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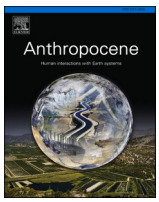

# Lipid biomarkers in high mountain lakes from the Cantabrian range (Northern Spain): Coupling the interplay between natural and anthropogenic drivers

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ABSTRACT

The lipid content of a high mountain lake (Lake Isoba) allowed the reconstruction of the paleoenvironmental changes and anthropic influence in Northern Spain during the last 550 years. Fatty acids (FAs) and *n*-alkan-2-ones indicate little degradation of OM. Three units were delimited. During Unit A (ca. 1460–1780 CE) high carbon preference index values, predominance of high-molecular-weight saturated FAs, and good correspondence between the predominant *n*-alkane and saturated FA chains indicate higher OM input and evidence of minimal degradation, linked to the cold and dry Little Ice Age, that favoured the OM input derived mainly from land plants, and the reduced bacterial activity. In Unit B (ca. 1780–2006 CE) the *n*-alkane and saturated FA profiles showed a remarkable mismatch suggestive of preferential microbial synthesis of long chain saturated FAs from primary OM and/or bacterial activity (predominance of low-molecular-weight saturated FAs but with a bimodal distribution), in coincidence with a decrease in OM input, which could be linked to the global warming that started in the second half of the 19th century. Although OM continued deriving mainly from terrigenous plants, aquatic macrophytes increased their contribution to the OM indicating the amelioration of environmental conditions. Evidence of considerable phytoplankton productivity and microbial activity was significant in Unit C (ca. 2006–2018 CE) coinciding with the highest concentrations of *n*-alkanes and saturated FAs, linked to warmer and drier conditions, and to greater anthropogenic influence. In addition, organic sulfur and gammacerane indicates loss of oligotrophy, and the record of faecal stanols, particularly that of 24-ethylcoprostanol, strongly evidences notable and rising water pollution associated with increasing cattle ranching in the lake catchment during the past 10–15 years.

## 1. Introduction

Lakes, especially high mountain lakes, play a crucial role in providing valuable information about hydrogeological and environmental conditions. These lakes are particularly sensitive to environmental changes at regional and global scales and the climatic conditions vary significantly with altitude, and the water chemistry reflects even subtle changes in the environment (Catalán et al., 2013). Furthermore, these lakes are often protected from direct pollution sources, making them important indicators of environmental health. However, despite their remote locations, mountain lakes are not immune to anthropogenic influences. They can be affected by contamination from global or regional sources. Therefore, studying the sediments of these lakes can provide valuable records of past environmental changes and human activities (Smol, 1995; Cohen, 2003). These reconstructions are essential for understanding the natural reference conditions of lakes, predicting future ecological changes, and establishing effective environmental restoration and management policies.

The following approaches have been used to reconstruct palaeoclimate from lacustrine and palustrine sediments: palynology (Reille

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et al., 2000; Tzedakis et al., 2003,), species assemblages (Carbonel et al. 1988; Anadón et al., 1994), carbon and oxygen stable isotopes (Schwalb et al., 1999; Ortiz et al., 2006) and inorganic geochemistry (Smith and Bischoff, 1997; Gallego et al., 2019). In addition, lipid content can also be used to obtain paleoclimatic reconstructions from continental records (Meyers and Ishiwatari, 1993; Meyers, 1997, 2003; Schwark et al., 2002; Ortiz et al., 2016a). Changes in lipid composition reflect environmental changes in lakes, which are influenced by natural factors, such as climate variability, and human activities at local and regional scales (Bechtel and Schubert, 2009), even during the Holocene, when climatic shifts have been rather subtle (cf. Nott et al., 2000; Xie et al., 2004; Ortiz et al., 2010).

Interpreting organic geochemical signals in lake sediments may be challenging due to the complex nature of organic matter (OM) and its variable degree of preservation. Nevertheless, sedimentary OM still retains valuable information about its origin, transport, and deposition (Meyers, 2003) and therefore biomarkers are known to be a highly useful proxy. Among the molecular geochemistry proxies, n-alkanes are commonly used biomarkers in lake sediments. They are source-specific and less prone to microbial degradation compared to other OM components, as they lack reactive functional groups (Prahl and Carpenter, 1984; Meyers et al., 1995). Thus, the composition and distribution of *n*-alkanes are useful to determine the sources of OM and evaluate the degree of preservation, providing insights into palaeoenvironmental changes. Saturated fatty acids (FAs) in lake sediments also offer valuable information about OM provenance and preservation. While saturated FAs come from various sources like algae, aquatic macrophytes, and land plants, they are more susceptible to degradation and chemical modification (Meyers, 1993). Therefore, changes in saturated FA content can indicate both OM sources and the degree of preservation, aiding in the understanding of environmental changes. *n*-Alkan-2-ones also provide information about the origin of OM together with information about its degree of degradation (Volkman et al., 1981; Cranwell et al., 1987; Ortiz et al., 2011a, 2016b).

The sterol content is also a proxy for the source and diagenetic alteration of OM. The diversity of sterols and the stanols derived from them provides information about the origin and diagenesis of OM. The relative amounts of sterols are used to identify the contributions of different types of OM (Meyers, 2003), whereas the stanol/sterol ratio serves as a proxy for microbial reduction of OM (Wakeham, 1989). Moreover, the presence of 5β-stanols is typical in animal faeces, thus allowing the identification of faecal pollution in lake sediments (Leeming et al., 1984, 1996; Bethell et al., 1994; Shah et al., 2007; Ortiz et al., 2016a).

In this context, the mountains of Northwestern Iberia, located in the mid-latitude, provide an ideal setting to investigate past environmental changes. First, northern Iberia is located at the transition between the temperate and the subtropical realms, the latter being responsible for rainfall seasonality, and experiences a marked oceanic influence as a result of its proximity to the North Atlantic. Second, high-elevation areas of the Cantabrian Range have been subject to intense use as summer pastures by sheep transhumant herds for centuries (Morales-Molino et al., 2022), changing to staying cattle during the past few decades. These two major drivers of environmental change have been operating simultaneously in the Cantabrian Range for the past few centuries, making it challenging to disentangle the influence of climate variability and anthropogenic disturbances on environmental change using multi-proxy studies of lake sediments.

In this geographical setting, we selected Lake Isoba (province of León, NW Spain, Fig. 1), since: (i) previous research has demonstrated that is is highly sensitive to climate variability and local human activities, mainly linked to livestock (Gardoki et al., 2023), and (ii) the robust chronology of its sedimentary sequence, based on $^{14}C$, $^{210}Pb$ and $^{137}Cs$ (Gardoki et al., 2023) which allow matching the environmental changes inferred from the biomarker analysis to regional climatic and land use changes occurred during the last ca. 550 years (Gardoki et al., 2023).

Here, we have sought to reconstruct the palaeoenvironmental evolution of high mountain areas from the northern part of the Iberian Peninsula and the anthropic influence by interpreting the organic geochemistry of sediments retrieved from Lake Isoba. A sedimentary core was carefully sampled at high temporal resolution (2 cm intervals) in order to study the C and N content in the OM and various biomarkers, mainly *n*-alkanes, FAs, *n*-alkan-2-ones, sterols, organic sulfur, gammacerane, dehydroabietic acid and α- and β-amyrin. In addition, by characterizing the stanol content, which complemented existing data in palaeoclimate studies, we also assessed the influence of anthropogenic activities in the area.

## 2. Geographical setting

Lake Isoba (4768494 N, 311484 W, 1404 m asl.) is located in the western Cantabrian Range, northern Spain, within the "Montañas de Riaño and Mamprodre" Regional Park (Fig. 1A). The regional climate is characterized by being low orotemperate upper hyperhumid (Rivas Martínez et al., 2022). The study area is characterized by a mountainous oceanic climate, with high annual precipitation (1516 mm) and a mean

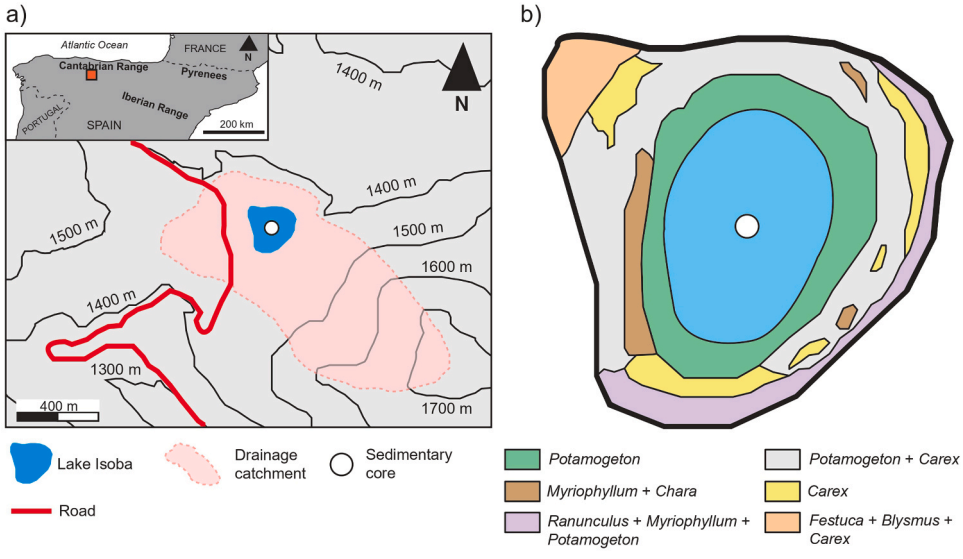


**Fig. 1.** A) Location of Lake Isoba with the position of the core together with B) the assemblages of aquatic vegetation (modified from Fernández-Aláez et al., 1987).

annual temperature of 5.8ºC (Ortega Villazán and Morales Rodríguez, 2015).

Lake Isoba has a surface area of 2.9 ha, a maximum depth of 6 m, and a catchment of 0.7 $km^2$. It presents two sub-environments: a littoral or shallow flooded platform (0–2 m deep), colonized by hydrophytic vegetation, and a more distal or deep zone (2–6 m deep) (Fig. 1B). The lake lays over Quaternary fluvio-glacial deposits, which are above black limestones of the Barcaliente Formation of Carboniferous age (Álvarez-Marrón et al., 1988). The catchment is mostly composed of mudstones, sandstones, marls and bioclastic limestones (Beleño, Barcaliente and Alba Formations) of Carboniferous age. The lake is mainly fed by laminar runoff, also receiving groundwater inputs (Jiménez-Sánchez et al., 2014).

## 3. Material and methods

Three sediment cores were drilled at the deepest area of Lake Isoba (6 m below water level) using an Uwitec® gravity corer during a sampling campaign in 2018 (Gardoki et al., 2023). They were split longitudinally into two symmetrical halves, photographed and stored at 4 ºC until analysis. For this study, we selected the 66 cm long core ISB18A-3 G (Fig. 1).

The Lake Isoba sediment record is characterized by the presence of black silts containing abundant calcite and OM. Several 1–2 mm thick layers of laminated brown silts with OM and carbonates are present at 15 cm, 16.6 cm, 17.2 cm, 47 cm and 51 cm depths. No evidence of hiatuses or bioturbation was observed. OM was characterized by amorphous accumulations without distinguishable structures together with remains with clear structures of vascular plants.

The chronology of the sequence is based on $^{210}Pb$ and $^{137}Cs$ dating for the uppermost 38.5 cm and AMS $^{14}C$ dating of terrestrial plant macrofossils for the lower part of the sequence (Fig. 1 Supplementary Information). Geochronological analyses were carried out in parallel cores and correlated with ISB18A-3 G thanks to the presence of lithostratigraphically distinct units (see Gardoki et al., 2023) for further details). Thus, the Lake Isoba sediment record analyzed in this paper extends from ca. 1460–2018 CE.

Sediment samples from ISB18A-3 G core were taken for total organic carbon (TOC) and total nitrogen (TN) content and lipid content analysis at 2 cm intervals. Additionally, living terrestrial plants, aquatic macrophytes and algae were collected for lipid analysis in order to use them as modern analoges.

