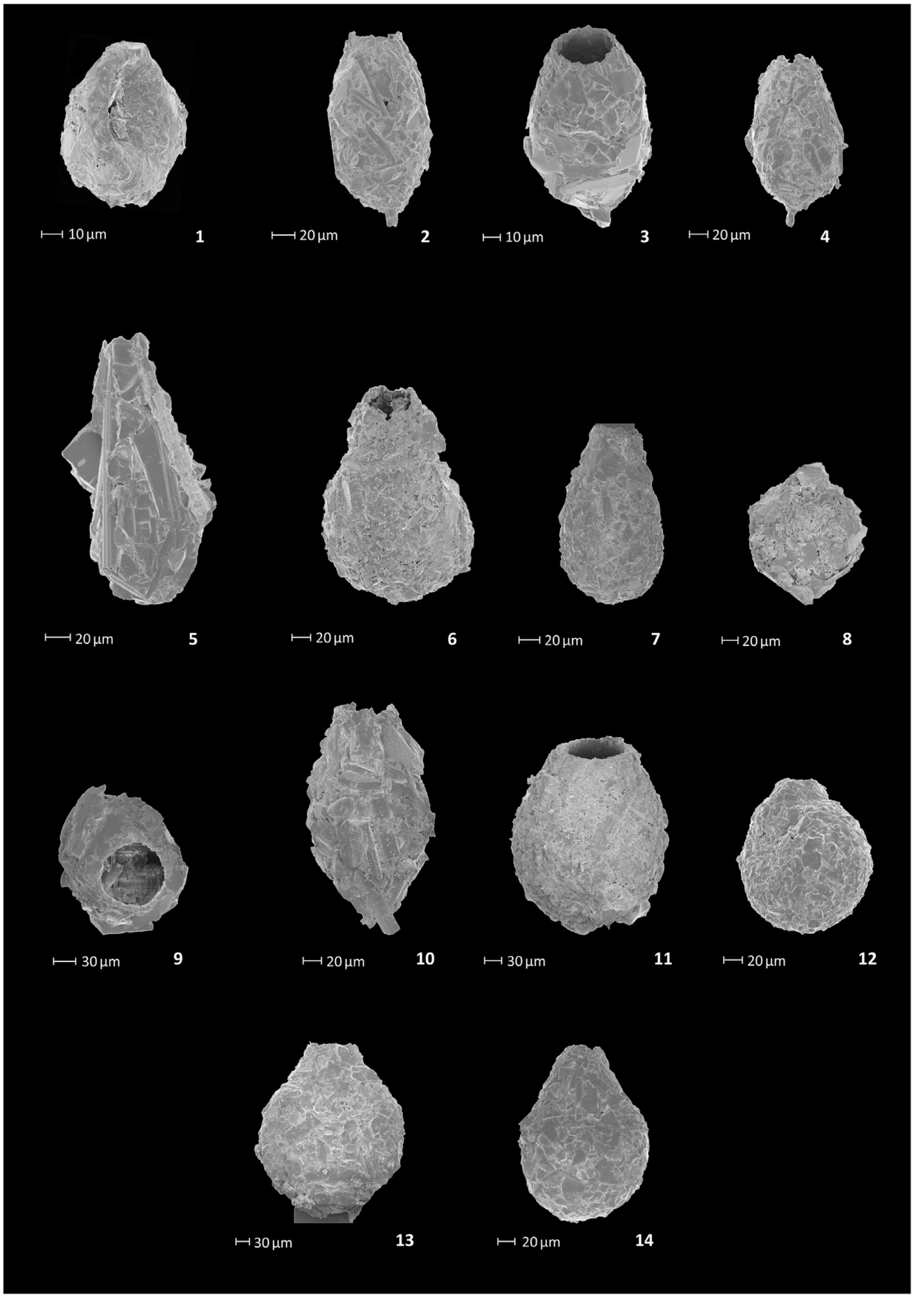

**Fig. 4.** Scanning electron microscope of selected arcellininid shells from the study lake (1 - *Centropyxis cassis*, lateral view; 2 - *Difflugia acuminata* strain "*brevicaulis*", lateral view; 3 - *Difflugia acuminata* strain "*levanderi*", lateral view; 4 - *Difflugia acuminata* strain "*inflata*", lateral view; 5 - *Difflugia lacustris*, lateral view; 6 - *Difflugia oblonga* strain "*longicollis*", lateral view; 7 - *Difflugia oblonga*, lateral view; 8 - *Difflugia rotunda*, lateral view; 9 - *Difflugia rotunda*, apertural view; 10 - *Difflugia pyriformis*, lateral view; 11 - *Difflugia viscidula*, lateral view; 12- *Lesquereusia modesta*, lateral view; 13—14 - *Pontigulasia bigibbosa*, lateral view).

### 3.3. *Testate amoebae-environment relationships*

All variable groups taken into consideration to explain the variation of the testate amoeba assemblages through the core were significant (Table 3) and explained 51.5% of the total variance (Fig. 6a, c). Among individual fractions, nutrients and trace elements explained most of the variation (respectively 26.2% and 24.2%). Sediment characteristics explained 13.4% whereas the presence of fish explained only 2.7%. The same analysis performed on the Chironomidae assemblages showed that all the variable groups were significant (Table 3) and explained 56.3% of the total variation (Fig. 6b, d). Analysis of the individual fractions highlighted that fish occurrence appeared to be associated with the highest percentage of explained variation (15.0%), followed by sediment characteristics (9.0%) and nutrients (7.2%). The lowest portion of the explained variation was associated with trace element concentrations (1.0%).