### 3.1. TOC and TN

A total of 33 samples were taken at 2 cm interval along the ISB core. The samples were homogenized with a mortar and pestle. TOC and TN concentration was measured using a LECO CNS 928 in the Laboratories of the Instituto Pirenaico de Ecología (IPE-CSIC, Zaragoza) after removal of inorganic carbon with HCl 1:1 (Morellón et al., 2008).

### 3.2. Lipid extraction and analysis (biomarker analysis)

Another set of 33 samples taken at 2 cm intervals was used for biomarker analysis. Additionally, sixteen living plants from the Lake Isoba and its surroundings were collected, including two different green algae, aquatic macrophytes (*Potamogeton* sp., *Myriophyllum* sp.), and plants living on the lake margins such as the sedge *Carex* sp., water mudworts (*Limosella* sp.), water crowfoot (*Ranunculus* sp.), spikerushe (*Eleocharis* sp.), spikemoss (*Sellaginella* sp.), fern (*Pteridium*), three grasses belonging to Poaceae, the heather *Erica tetralix*, broom (*Cytisus sp.*) and an unidentified moss (Fig. 1B).

Between 0.08 g and 0.91 g dried sample of sediment and between 0.05 g and 1.11 g dried sample of plant leaves (50 ºC, 24 h) were ground, and biomarkers were extracted with an accelerated solvent extractor (Dionex ASE 200). Free lipids were extracted with dichloromethane (DCM)/MeOH (2:1) at 1500 psi and 175 ºC. The heating phase was 8 min and the static extraction time 5 min.

The extract was concentrated using a rotary evaporator. Prior to analysis using gas chromatography-mass spectrometry (GC-MS), acidic and polar fractions were methlylated with trimethylsilyldiazomethane and silylated with a mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine at 70 ºC for 2 h. Samples were injected into an HP 6890 gas chromatograph equipped with a selective mass detector (HP 5973) and an ATM-5 column (250 ×0.25 mm; 0.20 μm). Prior to analysis, an internal standard (decafluorobiphenyl) with a concentration of 1 μg/l was added to the extracts in order to quantify the compounds. Helium was the carrier gas and the oven temperature was programmed from 60 to 300 ºC (held 20 min) at 6ºC/min and the injector was maintained at 275 ºC. Components were assigned with the Data Analysis program and the Wiley Library; *n*-alkane distributions were obtained from the *m/z* 57 chromatograms (base peak), the FAs from *m/z* 74, the *n*-alkan-2-ones from *m/z* 59, sterols from *m/z* 129, stanols from *m/z* 215, gammacerane from *m/z* 191, α- and β-amyrin from *m/z* 218, dehydroabietic acid from *m/z* 239, and organic sulfur from *m/z* 64.

### 3.3. Elemental proxies

#### 3.3.1. TOC and C/N ratio

The concentration of TOC is a fundamental proxy for describing the abundance of OM in sediments. The proportion of TOC (%) represents OM that escaped remineralization during sedimentation. It is influenced by both the initial production of biomass and subsequent degree of degradation, and integrates various sources of OM (Meyers, 2003).

C/N atomic ratio is a proxy for protein content (Müller and Mathesius, 1999). Proteins account for the greatest part of the OM in organisms, together with lipids and carbohydrates, but the proportional abundance varies. Thus, C/N provides information about the proportion of algal and land plant contribution to OM (Prahl et al., 1980; Meyers, 1994; Kaushal and Bindford, 1999). OM derived from lake algae has atomic C/N values typically between 4 and 10, whereas vascular plants usually have values equal or larger than 20 (Ertel and Hedges, 1985; Hedges et al., 1986; Meyers, 1994). Atomic C/N values of 12–17 suggest a mixture of algal and vascular plant input.

#### 3.3.2. Biomarker proxies

*3.3.2.1. n-Alkanes.* *n*-Alkane profiles were used to distinguish the diverse sources of OM, namely algal, aquatic or terrigenous. Each sample can be characterized by the predominant *n*-alkane chain length. Their distribution in phytoplankton and algae is dominated by low molecular weight (MW) *n*-alkanes, maximizing at $C_{17}$ (Gelpi et al., 1970; Blumer et al., 1971; Cranwell et al., 1987). Submerged/floating macrophytes maximize at $C_{21}$, $C_{23}$ and $C_{25}$ (Cranwell, 1984; Ogura et al., 1990; Viso et al., 1993), while emergent macrophytes have a composition similar to that of terrestrial plants, peaking at $C_{27}$ and $C_{29}$ (Cranwell, 1984). Terrestrial plants contain high proportion of higher MW *n*-alkanes ($C_{27}$, $C_{29}$ and $C_{31}$) in their epicuticular wax (Eglinton and Hamilton, 1963, 1967; Eglinton and Calvin, 1967; Cranwell et al., 1987; Rieley et al., 1991; Nott et al., 2000; Pancost et al., 2002). Deciduous trees typically maximize at $C_{27}$, whereas in marsh plants and possibly grasses $C_{31}$ is dominant (Cranwell et al., 1987; Schwark et al., 2002; Ortiz et al., 2004, 2011a). However, data from a broad survey of modern plants show that *n*-alkane chain-length distributions are highly variable within plant groups, and chemotaxonomic distinctions between grasses and woody plants are difficult to make, with the exception of aquatic plants and *Sphagnum* moss (Bush and McInerney, 2013). In contrast, changes in chain length distributions are likely to be a result of temperature and/or humidity conditions (Bush and McInerney, 2015).

A number of indices calculated using *n*-alkane abundance are used to

discriminate the OM sources in lake sediments. Thus, the use of the average chain length (ACL; Poynter, 1989), calculated as [($C_i$ x i + $C_{i+1}$ x (i+1) + $C_{i+2}$ x (i+2).+ $C_n$ x n))/ ($\Sigma$ $C_{n+1}$ + $C_{n+2}$ +.+ $C_n$), with i= 13, n = 33], is a good proxy for distinguishing between the predominance of low vs. high MW *n*-alkanes (Pancost et al., 2002; Rommerskirschen et al., 2003).

The carbon preference index (CPI; Bray and Evans, 1961), calculated as ½ [($\Sigma$ $C_i$ + $C_{i+2}$ +.+ $C_{i+8}$) / ($\Sigma$ $C_{i-1}$ + $C_{i+1}$ +.+ $C_{i+7}$) + ($\Sigma$ $C_i$ + $C_{i+2}$ +.+ $C_{i+8}$) / ($\Sigma$ $C_{i+1}$ + $C_{i+3}$ +.+ $C_{i+9}$)], with i = 25, is commoly used to discriminate between mature and inmature OM in sediments, because it indicates the predominance of odd-over even numbered *n*-alkanes of a certain chain length. The index was also used by Zheng et al. (2007) to discriminate between palaeoenvironmental conditions, as the *n*-alkanes from the cuticular wax of higher plants have a strong odd predominance and have CPI values > 5. In contrast, *n*-alkanes from bacteria and algae, which have low CPI values of ca. 1 (Cranwell et al., 1987).

Silliman et al. (1996) defined the terrigenous/aquatic ratio, calculated as ($C_{31}$ + $C_{29}$+ $C_{27}$)/($C_{15}$ + $C_{17}$ + $C_{19}$), to distinguish between land plants and algal input.

The $P_{aq}$ index, calculated as ($C_{23}$ + $C_{25}$)/($C_{23}$ + $C_{25}$ + $C_{29}$ + $C_{31}$) ratio (Ficken et al., 2000), was postulated to reflect the relative contribution of emergent and submerged/floating aquatic macrophytes, which typically maximize at $C_{23}$ and $C_{25}$, and that of terrestrial plants. According to Ficken et al. (2000), values < 0.1 are linked to a dominant contribution from land plants, while values between 0.1 and 0.4 reflect a significant input from emergent macrophyte. Values > 0.4 are typical of sediments with a major *n*-alkane input from submerged/floating macrophytes.

*3.3.2.2. Fatty acids.* Like aliphatic hydrocarbons, FAs in lake sediments come from OM derived from plants and micro-organisms. Long chain saturated FAs ($C_{24:0}$ to $C_{30:0}$) with a predominance of even numbers are major components of the wax of land plant leaves, flowers and pollen (Eglinton and Calvin, 1967; Rieley et al.,. 1991; Meyers and Ishiwatari, 1993), while algae and bacteria maximize at shorter chain length, from $C_{12:0}$ to $C_{18:0}$ (Eglinton and Calvin, 1967; Cranwell et al., 1987).

The ratio of terrigenous to aquatic FAs ($TAR_{FA}$; Meyers, 1997) is a measure of the sum of long chain to short chain even saturated FAs, calculated as ($C_{28:0}$ + $C_{26:0}$+ $C_{24:0}$)/($C_{18:0}$ + $C_{16:0}$ + $C_{14:0}$), and is used to distinguish between land plant and algal sources. However, selective degradation and diagenetic processes commonly overprint FA distributions. Short chain acids are often preferentially degraded by microbes during early diagenesis (Cranwell, 1974, 1976; Haddad et al., 1992; Ho and Meyers, 1994) and they produce higher $TAR_{FA}$ values (Tenzer et al., 1999). On the other hand, the microbial synthesis of secondary FAs from primary OM produces short chain components (Kawamura et al., 1987) and can depress $TAR_{FA}$ values.

*3.3.2.3. n-Alkan-2-ones.* *n*-Alkan-2-ones may have diverse origins, including direct input from plants (Arpino et al., 1970; Volkman et al., 1981; Ortiz et al., 2011a, 2016b), microbial oxidation of *n*-alkanes (Cranwell et al., 1987; Amblés et al. 1993; Jaffé et al., 1993, 1996; van Bergen et al., 1998; Ortiz et al., 2016b), microbial β-oxidation and decarboxylation of FAs (Volkman et al., 1983, Chaffee et al., 1986; de Leeuw, 1986; Quénéa et al., 2004), and bacterial material (López-Días et al. 2013b; Ortiz et al., 2016b).

*3.3.2.4. Sterols.* These compounds, especially β-sitosterol, stigmasterol and campesterol, have been reported to be good palaeoclimatic indicators, as they are typically constituents of higher plants (Goad and Goodwin, 1972; Goad 1991; Pancost et al., 2002), algae, bacteria, and diatoms (Volkman, 1986). In addition, other componets such as brassicasterol and dinosterol are mainly present in algae, diatoms and dinoflaggellates (Volkman, 1986; He at al., 2008; Rampen et al., 2010).

Sterols occur in animals and humans and provide information about their influence on the environment (Leeming and Nichols, 1996; Leeming et al., 1996; Bull et al., 2002).

*3.3.2.5. Stanols.* 5α-Stanols are produced through selective natural microbial reduction of their respective $\Delta^5$-sterols (Wakeham, 1989; Lehtonen and Ketola, 1993; Jaffé et al., 1996; Bull et al., 1999, 2002), and only a small fraction of the latter is transformed into 5β-stanols (Gaskell and Eglinton, 1976; Grimalt et al., 1990). Thus, the 5α-stanol/sterol ratio is used as a proxy for diagenetic processes in recent marine sediments (Wakeham, 1989; Ranjan et al., 2015), lakes (Jaffé et al., 1996), and peat bogs (Andersson and Meyers, 2012; Routh et al., 2014).

5β-Stanols are commonly formed as reduction products of cholesterol and the higher MW counterparts (campesterol, sitosterol and stigmasterol) in the intestinal tracts of most mammals (Bull et al., 2002) and can accumulate in sediments. Cholesterol, a typical sterol in animals—although also found in some microalgae (Volkman et al., 1999), is converted to 5β-coprostanol in the human digestive track (Murtaugh and Bunch, 1967; Leeming et al., 1984; Bethell et al., 1994; Shah et al., 2007). This compound is relatively less abundant in the faeces of other animals (Leeming et al., 1996; Mudge and Morrison, 2010). Small amounts of coprostanol can be generated from cholesterol in anaerobic sediments (Nishimura and Koyama, 1977; Müller et al., 1979; Mudge and Gwyn Lintern, 1999).