The application of the PERMANOVA on the testate amoebae dataset allowed for the detection of significant differences between the observed groups (Table 4). Group 5 (top of the core) and Group 2 (including section L21) differed from the others. The other groups did not differ. The SIMPER test highlighted that the observed dissimilarity was mainly related to *D. viscidula*, *D. Rotunda*, *D. acuminata* var *brevicaulis* and var. *levanderi*. The contribution of

**Table 2**
Densities (ind $g^{-1}$) of the testate amoeba species observed at each level of the Balma Lake core.

| Core section | L1 | L3 | L5 | L7 | L9 | L11 | L13 | L15 | L17 | L19 | L21 | L23 | L25 | L27 | L29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Core depth (cm) | 0–2 | 2–4 | 4–6 | 6–8 | 8–10 | 10–12 | 12–14 | 14–16 | 16–18 | 18–20 | 0–22 | 22–24 | 24–26 | 26–28 | 28–30 |
| *Difflugia* acuminata var. *Brevicaulis* (Thomas and Mabille, 1956) | 42.1 | 31.6 | 114.0 | 372.6 | 50.8 | 164.5 | 159.4 | 119.4 | 203.8 | 134.1 | | 65.5 | 10.2 | 227.4 | 210.0 |
| *Difflugia acuminata* var. *Inflata* (Penard, 1899) | 2.3 | | 1.8 | | | | | 9.0 | 28.1 | 27.4 | | | 0.3 | 13.9 | 47.7 |
| *Difflugia acuminata* var. *levanderi* (Playfair, 1914) | | | | 168.8 | 6.8 | 37.3 | | 72.1 | 23.4 | 140.2 | | 19.6 | 5.1 | 99.8 | 133.7 |
| *Difflugia difficilis* (Thomas, 1954) | | | 1.8 | 5.8 | | | | | | | | | | | |
| *Difflugia rotunda* (Dujardin, 1837) | 327.2 | 30.3 | 126.9 | 407.6 | 22.7 | 223.5 | 31.6 | 110.4 | 70.3 | 106.6 | | 117.8 | 12.1 | 110.9 | 187.7 |
| *Difflugia lacustris* (Penard, 1899) | 9.3 | 2.7 | | 5.8 | | 9.3 | 3.2 | 4.5 | 2.3 | 3.0 | | 8.7 | 0.3 | | 28.6 |
| *Difflugia oblonga* (Ehrenberg, 1838) | 30.4 | 4.7 | 44.1 | 116.4 | 17.4 | 40.4 | 6.3 | 38.3 | 30.5 | 33.5 | | 39.3 | 1.9 | 13.9 | 9.5 |
| *Difflugia oblonga* var. *longicollis* (Gassowsky, 1936) | 4.7 | 3.4 | 11.0 | 17.5 | | | 9.5 | 2.3 | | | | | | | |
| *Difflugia penardi* (Penard, 1890) | | | 3.7 | 17.5 | | | | 11.3 | | | | | 0.3 | | |
| *Difflugia pyriformis* (Perty, 1849) | 58.4 | 10.8 | 22.1 | 69.9 | 7.6 | 6.2 | 26.8 | 24.8 | 37.5 | 18.3 | | 24.0 | 6.4 | 16.6 | 38.2 |
| *Difflugia viscidula* (Penard, 1902) | 137.9 | 90.2 | 171.0 | 361.0 | 108.4 | 400.5 | 225.7 | 243.3 | 271.7 | 429.6 | 2.3 | 357.9 | 53.6 | 327.2 | 257.8 |
| *Lesquereusia modesta* (Rhumbler, 1895) | 23.4 | 4.7 | 25.7 | 52.4 | 3.0 | 3.1 | 3.2 | | | 3.0 | | | | | |
| *Pontigulasia bigibbosa* (Penard, 1902) | 60.8 | 21.5 | 23.9 | 133.9 | 8.3 | 43.5 | 7.9 | 36.0 | 32.8 | 18.3 | | 21.8 | 5.1 | 22.2 | 41.4 |
| *Centropyxis cassis* (Wallich, 1864) | 4.7 | 2.0 | 5.5 | 17.5 | 2.3 | 3.1 | | 4.5 | 2.3 | | | | 0.3 | | |
| Total | 701.1 | 201.9 | 551.6 | 1746.7 | 227.4 | 931.4 | 473.5 | 675.7 | 702.7 | 914.1 | 2.3 | 654.7 | 95.8 | 831.9 | 954.6 |

these species to the dissimilarity was up to 76% (Table 5).

The application of the PERMANOVA to the Chironomidae assemblages, based on the core section groups identified by the Q-mode PCA applied on testate amoebae dataset, also highlighted significant differences among the core section groups (Table 4). Only Group 5 significantly differed from the others, which did not differ among them. The SIMPER test highlighted that the dissimilarity was mainly due to *Paratanytarsus austriacus* type, *Micropsectra*, *Zavrelimyia*, *Psectrocladius*, *Corynocera oliveri* and *Heterotrissocladius marcidus* type. These taxa contributed to 95.3% of the observed dissimilarity (Table 5).