In contrast, herbivores tend to produce 24-ethylcoprostanol from β-sitosterol (abundant in plants). Likewise, β-sitosterol is also found in herbivore faeces (Leeming et al., 1996). Thus, using the stanol content, and specifically the type of coprostanol, human input can be distinguished from that of herbivores (Leeming and Nichols, 1996; Leeming et al., 1996). Recent studies have applied faecal stanols to identify ancient human settlements in Norway, Peru and the Yucatán Peninsula (Mexico and Guatemala) (D'Anjou et al., 2012; Arnold et al., 2021; Keenan et al., 2022, 2021).

*3.3.2.6. Dehydroabietic acid.* Dehydroabietic acid is a tricyclic diterpenoid mainly produced by conifers as major component of resin. Therefore, the presence of this compound in lake environments is unequivocally linked to the contribution of coniferous resins (Pereira et al., 1982). In this regard, Otto and Simoneit (2002) found dehydroabietic acid in pine wood extracts from Vancouver (Canada).

*3.3.2.7. α- and β-amyrin.* These compounds are two of the most popular pentacyclic triterpenes found in higher plants (Gómez-Pulido et al., 2022). In many cases, they are typical constituents of dicotyledoneous plant waxes (Chaffee et al., 1986; Jäger et al., 2009).

*3.3.2.8. Gammacerane.* Gammacerane is a non-hopanoid $C_{30}$ triterpene which indicates deposition of organic matter under highly reducing hypersaline conditions (ten Haven et al., 1985, Moldovan et al., 1985; Peters and Moldovan, 1993; Huang and Pearson, 1999). In addition, this biomarker can also indicate a stratified water body, with anaerobic conditions at the bottom (Sinninghe Damsté et al., 1995). In this regard, lacustrine deposits from middle latitudes, which are generally stratified during summer, may contain high amounts of gammacerane (Peters and Moldovan, 1993; Huang and Pearson, 1999; Chen and Summons, 2001).

*3.3.2.9. Organic sulfur.* Sulfur undergoes cyclic transformations, which can arise from different levels of organization and complexity. It is assimilated by most bacteria as well as by algae and other plants in the form of sulfate, which then undergoes an assimilatory reduction via sulfite to sulfide (Jørgensen, 1983; Killops and Killops, 1984). The reduced organic sulfur is released back into the environment after the death and decomposition of organisms (Jørgensen, 1983).

The microbial sulfur cycle begins with the sulfate input into the oceans or palustrine–lacustrine environments that is incorporated into the trophic chain by anaerobic sulfate-reducing bacteria that carry out

an assimilatory reduction via sulfite to sulfide (Jørgensen, 1983; Killops and Killops, 1984). Subsequently, anaerobic photosynthetic sulfur bacteria oxidize sulfide to elemental sulfur and then to sulfate, but the first step is faster than the second and so, sulfur accumulates (Gemerden, 1967; Killops and Killops, 1984; Fry, 1986; Luther and Church, 1992).

Among the main requirements for the development of sulfur-reducing bacteria are: reducing conditions, sulfate ions and suitable energy sources. Therefore, organic sulfur content is a proxy for anoxic/oxic conditions.

## 4. Results

### 4.1. TOC

TOC values varied between 13.8% (64 cm) and 25.8% (0 cm) in Lake Isoba record, showing an increasing trend towards the top (Fig. 2).

### 4.2. C/N

Atomic C/N values oscillated between 14.7 and 9.8 in the record of Lake Isoba, showing a decreasing trend towards the top (Fig. 2). Values were $< 12$ in the uppermost 8 cm, ranging from 12 to 14.7 between 8 and 64 cm.

### 4.3. Lipid biomarkers in plants

In order to discern the origin of *n*-alkanes and *n*-alkan-2-ones in the sediment, we analyzed the lipid content of living plants growing in the surroundings and from the lacustrine environment. Green algae maximized at $C_{17}$ and $C_{19}$ alkanes, the aquatic macrophyte *Potamogeton* sp. at $C_{23}$, *Ranunculus* sp. at $C_{25}$, whereas $C_{27}$ to $C_{31}$ alkanes were predominant in the rest of herbs, shrubs, mosses and ferns (Table 1). Similarly, green algae, together with *Potamogeton* sp., maximized at $C_{17}$ alkan-2-one, whereas $C_{27}$ and $C_{29}$ were predominant in the rest of species (Table 2).

### 4.4. Lipid biomarkers

#### 4.4.1. n-Alkanes

Typical chromatograms of *n*-alkanes from selected samples are shown in Fig. 3. All the samples from the core showed an odd carbon number predominance, with a chain length distribution ranging mainly from $C_{15}$ or $C_{17}$ to $C_{31}$ or $C_{33}$, maximizing either at low MW ($C_{17}$ Fig. 3A; Fig. 2 supplementary information) or at $C_{27}$, $C_{29}$ and $C_{31}$ (Fig. 3B, C; Figs. 3, 4 supplementary information), the former group being clearly predominant in the uppermost 12 cm of Lake Isoba record (Fig. 2). Remarkably, a bimodal distribution was detected in the samples.

The profiles of the various indexes related to the *n*-alkane content, namely the *n*-alkane predominant chain, the ACL, the carbon preference index (CPI), the terrigenous/aquatic ratio of hydrocarbons ($TAR_{HC}$) and the aquatic macrophyte proxy ($P_{aq}$), are shown in Fig. 2.

The predominant *n*-alkane chain varied between $C_{17}$ and $C_{27}$, $C_{29}$ or $C_{31}$. It was possible to identify three phases (Fig. 2), namely a period with a clear dominance of HMW alkanes from the bottom (64 cm) to 43 cm, another episode in which $C_{27}$ was the most abundant *n*-alkane with only two samples (42 cm and 16 cm) in which $C_{29}$ predominated, and $C_{17}$ dominance from 12 cm to the top, although with a bimodal distribution of *n*-alkanes. The ACL also showed three marked intervals (Fig. 2), with values ca. 28.0 in the lowermost 64–42 cm (coinciding with a dominance of long chain *n*-alkanes), ca. 26.0 between 42 and 12 cm (coinciding with a dominance of $C_{27}$), and between 24.3 and 20.0 in the uppermost 12 cm (together with predominance of short-chain *n*-alkanes).

CPI values along the Isoba sequence showed oscillations, with values $> 8$ in the lower part of the record ($> 52$ cm). In contrast, CPI values were between 4.0 and 6.0 in most of the record. $TAR_{HC}$ values markedly high ($> 17$) in the lowermost interval, with maxima of 122 and 116 at 54 and 65 cm, respectively, whereas values oscillated between 19.2 and 4.3 from 42 cm to 12 cm, being $< 1.0$ in the uppermost 12 cm, with the exception of 1.4 at 8 cm. In contrast to the $P_{aq}$ values showed the lowest values (0.29–0.17) in the lowermost interval (66–42 cm), increased to 0.51–0.32 between 42 cm and 12 cm, and ranged from 0.38 to 0.24 in the upper 12 cm.

The sum of the concentration of *n*-alkanes ranged between 446 (1 cm) and 28 mg/kg (36 cm) (Fig. 2), with the highest values at the lowermost and uppermost intervals, whereas between 42 cm and 12 cm, decreased.

#### 4.4.2. Fatty acids

All samples showed a strong predominance of even numbered acids, ranging from $C_{12:0}$ to $C_{32:0}$, and maximizing at $C_{16:0}$ (Fig. 3D), $C_{24:0}$ and $C_{26:0}$ (Fig. 3E), sometimes with a bimodal distribution (Fig. 3E, F). The predominant saturated FA chain and the ratio of terrigenous to aquatic FAs ($TAR_{FA}$) are shown in Fig. 2, together with the percentage of saturated, branched saturated and monounsaturated FA. Notably, saturated FAs were markedly more dominant than saturated branched (dominated by *iso*-$C_{15:0}$; *anteiso*-$C_{15:0}$ followed by *iso*-$C_{17:0}$; *anteiso*-$C_{17:0}$) and

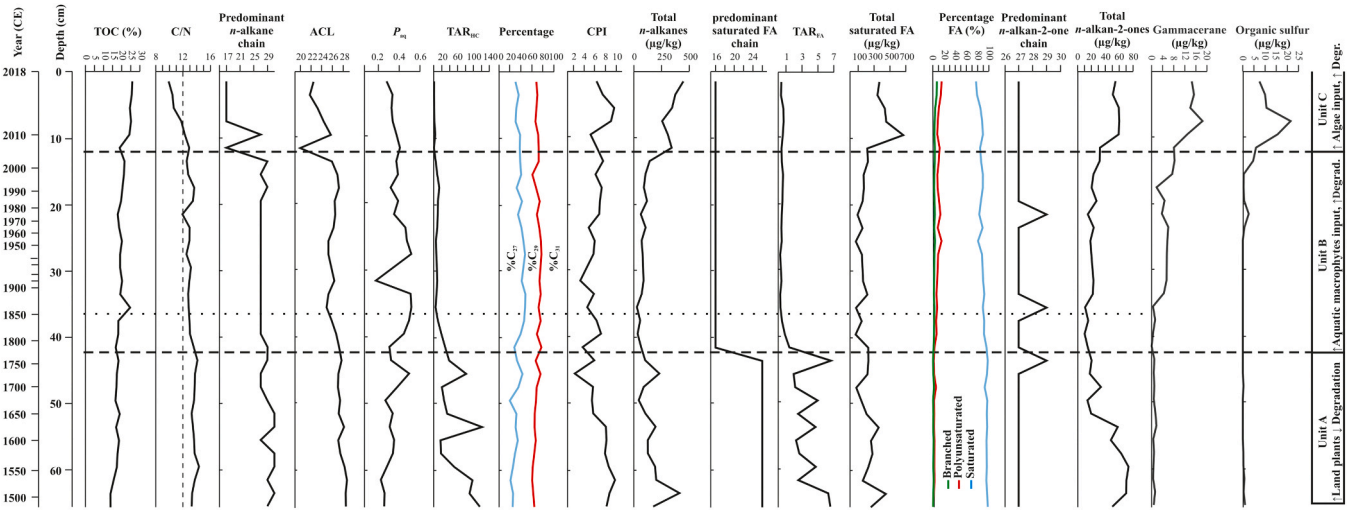


**Fig. 2.** Profiles of TOC%, C/N (atomic), predominant *n*-alkane chain, ACL, $TAR_{HC}$, $P_{aq}$ index, predominant saturated FA acid chain, $TAR_{FA}$, predominant *n*-alkan-2-one, and concentration of *n*-alkanes, *n*- saturated FAs, *n*-alkan-2-ones, organic sulfur and gammacerane in Lake Isoba core.

**Table 1**
Concentration (n.d., not detected) of *n*-alkanes in plants from Lake Isoba (highest values in bold).

| | Alkane $C_{no}$ (μg/g dry plant matter) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 |
| Algae 1 | 19.79 | 0.36 | **61.79** | 2.04 | 35.27 | 4.42 | 23.79 | 3.74 | 20.97 | 1.89 | 15.92 | 0.93 | 14.43 | 1.12 | 36.31 |
| Algae 2 | **40.73** | 0.82 | 6.74 | 6.65 | 2.18 | 3.91 | 9.18 | 1.85 | 38.07 | 1.76 | 21.86 | 2.45 | 1.25 | 1.76 | 18.13 |
| *Potamogeton* | 17.04 | 4.70 | 16.87 | 4.86 | 41.12 | 29.08 | **65.20** | 18.52 | 50.87 | 8.48 | 33.53 | 3.19 | 43.76 | 5.16 | 6.28 |
| *Ranunculus* | 0.45 | 0.31 | 0.29 | 0.28 | 2.68 | 0.64 | 3.38 | 2.65 | **180.96** | 11.68 | 92.76 | 3.92 | 3.99 | 3.67 | 14.87 |
| *Myriophyllum* | 5.83 | 6.71 | 2.14 | 2.77 | 46.50 | 5.01 | 23.26 | 8.89 | 24.17 | 7.00 | 25.44 | 6.05 | 14.41 | 4.63 | **47.72** |
| *Limosella* | 0.89 | 0.64 | 0.63 | 0.72 | 4.91 | 1.23 | 3.83 | 2.90 | 8.73 | 8.73 | **11.81** | 4.67 | 3.68 | 1.47 | 11.32 |
| Moss | 7.70 | 9.95 | 3.18 | 7.59 | 34.57 | 10.63 | 26.60 | 17.23 | 66.27 | 43.05 | 76.89 | 76.89 | 28.65 | 22.42 | **287.80** |
| *Eleocharis* | 0.10 | 0.17 | 0.05 | 3.55 | 5.02 | 0.06 | 0.16 | 0.08 | 0.28 | 0.21 | 1.41 | 0.24 | **10.63** | 0.52 | 4.31 |
| *Sellaginella* | 4.41 | 0.26 | 0.99 | 0.64 | 0.76 | 1.30 | 2.81 | 1.68 | 19.59 | 2.23 | 19.59 | 1.69 | **123.12** | 2.13 | 47.31 |
| *Cytisus* | 0.32 | 0.15 | 0.08 | 0.29 | 1.29 | 3.34 | 70.69 | 39.57 | **396.40** | 62.65 | 268.81 | 6.92 | 32.65 | 0.57 | 4.85 |
| *Carex* | 0.84 | 0.30 | 0.56 | 0.88 | 20.79 | 1.88 | 22.18 | 2.18 | 44.03 | 3.02 | 96.96 | 2.00 | 33.42 | 3.29 | **321.56** |
| *Erica* | 0.42 | 0.39 | 0.26 | 0.28 | 4.31 | 0.65 | 7.92 | 3.94 | 59.89 | 10.90 | 166.57 | 21.60 | 264.22 | 37.01 | **461.85** |
| *Pteridium* | 0.72 | 0.67 | 0.60 | 1.26 | 12.28 | 1.35 | 3.12 | 2.38 | 12.26 | 4.51 | 35.32 | 5.06 | 5.40 | 3.06 | **31.62** |
| Poaceae 1 | 1.59 | 1.61 | 0.83 | 1.98 | 11.22 | 1.14 | 3.66 | 2.10 | 16.24 | 1.76 | 14.50 | 3.93 | 8.32 | 2.73 | **67.43** |
| Poaceae 2 | 0.31 | 0.42 | 0.45 | 0.56 | 3.30 | 1.28 | 4.01 | 2.57 | 17.80 | 3.45 | 6.50 | 5.28 | 50.47 | 2.49 | **31.70** |
| Poaceae 3 | 1.89 | 0.70 | 8.96 | 1.21 | 13.22 | 2.45 | 14.87 | 5.75 | 13.33 | 5.25 | 21.99 | 3.65 | 10.53 | 1.00 | **21.88** |

**Table 2**
Concentration (n.d., not detected) of *n*-alkan-2-ones in plants from Lake Isoba (highest values in bold).

| | Alkan-2-one $C_{no}$ (μg/g dry plant matter) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | total |
| Algae 1 | **5.27** | 0.00 | 1.39 | 1.77 | 3.01 | 0.00 | 1.29 | 0.00 | 2.47 | 1.29 | 2.15 | 0.00 | 0.99 | 0.00 | 1.55 | 21.18 |
| Algae 2 | **3.44** | 0.00 | 0.70 | 0.64 | 3.08 | 0.23 | 1.00 | 0.33 | 1.56 | 0.48 | 1.50 | 0.50 | 1.01 | 0.00 | 0.84 | 15.32 |
| *Potamogeton* | **6.05** | 0.00 | 0.52 | 0.00 | 0.46 | 1.25 | 0.78 | 1.94 | 2.16 | 1.75 | 1.53 | 0.45 | 0.88 | 0.00 | 0.48 | 18.24 |
| *Ranunculus* | 0.71 | 0.00 | 0.13 | 0.27 | 0.34 | 0.38 | 0.92 | 0.54 | 4.91 | 1.06 | **12.10** | 1.47 | 7.66 | 0.31 | 1.39 | 32.20 |
| *Myriophyllum* | 3.07 | 0.00 | 1.72 | 1.51 | 2.50 | 0.73 | 4.26 | 1.51 | **11.36** | 0.86 | 9.09 | 0.98 | 3.36 | 0.00 | 0.94 | 41.87 |
| *Limosella* | 0.69 | 0.00 | 0.40 | 0.79 | 1.08 | 0.37 | 1.10 | 0.78 | 4.15 | 0.76 | **4.21** | 0.99 | 2.84 | 0.27 | 0.78 | 19.21 |
| Moss | 6.35 | 0.00 | 4.41 | 5.27 | 4.57 | 3.00 | 12.79 | 6.53 | **43.59** | 4.10 | 22.93 | 4.83 | 14.25 | 0.00 | 13.31 | 145.90 |
| *Eleocharis* | 0.04 | 0.00 | 0.00 | 0.00 | 0.03 | 0.08 | 0.06 | 0.04 | 0.10 | 0.03 | **0.21** | 0.02 | 0.19 | 0.00 | 0.11 | 0.90 |
| *Sellaginella* | 1.25 | 0.00 | 0.55 | 0.60 | 1.09 | 0.25 | 0.96 | 0.60 | 2.74 | 0.61 | **3.18** | 1.19 | 3.12 | 0.32 | 2.03 | 18.49 |
| *Cytisus* | 0.08 | 0.00 | 0.34 | 0.00 | 0.68 | 0.80 | 0.06 | 1.05 | **2.52** | 0.52 | 2.42 | 0.19 | 0.20 | 0.00 | 0.04 | 8.91 |
| *Carex* | 3.37 | 0.00 | 0.61 | 0.87 | 1.71 | 0.23 | 0.98 | 0.39 | 1.75 | 1.09 | **8.75** | 0.55 | 0.88 | 0.61 | 0.39 | 22.18 |
| *Erica* | 0.33 | 0.00 | 0.59 | 1.99 | 0.69 | 0.22 | 2.74 | 0.69 | **5.65** | 0.63 | 3.33 | 0.00 | 0.00 | 0.00 | 0.81 | 17.67 |
| *Pteridium* | 0.27 | 0.00 | 0.93 | 0.53 | 0.53 | 0.37 | 1.71 | 0.85 | **6.13** | 0.46 | 3.71 | 0.84 | 2.05 | 0.30 | 0.86 | 19.55 |
| Poaceae 1 | 1.78 | 0.00 | 0.93 | 0.60 | 0.72 | 0.91 | 1.68 | 0.92 | **5.68** | 1.41 | 5.36 | 4.93 | 3.88 | 0.99 | 2.45 | 32.26 |
| Poaceae 2 | 0.20 | 0.00 | 0.19 | 0.17 | 0.62 | 0.46 | 5.92 | 1.28 | 13.26 | 1.65 | **16.92** | 1.42 | 10.33 | 0.43 | 1.98 | 54.82 |
| Poaceae 3 | 0.00 | 0.00 | 0.00 | 0.00 | 0.81 | 0.00 | 0.56 | 0.37 | **2.11** | 0.00 | 1.76 | 0.00 | 1.45 | 0.00 | 0.00 | 8.46 |

monounsaturated FA, the latter dominated by $C_{16:1(n-7)}$ and $C_{16:3(n-9,\ 12,\ 15)}$.

Coinciding with the intervals identified from the *n*-alkane indexes, the predominant saturated FA was $C_{26:0}$, found from 66 cm to 42 cm, while $C_{16:0}$ was the most abundant homolog in the uppermost 42 cm (Fig. 2). Likewise, $TAR_{FA}$ values were > 2 in the lowermost 66–42 cm, falling to < 1.0 from 42 cm to the top.

The FA concentration was higher than that of *n*-alkanes, with values between 67 mg/kg and 667 mg/kg, reaching the highest concentrations in the uppermost 4 cm (Fig. 2).

#### 4.4.3. *n-Alkan-2-ones*

The *n*-alkan-2-ones ranged mainly from $C_{19}$ to $C_{33}$, with a strong odd/even predominance. All the samples maximized mainly at $C_{27}$ (Fig. 4 A, B) or $C_{29}$ (Fig. 4C) with only some samples with a certain bimodal distribution, especially at the uppermost part of the record (Fig. 4A).

The *n*-alkan-2-one concentration was lower than those of *n*-alkanes and FAs, with values between 10 mg/kg and 73 mg/kg, reaching the highest concentrations between 52 and 66 cm and in the uppermost 12 cm (Fig. 2).

#### 4.4.4. *Sterols*

The major sterols identified in the plants are shown in Table 3, 24-ethylcholest-5-en-3β-ol (β-sitosterol), 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol) and 24-methylcholest-5α-en-3β-ol (campesterol) being