## 4. Discussion

### 4.1. Core chemistry and sediment characteristics

The grain size analyses highlighted the occurrence of sandy silt for the deep area of Balma Lake, with sedimentological variations observed along the core sections. These results are likely related to high contribution from the shores with mixed particle size due to increased/intense precipitation or possible short flash flood events. Depositional trends in lakes depend on the relationship between the catchment extension and the lake surface. Lakes with small catchments generally show low sedimentation rates (Anderson et al., 2011; Ilyashuk et al., 2011; Arnaud et al., 2016), with values of approximately 0.01 cm $year^{-1}$, due to the absence of notable terrigenous sediment contributions and low primary productivity. This is likely the case of Balma Lake, which showed a sedimentation rate equal to 0.018 cm $year^{-1}$ (Perilli et al., 2020). In this condition, erosion flux largely depends on the occurrence of extreme events (Arnaud et al., 2016). For instance, in high altitude systems dominated by terrigenous sediment, few events play an important role for the export of sediment from the catchment area exclusively triggered by flash-flood events such as recorded in Lake Anterne sediments (Giguet-Covex et al., 2012).

The deposition of TOC is affected by multiple processes including nutrient inputs, which could increase primary production (Anderson et al., 2013; Dietz et al., 2015; Gallant et al., 2020). In addition, increasing water temperatures and high oxygen exposition of the sediments contribute to increase TOC mineralization and $CO_2$ production (Sobek et al., 2009; Gudasz et al., 2010; Marotta et al., 2014; Beaulieu et al., 2019). Moreover, TOC deposition is influenced by variations regarding the deposition of terrigenous components due to changes in the landscape (Anderson et al., 2013; Alcocer et al., 2020). Values of TN and TOC observed in Balma Lake core varied across the sections and drop-down events observed in levels L5, L15 and L25 could be related to decreasing temperatures due to climate changes which occurred in the late Holocene. On the other hand, increasing temperatures and insolation could positively affect organic production, as TOC content in alpine lakes is mainly influenced by climatic factors (Rosén, 2005). The range of C/N ratio values observed for Balma Lake (9.1–11.2) overlap with those related to the occurrence of phytoplankton (4–10), similar to those related to aquatic macrophytes (10–20), and notably lower than those related to terrestrial plants (>20) (Meyers, 2003; Zhao et al., 2020).

Regarding trace element concentrations in Balma Lake core, the results from this study were consistent with those reported for similar alpine lakes in France, Switzerland and Germany. Indeed, several studies confirmed that the amount of trace elements in lake sediments has notably increased since the end of the XIX century (Shotyk et al., 2001, 2003; Arnaud et al., 2003; Arnaud, 2003; Aboud and Nandini, 2009). In detail, the highest trace element levels were recorded between the 60's and 70's due to the rapid industrial development in Europe after World War II. In