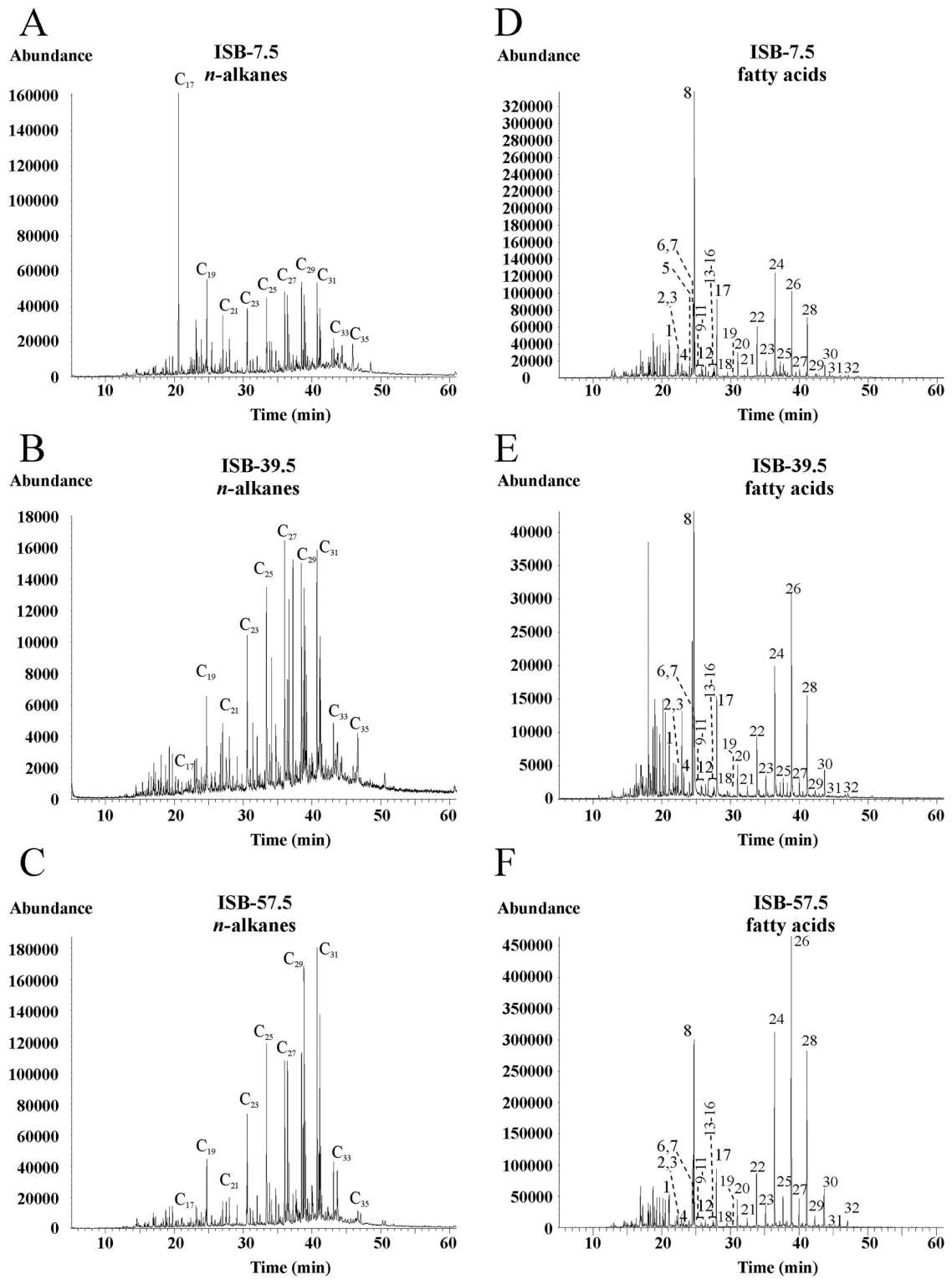


**Fig. 3.** Typical chromatograms of *n*-alkanes and FAs from selected samples in Lake Isoba core. Samples maximized: A) at low molecular weight *n*-alkanes ($C_{17}$); B-C) at high molecular weight *n*-alkanes ($C_{27}$ and $C_{31}$); D) at low molecular weight saturated FA ($C_{16:0}$); E) at a bimodal distribution, but maximizing at low molecular weight saturated FA ($C_{16:0}$); F) at a bimodal distribution, but maximizing at high molecular weight saturated FA ($C_{26:0}$). Peak identification of FA: (1) $C_{14:0}$; (2) *iso*-$C_{15:0}$; (3) *anteiso*-$C_{15:0}$; (4) $C_{15:0}$; (5) *iso*-$C_{16:0}$; (6) $C_{16:1(n-7)}$; (7) $C_{16:1(n-11)}$; (8) $C_{16:0}$; (9) 10-methyl $C_{16:0}$; (10) *iso*- $C_{17:0}$; (11) *anteiso*-$C_{17:0}$; (12) $C_{17:0}$; (13) *iso*-$C_{18:0}$; (14) $C_{18:2(n-9,12)}$; (15) $C_{18:3(n-9,12,15)}$; (16) $C_{18:1(n-8)}$; (17) $C_{18:0}$; (18) $C_{19:0}$; (19) $C_{19:1(n-9)}$; (20) $C_{20:0}$; (21) $C_{21:0}$; (22) $C_{22:0}$; (23) $C_{23:0}$; (24) $C_{24:0}$; (25) $C_{25:0}$; (26) $C_{26:0}$; (27) $C_{27:0}$; (28) $C_{28:0}$; (29) $C_{29:0}$; (30) $C_{30:0}$; (31) $C_{31:0}$; (32) $C_{32:0}$.

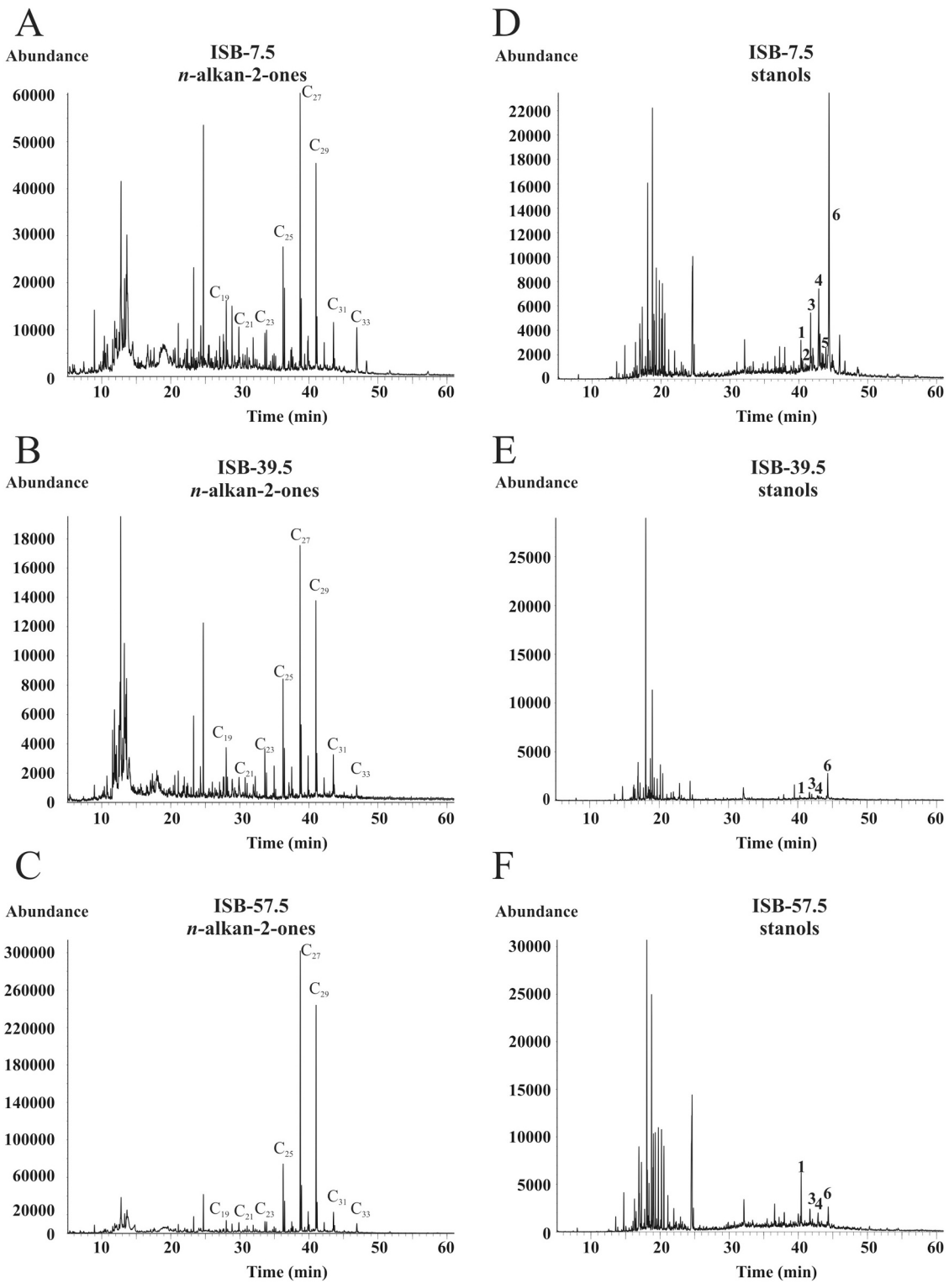


**Fig. 4.** Typical chromatograms of *n*-alkan-2-ones and stanols from selected samples in Lake Isoba core. Samples maximized: A) at high molecular weight *n*-alkan-2-ones ($C_{27}$); B-C) at high molecular weight *n*-alkan-2-ones ($C_{27}$) with a certain bimodal distribution. Peak identification of stanols D-F): (1) coprostanol; (2) epicoprostanol; (3) α-cholestanol; (4) 24-ethylcoprostanol; (5) 24-ethylepicoprostanol; (6) β-sitosterol.

the most abundant. The presence of cholest-5-en-3β-ol (cholesterol) in most species should be highlighted. In addition, 24-methylcholesta-5,22-dien-3β-ol (brassicasterol) was exclusively present in algae and aquatic macrophyes from Lake Isoba (Table 3).

The following major sterols were identified in the core (Fig. 5): β-sitosterol, stigmasterol, campesterol and cholesterol, coinciding with those observed in living plants (Table 3), although their concentration was much lower in the sediments. In addition, brassicasterol and 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol) were identified, especially in the uppermost 12 cm, which are typical sterols present in algae and diatoms (Volkman, 1986; He et al., 2008; Rampen et al., 2010). Of note, the uppermost levels of the core showed the highest

**Table 3**
Concentration (n.d., not detected) of sterols and α- and β-amyrin in living plants from Lake Isoba.

| | Campesterol | Stigmasterol | β-Sitosterol | Cholesterol | Brassicasterol | α-amyrin | β-amyrin |
|---|---|---|---|---|---|---|---|
| Algae 1 | 2.22 | 2.99 | 10.51 | 2.00 | 3.78 | 2.01 | 3.37 |
| Algae 2 | 0.74 | 1.11 | 6.18 | 0.93 | 1.63 | 3.74 | 5.57 |
| *Potamogeton* | 6.50 | 14.86 | 4.52 | 1.01 | 1.31 | 0.65 | 1.26 |
| *Ranunculus* | 0.48 | 0.79 | 3.34 | 0.69 | 0.11 | 2.91 | 4.53 |
| *Myriophyllum* | 3.89 | 2.33 | 8.92 | 2.98 | nd | 3.49 | 10.22 |
| *Limosella* | 0.61 | 1.13 | 5.36 | 1.07 | nd | 3.32 | 4.18 |
| Moss | 17.02 | 7.88 | 24.87 | 6.16 | nd | 122.76 | 199.20 |
| *Eleocharis* | 0.22 | 0.32 | 0.95 | 0.02 | nd | nd | 0.02 |
| *Sellaginella* | 3.90 | 1.74 | 5.92 | 1.21 | nd | 9.32 | 6.32 |
| *Cytisus* | 0.10 | 0.10 | 0.88 | 1.43 | nd | 0.27 | 0.06 |
| *Carex* | 3.58 | 2.59 | 14.39 | 0.89 | nd | 0.37 | 0.68 |
| *Erica* | nd | nd | 1.75 | 0.74 | nd | 424.76 | 417.01 |
| *Pteridium* | 1.28 | 0.54 | 14.15 | 1.04 | nd | 1.59 | 2.41 |
| Poaceae 1 | 3.48 | 2.66 | 15.24 | 1.21 | nd | 3.48 | 4.25 |
| Poaceae 2 | 0.36 | 0.34 | 4.05 | 3.12 | nd | 3.67 | 3.41 |
| Poaceae 3 | 2.30 | 1.80 | 18.03 | 2.93 | nd | 2.65 | 5.97 |

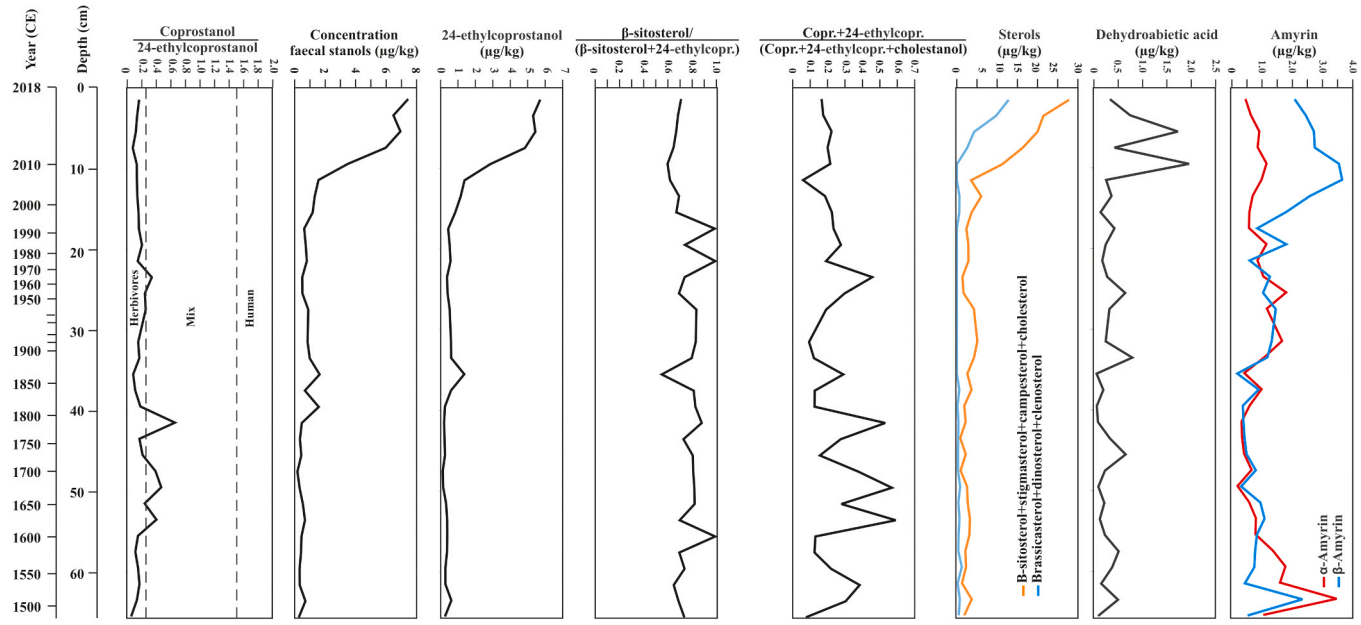


**Fig. 5.** Profiles of the [(coprostanol + epicoprostanol)/ (24-ethylcoprostanol + 24-ethylepicoprostanol)] ratio, concentration of faecal stanols and 24-ethylepicoprostanol, [(coprostanol + epicoprostanol)/(coprostanol + epicoprostanol+5α-cholestanol)] and [β-sitosterol/(β-sitosterol + 24-ethylcoprostanol + 24-ethylepicoprostanol)] ratios, concentration of sterols, and concentration of dehydroabietic acid and α- and β-amiryn in Lake Isoba core.

sterol abundance.