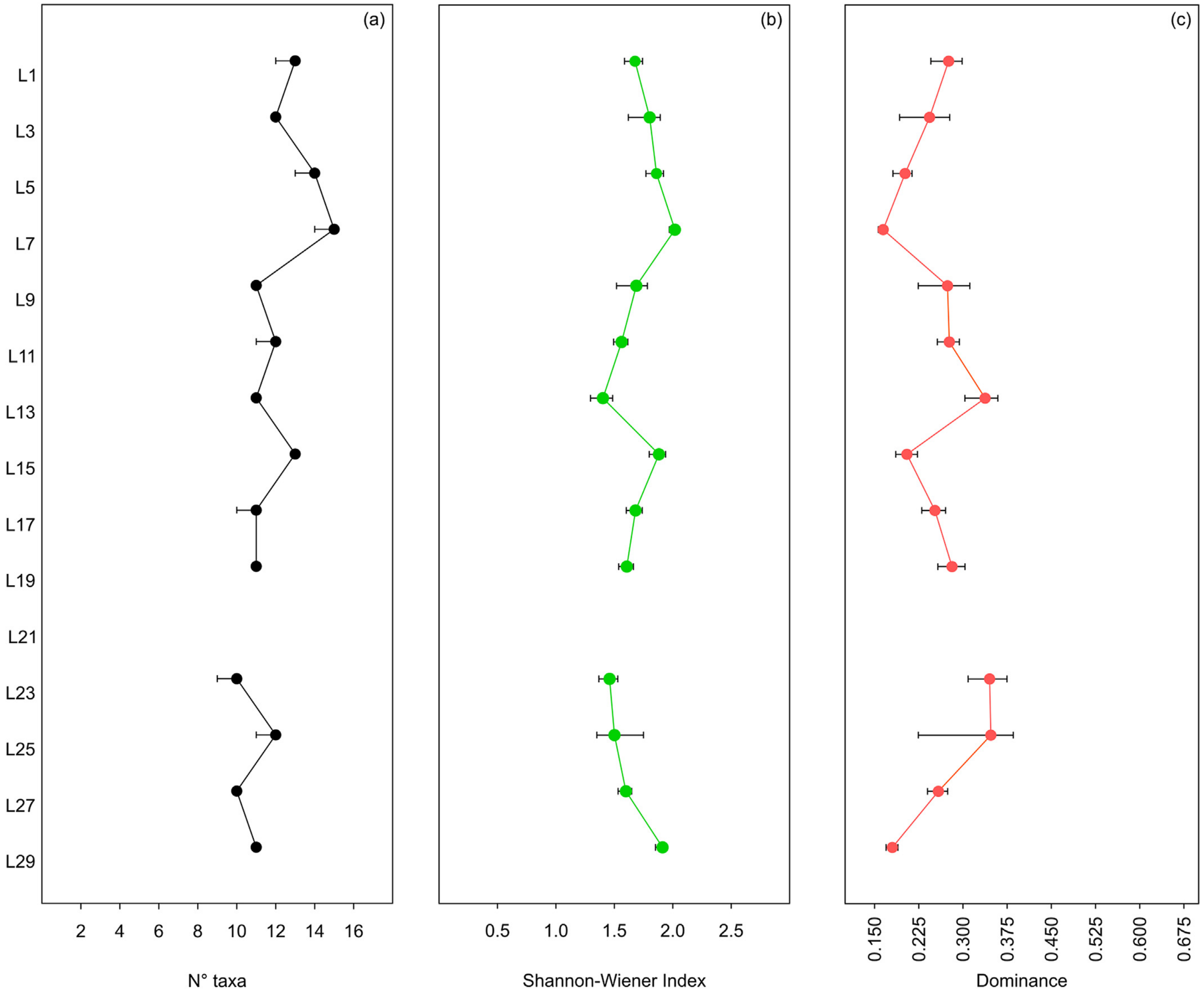

**Fig. 5.** Values of main community indices calculated for testate amoebae assemblages observed in the Balma Lake sediment core. Core section L21 was omitted, as only one species was observed.

**Table 3**
Results of the Redundancy analysis (RDA) and variance partitioning (VPA) performed on testate amoebae species observed in the Balma Lake sediment core samples. Results of the RDA performed on Chironomidae taxa observed in a core sample collected in the same lake by Perilli et al. (2020) are also reported. VPA groups of variables were associated with nutrients ($C_{tot}$, C/N e TN), trace elements (Pb, As), sediment characteristics (first percentile Cμ and median diameter Mμ) and the presence of fish. Results of the VPA applied to the Chironomidae assemblages observed in the Balma Lake sediment core are also reported.

| RDA | Testate amoebae | | Chironomids | |
|---|---|---|---|---|
| | RDA1 | RDA2 | RDA1 | RDA2 |
| Eigenvalue | 0.041 | 0.024 | 0.067 | 0.007 |
| Proportion Explained | 0.289 | 0.169 | 0.525 | 0.057 |
| Cumulative Proportion | 0.289 | 0.458 | 0.525 | 0.582 |
| Significance (999 permutations) | **$p < 0.001$** | **$p < 0.001$** | $p <$ **0.001** | $p =$ **0.025** |

| **VPA** | **Testate amoebae** | | | | | **Chironomids** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | $r^2$ | **Adjusted $r^2$** | **d.f.** | $F$ | ***p*-level** | $r^2$ | **Adjusted $r^2$** | **d.f.** | $F$ | ***p*-level** |
| Nutrients + Trace elements + Sediment + Fish | 0.603 | 0.515 | 8 | 6.848 | < **0.001** | 0.643 | 0.563 | 8 | 8.092 | < **0.001** |
| Nutrients | 0.239 | 0.184 | 3 | 4.299 | < **0.001** | 0.083 | 0.016 | 3 | 2.265 | **0.047** |
| Trace elements | 0.207 | 0.169 | 2 | 5.478 | < **0.001** | 0.29 | 0.257 | 2 | 8.597 | < **0.001** |
| Sediment | 0.078 | 0.034 | 2 | 2.165 | **0.049** | 0.108 | 0.066 | 2 | 2.547 | **0.039** |
| Fish | 0.12 | 0.099 | 1 | 5.861 | < **0.001** | 0.45 | 0.438 | 1 | 35.247 | < **0.001** |

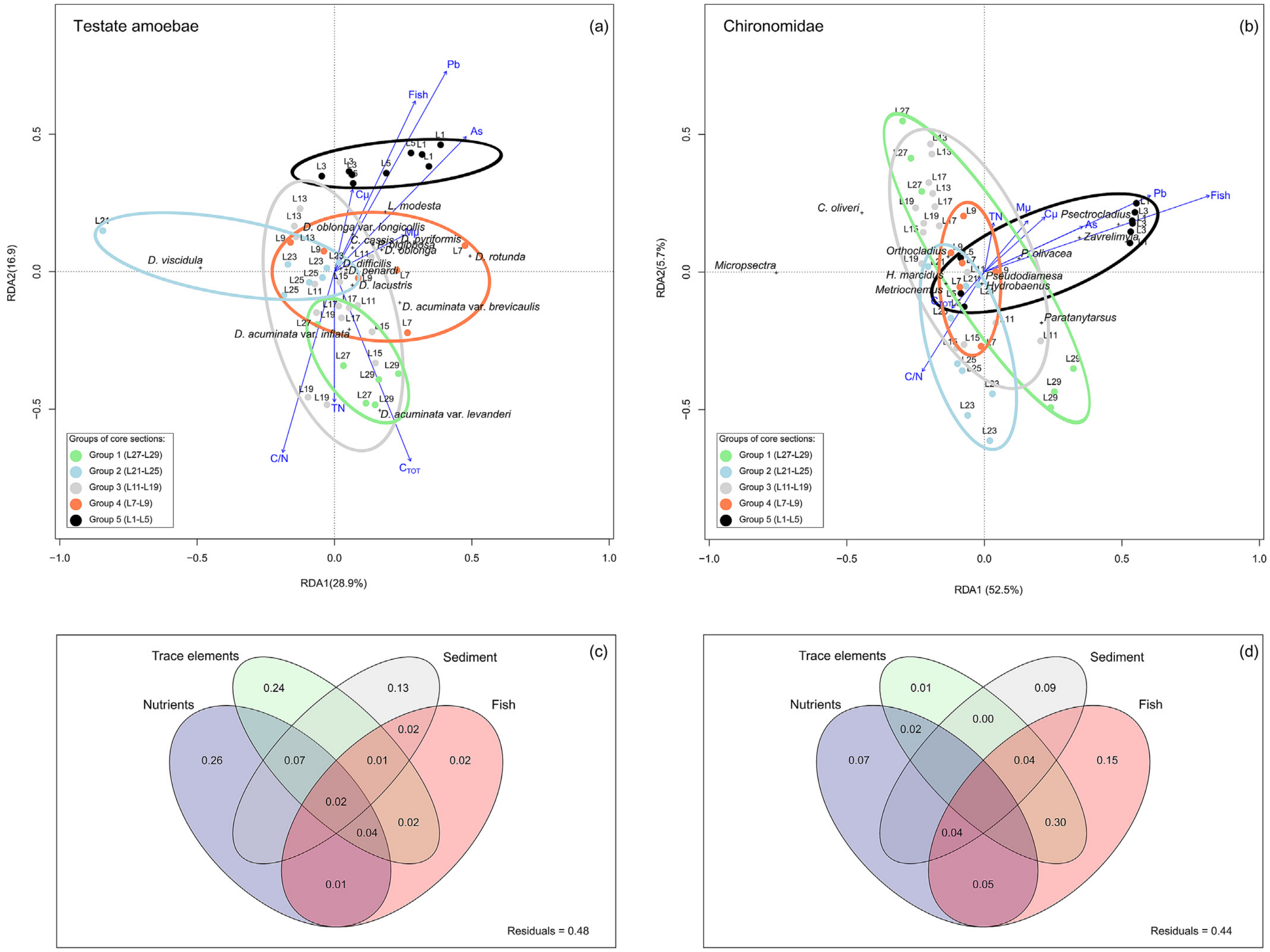

**Fig. 6.** Redundancy analyses (RDA) showing the relationships between testate amoebae species and considered variables (a) and between Chironomidae and considered variables (b). Venn diagrams show the variance partitioning (VPA) results for the four variable groups associated with nutrients ($C_{tot}$, C/N e TN), trace elements (Pb, As), sediment characteristics (first percentile Cμ and median diameter Mμ) and the presence of fish in relation to the testate amoebae RDA (c) and the Chironomidae RDA (d). Unexplained variance and explanations <1% are not shown.

**Table 4**
Results of PERMANOVA tests based on testate amoebae and chironomid assemblages observed in the Balma Lake sediment core for the sample groups identified by Q-mode PCA with VARIMAX rotation applied to the testate amoebae dataset.

| Testate amoebae PERMANOVA | | | | | | |
|---|---|---|---|---|---|---|
| Factor | d.f. | Sum of squares | Mean square | *F* | $r^2$ | *p*-value |
| Core section groups | 4 | 1.26 | 0.315 | 3.663 | 0.268 | **0.002** |
| Residuals | 40 | 3.439 | 0.086 | | 0.732 | |
| Total | 44 | 4.699 | | | 1 | |
| **Chironomidae PERMANOVA** | | | | | | |
| **Factor** | **d.f.** | **Sum of squares** | **Mean square** | ***F*** | $\mathbf{r^2}$ | ***p*-value** |
| Core section groups | 4 | 1.534 | 0.384 | 8.348 | 0.455 | < **0.001** |
| Residuals | 40 | 1.838 | 0.046 | | 0.545 | |
| Total | 44 | 3.372 | | | 1.000 | |

particular, elements such as Zn, Cu, and Pb showed the highest increasing trends, followed by Hg and Cr (Nedjai et al., 2021). The rapid decrease of the trace element content observed along the sediment core of Balma Lake could likely be related to the implementation of stricter emission standards and the introduction of lead-free petrol (Brännvall et al., 2001; Renberg et al., 2001; Siver and Wonziak, 2001). Indeed, since the 1970s, Pb emissions have decreased by 60–70% (Pacyna et al., 2007; Renberg et al., 2002; Thevenon et al., 2011), also showing a strong declining trend in Italy (ISPRA, 2009). In addition, stricter emissions for industrial activities were introduced in the same periods (Ministerial Decree July 12th, 1990).