The wide variety of sterols and their derivatives are important palaeoenvironmental indicators. As observed in living plants from the Lake Isoba and surroundings, the major sterol was β-sitosterol, followed by stigmasterol and campesterol, coinciding with observations by Goad and Goodwin (1972), Nishimura and Koyama (1977), Nishimura (1977), Volkman (1986), Gülz et al. (1987), Goad (1991) and Laureillard and Saliot (1993) for vascular plants. We also found that β-sitosterol was predominant in green algae, as observed by Volkman (1986), and abundant in the aquatic macrophyte *Potamogeton* sp., together with stigmasterol and campesterol. Therefore, the presence of these sterols along the core is not unusual.

#### 4.4.5. Stanols

The main stanols identified in the sediments included 24-ethyl-5β-cholestan-3β-ol (24-ethylcoprostanol), 5β-cholestan-3β-ol (coprostanol), 5α-cholestan-3β-ol (cholestanol) and 24-ethyl-5α-choles-22E-en-3β-ol (5α-stigmastanol) among others (Fig. 4D-F). In some levels we also determined 5β-cholestan-3α-ol (epicoprostanol) and 24-ethyl-5β-cholestan-3α-ol (24-ethylepicoprostanol) in low amount. Of note, the uppermost levels of the core showed the highest faecal stanol abundance (Fig. 5). 24-ethylcoprostanol and coprostanol were found in the core, the former being predominant (Fig. 5).

#### 4.4.6. Dehydroabietic acid

Dehydroabietic acid was found throughout the core with concentrations ranging from 0.1 to 1.9 mg/kg, being specilly abundant at 45, 33, 25, 9 and 5 cm (Fig. 2).

#### 4.4.7. α- and β-amyrin

These pentacyclic triterpenoids were observed in the plants from Lake Isoba, although thye were markedly abundant in *Erica* and moss (Table 3). In contrast, in other taxa (*Eleocharis*, *Cystisus*, *Carex*) were present in very low amounts.

These compunds were observed in the whole record (Fig. 2), showing concentrations between 0.2 mg/kg and 3.9 mg/kg. Both compounds were abundant at the bottom of the core, and β-amyrin increased at the top of the record.

#### 4.4.8. Gammacerane and organic sulfur

The gammacerane abundance was low, with values between 0.5 mg/kg and 1.7 mg/kg, between the bottom of the core and 35 cm. Between 35 and 12 cm the concentration ranged between 1.8 and 8.2 mg/kg, and reaching high concentrations (> 8.0 mg/kg) in the uppermost 12 cm (Fig. 2).

Organic sulfur is absent or present in low concentrations (0.3–1.0 mg/kg) with the exception of the uppermost 12 cm, in which the abundance was > 5.0 mg/kg (Fig. 2). In our view, the organic sulfur content in Lake Isoba record is a more reliable proxy for reflecting the oxic/anoxic conditions than other proxies since the OM is mainly of terrestrial origin and was transported, it could have undergone diagenetic processes (oxidation) prior to deposition, whereas the organic sulfur originates directly from the medium.

## 5. Discussion

### 5.1. Origin and preservation of OM-Palaeoenvironmental implications

We used the TOC content, C/N and *n*-alkane profiles to distinguish the various sources of OM in the record of Lake Isoba. The predominant *n*-alkane chain can provide information about the input of OM from algae ($C_{17}$ max.), aquatic macrophytes (maximizing at $C_{21}$, $C_{23}$ and $C_{25}$) and terrigenous plants (maximizing at $C_{27}$, $C_{29}$ and $C_{31}$). In this regard, the *n*-alkane profiles of the living plants collected around Lake Isoba (Table 1) support this distinction.

Overall, HMW alkanes dominate in the sediment core, indicating a major input from terrestrial plants. However, the bimodal distributions of *n*-alkanes in some samples together with $TAR_{HC}$ values suggest that algae also contributed significantly to OM input. Indeed, there was generally good correspondence between $TAR_{HC}$, the predominant *n*-alkane chain, ACL and $TAR_{FA}$ values in Lake Isoba core (Fig. 2). The observed trend pointed to a low microbial degradation of *n*-alkanes in the sediments of the record.

However, there was a lack of correlation or a certain degree of inverse covariation between $P_{aq}$ and other indices (Table 4), suggesting that aquatic macrophytes did not make a significant contribution to the OM. Nevertheless, a well developed ring of aquatic vegetation dominated by *Potamogeton* characterizes Lake Isoba today (Fig. 1B; Fernández Aláez et al. 1987; García-Criado et al., 2005).

Of note, the $P_{aq}$ index showed a good correspondence with the relative percentage of $C_{27}$ alkane which could be linked to wetter conditions, i.e. $\%C_{27}$ usually reflects increasing rainfall, and $P_{aq}$ the contribution of aquatic macrophytes, thereby suggesting that oscillations of the water level of Lake Isoba were linked to climatic (wetter vs. drier) conditions. In general, deciduous tree assemblages (dominated by $C_{27}$) are more abundant during humid climatic phases, while grasses (high concentrations of $C_{31}$) dominante during deforestation periods and drier phases (Schwark et al., 2002; Ortiz et al., 2004, 2011b; Sachse et al., 2006). Thus, the $C_{27}$ alkane content in sediments with considerable input from vascular plants would increase under more humid conditions, whereas drier climates would produce enrichment in $C_{31}$. The strong negative correlation between the percentages of $C_{27}$ and $C_{31}$ throughout the Isoba record (Table 4) may be related to wet/dry phases. In addition, the $P_{aq}$ values were originally defined and used to determine the relative contribution of aquatic macrophytes and terrestrial plants in some African lakes (Ficken et al., 2000), with higher values indicating the increasing contribution of the former ones. Thus, we interpret the high correlation coefficient (0.80) between $P_{aq}$ values and the $C_{27}$ content as indicative of increasing abundances of macrophytes in turn linked to high water-level phases under more humid conditions.

**Table 4**
Correlation coefficients and significant levels (correlations statistically significant at the level of p<0.001 are in bold) for ACL, $TAR_{HC}$, $P_{aq}$ and $TAR_{FA}$ indexes in Lake Isoba.

| | CPI | $P_{aq}$ | $TAR_{HC}$ | $\%C_{27}$ | $\%C_{29}$ | $\%C_{31}$ | $TAR_{FA}$ |
|---|---|---|---|---|---|---|---|
| ACL | 0.05 | -0.29 | **0.63** | -0.37 | 0.17 | 0.39 | **0.62** |
| CPI | | -0.37 | 0.23 | -0.50 | 0.13 | **0.64** | 0.27 |
| $P_{aq}$ | | | -0.38 | **0.80** | **-0.58** | **-0.61** | -0.48 |
| $TAR_{HC}$ | | | | -0.51 | 0.30 | 0.46 | **0.75** |
| $\%C_{27}$ | | | | | **-0.77** | **-0.72** | **-0.65** |
| $\%C_{29}$ | | | | | | 0.10 | 0.41 |
| $\%C_{31}$ | | | | | | | 0.57 |

The CPI values observed in the cores showed some oscillations, but overall indicated low degradation of *n*-alkanes, which might be interpreted in terms of the origin of OM input (cf. Hedges and Prahl, 1993; Zheng et al., 2007). However, the results indicate that the correlation coefficients between the CPI and the other indices (ACL, $P_{aq}$, $TAR_{HC}$) were not significant (Table 4). This fact could be due to mixed sources of OM along the core, although a certain degree of diagenetic alteration cannot be ruled out in some periods (Thornton and McManus, 1994; Graham et al., 2001; Liu et al., 2006). In this regard, there was good correlation between $TAR_{FA}$ values and $TAR_{HC}$ (Table 4), which points to a low microbial degradation of *n*-alkanes in these sediments.

Nevertheless, samples in the uppermost 42 cm of Lake Isoba record showed a strong predominance of even-numbered FAs, maximizing at $C_{16:0}$ or $C_{18:0}$ (Fig. 2), with a bimodal distribution (Fig. 3E,F). This interval showed low $TAR_{FA}$ values (< 1.0), and a bimodal distribution of saturated FA, maximizing mainly at $C_{16:0}$. In addition, the most abundant alkanes were $C_{27}$ and $C_{29}$, with the exception of some samples in the uppermost 12 cm, that maximize at $C_{17}$ alkane. This trend implies a mixed source of OM input (algae, bacteria and land plant) or microbial degradation of high molecular weight saturated FAs.

The long-chain saturated FAs ($C_{24:0}$ to $C_{30:0}$) with a predominance of even numbers are major components of the wax of the leaves, flowers and pollen derived from land plants (Eglinton and Calvin, 1967; Rieley et al., 1991; Meyers and Ishiwatari, 1993), while algae and bacteria maximize at shorter chain lengths, from $C_{12:0}$ to $C_{18:0}$ (Eglinton and Calvin, 1967; Cranwell et al., 1987). Furthermore, $C_{16:0}$ is predominant in algae and accompanied by $C_{14:0}$ and/or $C_{18:0}$ and diverse monounsaturated FAs, mainly $C_{16:1(n-7)}$ or $C_{18:1(n-9)}$, and polyunsaturated FAs, including $C_{18:3(n-3)}$, $C_{18:2(n-6)}$, $C_{20:5(n-3)}$ and/or $C_{22:6(n-3)}$, depending on the algal class (diatoms, eustigmatophytes, green algae, dinoflagellates, haptophytes) (Volkman et al., 1980, 1993, 1999, 2008; Cranwell et al., 1990; Dunstan et al., 1994; Mansour et al., 1999; Olofsson et al., 2012, 2014; Mitra et al., 2015; Rezanka et al., 2017; Volkman, 2018). In this regard, the significant concentration of $C_{18:0}$ FA and $C_{18}$ polyunsaturated FAs in the cores indicated contributions from green algae, specially at the top of the record (Cranwell et al., 1990; Volkman et al., 2008).

The FA distributions reflected the significant proportion of branched FAs ($C_{15}$ and $C_{17}$ iso- and anteiso-FAs predominated in this group), which, together with $C_{16:1(n-7)}$, are indicative of bacterial biomass (Volkman et al., 1980; 2008; Cranwell, 1982; Jaffé et al., 1995; Mudge and Norris, 1997). Given the abundance of branched acids derived from bacteria, it is highly likely that a certain proportion of the $C_{16:0}$ and $C_{16:1(n-7)}$ acids were also from these organisms (Volkman et al., 1980). Thus, the FA data point to certain bacterial reworking of the OM deposited in the sediments (Haddad et al., 1992), which showed an increasing trend towards the top of the record.