**Table 5**
Results of SIMPER tests based on testate amoebae and chironomid assemblages observed in the Balma Lake sediment core for the sample groups identified by Q-mode PCA with VARIMAX rotation applied to the testate amoebae dataset.

| Testate amoebae | | | | Chironomidae | | | |
|---|---|---|---|---|---|---|---|
| Taxon | Av. dissim | Contrib. % | Cumulative % | Taxon | Av. dissim | Contrib. % | Cumulative % |
| *Difflugia viscidula* | 12.07 | 29.88 | 29.88 | *Paratanytarsus austriacus* type | 18.68 | 48.43 | 48.43 |
| *Difflugia rotunda* | 7.46 | 18.45 | 48.33 | *Micropsectra* | 11.32 | 29.34 | 77.77 |
| *Difflugia acuminata* var. *Brevicaulis* | 7.25 | 17.94 | 66.27 | *Zavrelimyia* | 3.46 | 8.96 | 86.74 |
| *Difflugia acuminata* var. *levanderi* | 3.93 | 9.72 | 75.98 | *Psectrocladius* | 1.68 | 4.36 | 91.09 |
| *Pontigulasia bigibbosa* | 2.16 | 5.34 | 81.33 | *Corynocera oliveri* | 0.87 | 2.26 | 93.36 |
| *Difflugia oblonga* | 2.07 | 5.13 | 86.46 | *Heterotrissocladius marcidus* | 0.76 | 1.96 | 95.31 |
| *Difflugia pyriformis* | 1.76 | 4.34 | 90.8 | *Orthocladius* | 0.67 | 1.73 | 97.04 |
| *Lesquereusia modesta* | 1.03 | 2.55 | 93.35 | *Metriocnemus* | 0.58 | 1.51 | 98.55 |
| *Difflugia acuminata* var. *Inflata* | 0.95 | 2.35 | 95.7 | | | | |

### *4.2. Temporal changes in testate amoeba assemblages*

Variations recorded for testate amoeba assemblages in Balma Lake sediment core allowed for the identification of different time periods/level groups that could be summarized as follows:

- Group 1 (L29-L27cm/158–260 AD): sediment parameters (Mμ and Cμ) did not allow for the highlighting of abundant inputs of coarse sediment in Balma Lake catchment, but this group is related to the highest C/N values observed along the core, which indicates the increasing contribution of land-plant organic matter in Balma Lake (Meyers, 2003). The dominant species were *D. viscidula* and *D. acuminata* with its different variants. The latter species prefers alkaline pH levels (Qin et al., 2013; Todorov and Bankov, 2019) and high nutrient concentrations (Schwind et al., 2017). The functional traits of *D. acuminata* could help the species for stabilization and predation (Han et al., 2008) and/or act as defense against predation (Gomaa et al., 2015). *D. viscidula*, which has one of the greatest shells observed in the present study (~320 μm) (Fig. 4). This is a functional trait that could be related to a high sensitivity to environmental changes, as larger shells need more time to recover compared to small organisms (Marcisz et al., 2020). These findings allow one to suppose that the Group 1 period was characterized by stability in precipitation regimes. These data suggest the occurrence of exogenous nutrient inputs in Balma Lake due to high precipitation and rainfall, in agreement with Büntgen et al. (2016, 2021) defining this period as "Late Roman Pluvial". This finding is supported by the results of the RDA and VPA application that highlighted how nutrient features explain most of the observed variance in testate amoeba assemblages.
- Group 2 (L25-L21 cm/260–675 AD): sediment texture parameters did not highlight the presence of exogenous terrigenous inputs, whereas TOC, TN and C/N values decreased from levels observed in the previous group. The most abundant species is *D. viscidula*, which showed a decreasing trend in the L25 section and then increase densities in L23, indicating again stability in precipitation regimes. On the other hand, in section L21 testate amoebae almost disappeared. This dramatic reduction of the amoebae assemblage could be related to a marked decrease in water temperatures due to the "Late Antique Little Ice Age" (LALIA) (536–660 AD), in association with drought time periods (Büntgen et al., 2016, 2021). The L21 period was dated as 513–626 AD (Perilli et al.,0.2020; Cantonati et al., 2021) and was associated with the presence of diatom species belonging to genus *Psammothidium*, which includes cryophilic species such as the cold stenothermic *Psammothidium curtissimum* (Cantonati et al., 2021), in agreement with the LALIA period.
- Group 3 (L19-L11 cm/675–1444 AD): trends observed for Mμ and C/N showed high variability and highlighted the presence of exogenous inputs. The dominant species were *D. viscidula*, *D. rotunda* and *D. acuminata*, whereas *Lesquereusia modesta* appeared. The latter species shows a shell made both of exogenous particles and by biogenic siliceous structures (Beyens and Meisterfeld, 2001; Todorov and Bankov, 2019) and prefers alkaline pH levels (Payne et al., 2008). Moreover, *L. modesta* could be found in habitats with a presence of high detritus content and macrophytes (Lansac-Tôha et al., 2014; Siemensma, 2021), and it has been recognized that the species could be considered an indicator of both macrophytes and periphyton (Tran et al., 2021). In addition, the occurrence of *D. rotunda* is related to habitats with epiphytic vegetation and/or planktonic organisms (Marcisz et al., 2020). The association between *D. acuminata* and *L. modesta* could be related to nutrients and plant particle inputs from the shoreline during this period, in agreement with values of sediment texture parameters and C/N trends. The exogenous inputs were likely related to increased precipitation and alluvial events, which could have caused an increase both in the hydrometric level of the lake and in the material flow to the center of the lake. In fact, the period corresponding to the L19 core level is reported by Büntgen et al. (2021) as "Early Medieval Pluvial". This period was followed by phases called "Medieval Drought" and "Late Medieval Pluvial" (Fig. 3b). The variability observed in the Group 3 period from core sections L19 to L11 (nutrients, sediment grain size and composition and fluctuating densities of testate amoeba species) could likely be related to the alternating phases identified in the medieval period (Trouet et al., 2009).
- Group 4 (L9-L7/1444–1843 AD): grainsize parameters showed reduced variability in relation to possible low fluxes of exogenous material. Total carbon ($C_{tot}$), TN and TOC showed fairly constant concentrations, with the lone exception of the L9 dropdown point, whereas values of the C/N ratio indicate a high contribution of vegetal organic matter. This is confirmed by the density of *L. modesta* which reached its highest density in Balma Lake sediments, as the dominant species were *D. acuminata*, *D. viscidula*, and *D. rotunda.* However, all testate amoebae species increased in density except *D. acuminata* var. *Inflata.* Moreover, the L7 core section showed the highest density value of testate amoebae along the sediment core. The general development observed for the testate amoeba assemblages is also confirmed by the community indices, with the highest values observed for species number and the Shannon-Wiener index and the lowest value recorded for dominance (Fig. 5b). The period corresponding to core section L7 is known as "Little Ice Age Pluvial" (Büntgen et al., 2006, 2011, 2021; Frank et al., 2010) which was recorded between the so-called minimums