Thus, our findings may also indicate the occurrence of some microbial degradation of high molecular weight saturated FAs from primary OM, a process that produces short-chain homologues (Kawamura et al., 1987). Indeed, saturated FAs are more sensitive to degradation and modification than other lipid biomarkers (Meyers and Eadie, 1993). In any case, these results indicate the usefulness of *n*-alkane indices for the interpretation of paleoenvironmental conditions in Lake Isoba.

In Lake Isoba, there was a predominance of $C_{27}$ and $C_{29}$ alkan-2-ones, with the LMW homologues present in low amounts (Fig. 2), which could be attributed to an important input of land plants, as HMW *n*-alkan-2-ones between $C_{23}$ and $C_{31}$ are mainly present in *Sphagnum* moss

(Morrison and Bick, 1967; Baas et al., 2000; and Nichols and Huang, 2007; Ortiz et al., 2011), and in other land plants (Table 2; Ortiz et al., 2011a; 2016b).

Nevertheless, according to Amblés et al. (1993), Jansen and Nierop (2009) and Ortiz et al. (2011a), (2016b), direct input of *n*-alkan-2-ones from plants may not be the dominant contributor to the distributions in certain environments. In this regard, the abundance of *n*-alkan-2-ones in living plants (Table 2) was similar or slightly lower than in Lake Isoba sediments (Fig. 2). Thus, a possible enrichment process cannot be discarded. In this regard, microbial oxidation of the related *n*-alkanes (Amblés et al. 1993; van Bergen et al., 1998; Ortiz et al., 2016b) is the most widely suggested origin for *n*-alkan-2-ones in sediments, supported by similar chain length distribution profiles of both compound classes and lower abundance of the *n*-alkan-2-ones, i.e., in Lake Isoba both $C_{27}$ and $C_{29}$ homologues were the most abundant alkanes and alkan-2-ones (Fig. 2). In addition, oxidative decarboxylation of FAs may have also be another source for the *n*-alkan-2-ones in the sediments of Lake Isoba, as a dominance of FAs of even chain length with one additional carbon vs. the associated ketones occurred (Püttmann and Bracke, 1995). In this regard, between 66 and 42 cm, FAs maximized at $C_{26}$, being $C_{27}$ the most abundant *n*-alkan-2-one. In addition, $C_{26}$ homologue was still an abundant FA in the uppermost 40 cm although a bimodal distribution of FAs occurred. In contrast, bacterial activity was not expected to be the main source of alkan-2-ones, as $C_{27}$ and $C_{29}$ were the most abundant *n*-alkane-2-ones whereas the $C_{19}$ homologue, indicative of bacterial degradation, was present in very low amounts (cf. López-Días et al., 2013). Thus, despite some oxidation processes may have occurred, degradation of OM was not significant.

In addition, the concentration of sterols in sediments was higher in the uppermost levels of the core and decreasing sharply below ca 12 cm. In this regard, the typical algal-sterols were especially abundant at the top of the record. These observations may be attributed to the high contribution of algae in the uppermost 12 cm, and certain microbial degradation below this depth. The following processes may be responsible for the loss of sterols: complete mineralization, chemical conversion to modified sterols and/or condensation of steroid moieties to form non-volatile constituents (van Bergen et al., 1997). However, sterols are relatively more resistant to degradation than other lipids, and we did not find altered compounds derived from them, such as keto- or hydroxylated products. In contrast, important diatom productivity was identified at the top of the core (Gardoki et al., 2023). Likewise, according to Bull et al. (2000), sitosterol rapidly diminishes in soil, possibly as a result of assimilation by soil-dwelling invertebrates (cf. Svoboda and Thompson 1985; Nes et al., 1997) and rapid oxidative degradation. In fact, we found certain evidence of the degradation of saturated FAs and the presence of bacterial activity.

The FA and alkan-2-ones profiles were used to confirm the information obtained from the above mentioned proxies, and also to identify the microbial degradation of OM. Based on the *n*-alkane and FA indices, three geochemical units were identified: A, B and C (Fig. 2).

**Unit A (ca. 1460–1780 CE)**

This unit was identified in the lowermost part of Lake Isoba record (66–42 cm; Fig. 2). According to the chronology, this unit covered ca. 1460–1780 CE.

C/N values (13.1–14.2) showed that OM had a mixed source comprising vascular plants and algae (Hedges et al., 1986; Meyers, 1994). The predominant *n*-alkane chain ($C_{27}$, $C_{29}$, $C_{31}$), the high ACL (27.0–28.4), CPI (>5.3) and $TAR_{HC}$ (16.8–121.8) values, and the homogenous $P_{aq}$ index profile (0.18–0.36) allow specifying that continuous and considerable input of OM came from land plants and a small input from aquatic macrophytes or algae. The scarce presence of typical algal-derived sterols, together with the high abundance of α- and β-amyrin, which are mainly linked to higher plants, as well as dehydroabietic acid, derived from coniferous resins, corroborated this interpretation.

The predominant saturated FA, maximizing at $C_{26:0}$, the higher $TAR_{FA}$ values (> 1.9) than in the other units, and high CPI pointed to minimal microbial degradation of OM. This observation was accompanied by evidence of substantial OM input in this unit, as the *n*-alkane and FA contents were higher than in other parts of the records. These findings (little degradation of organic compounds and increased OM input through runoff) could be linked to the climatic conditions (colder temperatures) that occurred during the Little Ice Age (LIA), which ended in the 19th century (Jones et al., 2001, 2009), when humidity generally increased at the Iberian Peninsula (Moreno et al., 2008, 2010; Martín-Puertas et al., 2008, 2010; Morellón et al., 2009, 2012; Nieto-Moreno et al., 2011), leading to higher runoff. Moreover, colder conditions during LIA could have inhibited bacterial activity and diagenesis of OM (cf. Zheng et al., 2007). In addition, organic sulfur was identified in some levels of this unit, pointing to low oxygen conditions. In this regard, the maximum abundances of small fragilaroid diatoms occurred between 50 and 42 cm (Gardoki et al., 2023) pointing to the occurrence of prolonged periods of ice cover. Indeed, the proliferation of these taxa has been recorded in shallow lakes at high elevation under cold conditions (Karst-Riddoch et al., 2009). Interestingly, lipid proxies in Lake Enol record, also located in the Cantabrian region, show a remarkably similar behavior during the LIA (Ortiz et al., 2016a). Taken together, these results suggest that reduced mixing of the water column was a widespread phenomenon in Cantabrian mountain lakes during this period. Indeed, the percentage of the $C_{27}$ *n*-alkane showed low values in this Unit pointing to relatively drier conditions, although two short episodes with increasing humidity should occur between 59 and 55 cm (ca. 1570–1600 CE) and 48–46 cm (1740–1750 CE). These conditions were also inferred from the diatom stratigraphy and the inorganic geochemistry of Lake Isoba record, particularly during the first episode (DZ1, Gardoki et al., 2023), also characterized by relatively high lake levels and runoff together with variable macrophyte coverage driven by moister climate conditions.

In the Iberian Peninsula, the LIA was characterised by general cold conditions and substantial hydrological variability considering records from the Cantabrian region, Sierra Nevada, the Pyrenees and the Iberian Central Range (Morellón et al., 2011, 2012; López-Merino et al., 2011; Roberts et al., 2012; Ortiz et al., 2016a; Oliva et al., 2018). According to Castro et al. (2020) Late 16th and 17th centuries in NW Iberia were cold and wet but with several dry episodes.

**Unit B (ca. 1780–2006 CE)**

This unit was defined in the intermediate part of Lake Isoba record (42–12 cm; 1780–2006 CE; Fig. 2).

The atomic C/N values, which were slightly lower than in the previous unit (11.8–13.4), also indicated a mixed input of terrestrial plants but with a greater influence of OM derived from algae than in unit A.

The predominant *n*-alkane chain ($C_{27}$), ACL (24.8–27.1), CPI (>4.6) and $TAR_{HC}$ (4.7–19.2) values reflected the presence of OM derived mainly from terrestrial plants. However, ACL and $TAR_{HC}$ values were lower than in unit A, indicating that other kinds of material (probably aquatic macrophytes and algae) contributed to the OM. The increase in the $P_{aq}$ index (0.28–0.51) confirmed a relatively higher contribution from aquatic macrophytes than in Unit A. Indeed, the slight increase in the percentage of the $C_{27}$ *n*-alkane points to episodes of more humid conditions since ca. 1850 CE (subunit B2; Fig. 2), which could have favoured macrophyte development. The notable increase of periphytic and epiphytic diatoms also showed that macrophytes expanded during this phase, suggesting higher lake levels and warmer temperatures (Gardoki et al., 2023).

It is worth noting that climatic amelioration towards temperate conditions and increasing humidity occurred gradually. Indeed, between ca. 1780 and 1850 CE (subunit B1) there was a transitional period in which ACL and $TAR_{HC}$ ratios showed a decreasing trend whereas $P_{aq}$ values as well as the percentage of $C_{27}$ *n*-alkane progressively increased, which has a good correspondence with the end of the LIA. In addition, low *n*-alkane and FA contents revealed less OM input and/or higher degradation of organic compounds during ca. 1780–2006 CE compared

to the previous unit. In this regard, organic sulfur did not occurred during this unit, likely indicating oxigenated conditions.

The low $TAR_{FA}$ values (< 1.0), and the bimodal distribution of saturated FAs, maximizing mainly at $C_{16:0}$, and the most abundant alkane being $C_{27}$ (Fig. 3E), pointed to the occurrence of microbial degradation of high MW saturated FAs from primary OM, a process that produces short chain homologs (cf. Kawamura et al., 1987). This process was probably linked to global warming that began at the 19th century and continued during the 20th century, being specially noticeable in the decrease of $TAR_{FA}$ values and increase of branched and polyunsaturated FAs since ca. 1850 CE (subunit B2; Fig. 2). It is also noticeable the slight increase of gammacerane from ca. 1850 CE to the top of this unit pointing to certain stratification of the water column. Similar palaeoenvironmental conditions were reconstructed in Lake Enol record during this period from the *n*-alkane and FA content (Ortiz et al., 2016a).

Of note, a warming trend starting at the end of the LIA at ca. 1850 CE has been reconstructed in other areas of the Cantabrian region (López-Merino et al., 2011; Martín-Chivelet et al., 2011; Ortiz et al., 2016a) and the Mediterranean area (Calò et al., 2013). In addition, there is evidence of glaciers melting until their complete disappearance during the first half of the 20th century (Oliva et al., 2018; Serrano et al., 2018).

However, anthropogenic influence cannot be ruled out in this area and changes in livestock management were considerable during the 20th century—a period marked by a decline in sheep and goats. Furthermore, cattle, which were used mostly for meat, were replaced by milk-producing breeds, which spent more time in the valleys rather than in the mountains (Suárez Antuña et al., 2005). Indeed, an increase in the stanol concentration was observed since ca. 1850, although between ca. 1960 and 1990 a decrease occurred.

**Unit C (ca. 2006–2018 CE)**

This unit was identified only in the uppermost part of Lake Isoba record (12–0 cm; Fig. 2), representing the last ca. 12 yr (2006–2018 CE).

The low atomic C/N values (< 12.0), indicated a substantial algal source. There was also good correspondence with the interpretation derived from the predominant *n*-alkane chain and ACL profiles, i.e. the dominance of short chain homologues ($C_{17}$) and the low ACL values (20.0–24.3) in this unit bring further support to the algal contribution to the OM. In addition, the abundance of typical algal-derived sterols, such as brassicasterol and dinosterol confirmed this interpretation. Nevertheless, most of the samples showed a bimodal distribution of *n*-alkanes, thereby pointing to a mixed OM input.