of Maunder and Dalton (Büntgen et al., 2006; Delaygue and Bard, 2010). The conditions corresponding to the Group 4 period seemed to promote the development of testate amoeba assemblages.

- Group 5 (L5-L1/1843–2017 AD): at the top of the sediment core, grainsize parameters (Cμ and Mμ) are notably higher than in other groups and reach the maximum values at the top section (L1), indicating an increase in coarse particles from the lake catchment. This could be exclusively triggered by flash-flood events as previously recorded in Lake Anterne (Giguet-Covex et al., 2012). *D. viscidula* and *D. acuminata* showed marked decreasing trends, while *D. rotunda* became dominant at the top of the core. However, in this group a general decreasing trend was observed for the whole testate amoebae assemblage. Anthropogenic impact characterizes the Group 1 core sections. As highlighted by the RDA and VPA analyses, after nutrients, trace elements were found to be one of the most important factors in shaping the amoeba assemblages in Balma Lake. It is known that the occurrence of potentially toxic trace elements could affect testate amoeba assemblages (Asioli et al., 1996; Patterson, 1996; Sigala Regalado et al., 2018; Wanner et al., 2019; Ndayishimiye et al., 2021). Nguyen-Viet et al. (2007) observed a general decrease in testate amoebae abundances in zones characterized by elevated concentrations of potentially toxic trace elements. However, The increase in TOC and TN values and the decrease in the C/N ratio indicate the prevalence of aquatic (algae) origin of sedimentary organic matter than in previous sections and a concurrent high terrigenous input to the sediments with elevated sedimentation rates and short transport of the sinking particles (and hence low exposure to water column oxidation). This could also be related to softer precipitation regime linked to the drying trend observed by Büntgen et al. (2021). The influence of the temperature increase was also highlighted by the size of the amoebae, which were smaller than those observed in the other core sections. Indeed, it has been demonstrated that decreasing trends in amoebae shell dimension is correlated to a temperature increase (Wanner, 1994; Wall et al., 2010a). The observed changes in species richness as recorded by Wall et al. (2010a) in the sediment core of the Lake Lautrey, are related to the response of testate amoeba assemblages to climate change and seems to be more rapid and marked in assemblage structure and composition and particularly in terms of species dominance (Wall et al., 2010a; Ndayishimiye et al., 2020). Wall et al. (2010b), besides, reported during warmer climatic periods the dominance of small species such as *Paraquadrula irregularis*, unrecovered in Balma Lake sediment record. The use of mesh-size filters greater than the 25 μm mesh-size could have partially reduced intersample differences and altered the species richness and assemblage structure (Wall et al., 2010b), even if numerous studies have highlighted that it is adequate a 40–55 μm screen to retain testate amoebae because most specimens are found in the 44–174 μm size fraction (Ellison, 1995; Patterson and Kumar, 2000, 2002).

As highlighted by the application of the PERMANOVA, testate amoeba assemblages significantly differed from those observed in the rest of the sediment core and were characterized mainly by decreasing trends for all the observed species, in particular for *D. acuminata*. The dominant species is *D. rotunda* characterized by a small test, which could be considered a better adaptation to survive under dry conditions and/or in sites following direct human-induced impact (McKeown et al., 2019; Marcisz et al., 2016). Interestingly, *Centropyxis cassis* was observed among the species that maintained good density values in the top sediment core section. It was reported that most *Centropyxis* taxa are opportunistic and capable of existing in water systems where elevated concentrations of trace elements occurred, as well as in oligotrophic water with low organic content (Patterson, 1996; Kauppila et al., 2006; Kihlman and Kauppila, 2009, 2012; Qin et al., 2016), such those of Balma Lake.

### *4.3. Testate amoebae and other paleolimnological proxies*

The results presented in this study were found to be consistent with those obtained using other paleolimnological proxies, in particular regarding the trophic condition of Balma Lake, which could be considered an oligotrophic environment still unaffected by acidification processes. Based on the chironomid assemblage analysis, Perilli et al. (2020) reported that Balma Lake has maintained an oligotrophic status over the past 2000 years. Moreover, Cantonati et al. (2021), based on a diatom assemblage study, stated that slight alterations in the trophic level of Balma Lake might have occurred or are still occurring, but it can still be considered an oligotrophic environment. This is highlighted by the occurrence of some testate amoeba species such as *Difflugia pyriformis*, which was found all along the sediment core. *D. pyriformis* is characterized by a large shell with a piriform shape (Fig. 4), commonly associated with well oxygenated benthic habitats and oligotrophic conditions (Gomaa et al., 2012; Lansac-Tôha et al., 2014; Lahr et al., 2019; Krashevska et al., 2020). Another species observed in all the sediment core sections is *Difflugia oblonga*, commonly associated with alkaline pH values, high oxygenation levels and oligotrophic environments (Velho et al., 2003; Qin et al., 2013). The presence of other species associated with alkaline pH levels such as *D. acuminata* (Todorov and Bankov, 2019), *L. modesta* (Payne et al., 2008) and *D. oblonga* suggest that Balma Lake generally maintained an alkaline condition over time, in agreement with findings by Cantonati et al. (2021).

Regarding the Chironomidae assemblages reported by Perilli et al. (2020), the PERMANOVA did not show significant differences among the core Groups presented in this study, except for the top core levels (Group 5). However, the factors involved in shaping the assemblages seem to be slightly different: testate amoebae seem to be more affected by nutrient loads and by trace element concentrations than chironomid assemblages, as highlighted by the RDA and VPA results. Chironomidae RDA seem to identify a gradient related to the first RDA axis (Fig. 6b) and VPA analysis highlighted that the presence of fish is the major factor influencing the variability observed in chironomid paleocommunities of Balma Lake sediment core, whereas the contribution of this factor in shaping testate amoeba assemblages is reduced. This could also be highlighted by the gradient observed in the chironomid RDA, related to the taxa distribution along the first axis. Among these taxa, *Micropsectra*, *Corynocera oliveri* and *Heterotrissocladius marcidus* type take place on the left side of the graph, whereas *Paratanytarsus austriacus* type, *Psectrocladius,* and *Zavrelimyia* can be found on the right side. This trend reflects the changes chironomid assemblages due to the presence of fish, described by Perilli et al. (2020), but an indication due to the water temperature increase (related to climate change) could also be noticed. *Micropsectra*, *Corynocera oliveri* and *Heterotrissocladius marcidus* types are cold stenothermic taxa, generally associated with oligotrophic conditions (Brooks et al., 2007; Moller Pillot, 2009), whereas *P. austriacus* type is often related to a macrophyte presence or non-vascular plant vegetation (Buskens, 1987; Brodersen et al., 2001; Brooks et al., 2007), and *Psectrocladius* is generally associated with macrophyte presence and/or productive lakes (Brodersen et al., 2001; Langdon et al., 2010; Axford et al., 2017). Finally, *Zavrelimyia* is adapted to warmer temperatures and shallow habitats and its presence could be related to an increase in lake productivity especially associated with warm summers (Perilli et al., 2020). The effect of fish introduction as a source of impact on lakes is well known (Sánchez-Hernández et al., 2015; Milardi et al., 2016; Raposeiro et al., 2017), and it likely contributes to shaping the chironomid assemblages in Balma Lake. On the other hand, it may also be considered a complementary factor with other environmental impacts, in particular with climate change which has heavily affected chironomid assemblages in alpine lakes (Perilli et al., 2020). However, this feature seemed to have less effect on testate amoeba assemblages in Balma Lake, which appeared to be more affected by other factors such as nutrients and trace element occurrence.

## 5. Conclusions

The present study reports the results of paleolimnological analysis carried out using testate amoebae in Balma Lake, an alpine high altitude lotic environment in the Western Italian Alps and represents the first attempt to investigate temporal changes in testate amoeba assemblages in the European Alps, since little information regarding changes of testate amoeba assemblages in high altitude lakes is currently available. Results from this research confirmed the trends previously observed in Balma Lake through the study of other paleolimnological proxies, but also highlighted that the testate amoebae could be used as an integrating proxy, as a useful tool to detect some effects caused by pollution and/or nutrient alterations in paleolimnological reconstructions.

## Author statement

**Gianguido Salvi**: Investigation, Conceptualization, Writing – review & editing, Formal analysis, **Marco Bertoli**: Writing - Conceptualization; Investigation; Data curation; Methodology; Writing – original draft; Formal analysis, **Cecilia Giubileo**: Investigation; Data curation, **Paolo Pastorino**: Data curation; Methodology; Investigation; Writing – review & editing. **Elena Pavoni**: Investigation; Methodology; Writing – review & editing, **Matteo Crosera**: Investigation; Methodology; Writing – review & editing, **Marino Prearo**: Writing – review & editing, **Elisabetta Pizzul**: Conceptualization; Data curation; Investigation; Supervision; Writing – review & editing

## Declaration of competing interest

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

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