Moreover, low $TAR_{HC}$ values (< 1), were linked to significant algal input or bacterial activity. As in the other units, $P_{aq}$ values ranged between 0.24 and 0.38, suggesting some input of aquatic macrophytes, although much smaller than that of OM from land plants and algae. CPI values (8.0–10.0) confirmed that the contribution of terrestrial plants to the OM accumulation was less significant than in other units (cf. Hedges and Prahl, 1993).

Coinciding with the interpretation derived from the *n*-alkane indices, low $TAR_{FA}$ values (< 0.5) and the predominance of short chain saturated FAs indicated more substantial algal source. However, we cannot rule out that bacterial activity may have led to the production of low MW saturated FAs at the expense of long chain homologs (cf. Kawamura et al., 1987). In this regard, a marked increase of branched and polyunsaturated FAs was observed in this unit.

It is worth noting that the total sum of *n*-alkanes and FAs was significantly higher than in other units, coinciding with an increase in TOC (Fig. 2). Simultaneously, evidence of considerable algal input (high content of low MW *n*-alkanes and sterols) and some microbial degradation (predominance of low MW saturated FAs) was recorded. This observation can be partly interpreted in terms of climatic change as, according to available meteorological data from the region, the regional precipitation has decreased and temperature has increased since 1973 CE (López-Merino et al., 2011). These changes must have affected the hydrology of the lake catchment, reducing land plant input at boosting phytoplankton production. In this regard, the percentage of $C_{27}$ decrease in this unit, indicating drier conditions with respect to Unit B.

Furthermore, this may be also linked to anthropogenic influence, as significant changes in livestock composition and management occurred within the catchment during the last few decades. Summer transhumant merino sheep grazing occurred in the surroundings of Lake Isoba until year 2010 CE, when they were replaced by staying local cattle (Rodríguez, 2001; Ezquerra and Rey, 2011), characterized by longer presence in the catchment and usually leading to a higher erosion and faecal pollution (Bond et al., 2014; Burt et al., 2013). In addition, gammacerane and organic sulfur were markedly abundant in this unit, indicating a stratified water body with anaerobic conditions. In contrast, the absence or low abundance of organic sulfur in unit B could be linked to recirculation of water in the lake, which produced water column mixing and oxic conditions in previous periods. The high abundance of β-amyrin and dehydroabietic acid in Unit C could be linked to the anoxic conditions which prevented degradation of these compounds although the high concentrations of β-amyrin may also be attributed to algae, aquatic macrophytes and moss abundance although it is also very abundant in *Erica*. Dehydroabietic acid may also be related to pine reforestation in the area.

Similarly, López-Merino et al. (2011) inferred lower runoff, more input of nutrients and increasing lake productivity in Lake Enol from the presence of diatoms characteristic of mesotrophic and eutrophic waters since 1970. In the same lake, Ortiz et al. (2016a) found similar environmental changes based on organic geochemistry.

### 5.2. Pollution

For the interpretation of the measured results in terms of degradation effects, the following ratio was calculated (Fig. 5): (coprostanol + epicoprostanol)/(coprostanol + epicoprostanol+5α-cholestanol). Indeed, Bull et al. (1999), (2001) and Schroeter et al. (2020) use this ratio to estimate microbial degradation of steroids (5β-stanols, epi-5β-stanols and 5α-stanols), although it can also be used to identify human faeces input based on the presence of coprostanol (Wiedner et al., 2015). In this regard, 5α-cholestanol is a degradation product of cholesterol (the $\Delta^5$-sterol precursor), which is transformed by soil microorganisms (Prost et al., 2017). In Lake Isoba the values of this ratio (Fig. 5) suggest low human faeces input and little microbial degradation (Bull et al., 1999; Schroeter et al., 2020). We exclude a strong degradation of coprostanol in the samples due to the non-detected epicoprostanol (with the exception of the uppermost 3 samples) as a transformation product of coprostanol (Lerch et al., 2022). We also discard a significant degradation of 24-ethyl-coprostanol as the 24-epiethyl-coprostanol was not identified in the samples.

In addition, the ratio β-sitosterol/(β-sitosterol + 24-ethylcoprostanol + 24-ethylepicoprostanol) was also calculated for differentiation between plant-derived steroids (β-sitosterol) and livestock-derived ones (24-ethylcoprostanol+24-ethylepicoprostanol). According to Prost et al. (2017), β-sitosterol belongs to the typical $\Delta^5$-sterols, which are characteristic of plant biomass and thus normally occur at high abundance in soils. Lake Isoba samples yielded values of > 0.5, indicating a dominant content of plant-derived steroids (high β-sitosterol contents) over livestock-derived steroids in the sediments. Nevertheless, it has to be considered that faeces of ruminants can also contain high amounts of β-sitosterol due to the ruminants' plant-dominated diet (Haurrault et al., 2019; Schroeter et al., 2020).

The presence of 24-ethylcoprostanol, a biomarker of hervibores faecal pollution, as well as, coprostanol, linked to human faecal pollution in the recent sediments of Lake Isoba must be highlighted.

Bethell et al. (1994) and Evershed and Bethell (1996) proposed the use of the coprostanol/24-ethylcoprostanol ratio as proxy for distinguishing human vs. herbivores faecal input, with values > 1.5 considered indicative of human pollution and < 0.25 when herbivores faeces predominate. Leeming et al. (1997) proposed a refinement of this approach to estimate the relative contribution of different faecal sources

involving the use of the following index: 100x[(coprostanol)/(coprostanol+24-ethylcoprostanol)] to assess the relative contribution of human and herbivore inputs. According to this study, values > 73% would represent solely human contamination and values < 38% solely from herbivores. Here, we used the ratio proposed by Bull et al. (2002) (coprostanol + epicoprostanol)/(24-ethylcoprostanol + 24-ethylepicoprostanol). In this case, values > 1 would indicate predominant human source and < 1, ruminant. However, the epimers from both stanols (epicoprostanol and 24-ethylepicoprostanol) were weakly present, and we therefore calculated the ratio principally with the main stanols (Evershed and Bethell, 1996). At Lake Isoba values were consistently < 1 (Fig. 5), indicating a continuous pollution source derived mainly from cattle. In the last 20–30 yr, the concentration of 24-ethylcoprostanol was significantly higher than in the rest of the record (Fig. 5). The input of organic matter and nutrients derived from faecal pollution may have caused a change of the oligotrophic state in Lake Isoba (Fernández Aláez et al. 1987). This process seems to be widespread in the Cantabrian Range, since according to López-Merino et al. (2011), the most relevant change in the pollen record of Lake Enol in the last 200 yr was related to pastoral activity, as shepherding was, and continues to be, a major economic activity in the region (Domínguez Martín and Puente Fernández, 1995; Mayor López, 2002). The record of coprophilous fungi also supported this fact; as they were abundant, particularly during the 19th century, decreasing in the 20th century (López-Merino et al., 2011). The recent increase in stanols in Lake Isoba occurred since year 2006 CE coinciding with the end of trashumant merino sheep summer grazing and their replacement by staying cattle in the catchment (Rodríguez, 2001; Ezquerra and Rey, 2011). The subsequent increase in faecal pollution might have contributed to increasing the amount of faecal stanols. Consistently, this shift to cattle was also interpreted as the most likely cause for increasing nutrient enrichment and turbidity leading to an abrupt change in diatom assemblages, in terms of diversity and abundance, and maximum absolute diatom productivity reconstructed after year 2010 CE (Gardoki et al., 2023). In addition, coinciding with Gardoki et al. (2023), the previous natural ecological dynamics in Lake Isoba were disrupted in a very short period by cattle grazing. The effects of cattle grazing on the ecological quality status have also been reported from coastal to mountain lakes (García-Rodríguez et al., 2002; Van Colen et al., 2018), although Lake Isoba stands out because of its sharp change and its magnitude. Faecal pollution is known to be an extensive process capable of altering the trophic status and the overall environmental quality of freshwater ecosystems (Haller et al., 2009; Sherry, 1986; Vane et al., 2010). These changes are more pronounced in small and shallow mountain lakes, such as Lake Isoba, since they seem to be less resilient comparing with deep montane lakes (Kuefner et al., 2020).

The replacement of native cattle (used mainly for meat production) that pastured mostly the mountains by breeds (used for milk production) that spent long periods in the valleys (Rodríguez Castañón, 1996; Suárez Antuña et al., 2005), together with the decline in sheep and goats, did not lead to a decrease in the 24-ethylcoprostanol concentration in the sediments, as occurred with the reduction of coprophilous fungi at the expense of the Atlantic bushes *Erica* and *Cytisus/Ulex* (López-Merino et al., 2011). Moreover, our results showed that the contamination linked to herbivores began in the 15th century, perhaps even earlier.

In addition, it is worth noting the significant presence of gammacerane in the uppermost part of the record, indicating a stratified water body with anoxic conditions. There was a good correspondence between gammacerane and the organic sulfur log, which also pointed to the existence of anaerobic conditions in Unit C. In contrast, the absence of organic sulfur in Unit B can be linked to circulation of water in the water body, which produced water column mixing and oxic conditions.

Thus, the geochemical fingerprint of gammacerane and organic sulfur, together with the characteristics of other lipid biomarkers in Unit C (TOC, concentration of *n*-alkanes and FAs, sterols), which reflected an increase of OM input mainly derived from algae (predominance of $C_{17}$ alkane and presence of dinosterol and brassicasterol) and the marked abundance of faecal stanols, revealed a trend to low oxygen conditions and increase nutrient content (possible eutrophication) in the last 30 yrs due to anthropic activities. Indeed, a significant nutrient enrichment, and loss of oligotrophy has been observed since 1980s (Fernández Aláez et al. 1987; Gardoki et al., 2022).

## 6. Conclusions

The *n*-alkane, FA, *n*-alkan-2-one, sterol, α- and β-amyrin, dehydroabietic acid, gammacerane, organic sulfur and stanol content in the Lake Isoba record allowed to reconstruct the palaeoenvironmental evolution during the last ca. 550 yr. In this regard, we identified three units with distinct environmental conditions. There was good correspondence between the predominant *n*-alkane chain, ACL, $TAR_{HC}$ index and C/N values. The record showed dominant OM input from land plants (Unit A –ca. 1460–1780 CE –and Unit B –ca. 1780–2006 CE) as the predominant *n*-alkane was $C_{31,}$ whereas aquatic macrophytes did not contribute significantly, and only the uppermost levels (12 cm) of the core were characterized by considerable algal input linked to anthropic influence (Unit C, ca. 2006–2018 CE).

The lack of correspondence between the *n*-alkane and saturated FA indices in some intervals of the cores (Unit B) was interpreted as microbial synthesis of FAs from primary OM, the process being especially significant in the uppermost part of the core (Unit C).

The lowermost part of the record (Unit A) showed a higher concentration of land plants-derived *n*-alkanes and other lipids than in the other intervals. In our view, this could be linked to the palaeoenvironmental conditions, during the LIA that lessen the algae and macrophyte development and favoured the arrival of terrigenous OM to the lake via runoff. This OM was subject to less degradation because of colder conditions, which inhibited microbial action, and probably had some influence in more prolonged episodes of low oxygen conditions (presence of organic sulfur). Warmer and more humid conditions at the end of the 19th century and during the 20th century, interpreted from the increase of $P_{aq}$ and the percentage of $C_{27}$ *n*-alkane values (Ficken et al., 2000; Schwark et al., 2002; Ortiz et al., 2004, 2011b; Sachse et al., 2006)– also stated by Gardoki et al. (2023) using diatom assemblages and inorganic geochemistry– together with anthropogenic influence (increased presence of livestock in the catchment and the lake shores, afforestation) seemed to have produced a decrease in the input of OM to the lake and favoured the rise of the water level and microbial activity together with some stratification of the water column due to gammacerane increase (Unit B).

The last ca. 12 yr (uppermost 12 cm - Unit C) were characterized by considerable phytoplankton productivity (predominance of $C_{17}$ *n*-alkane and typical algal sterols) and microbial activity (predominance of $C_{16:0}$ saturated FA), accompanied by an increase in OM, organic sulfur and gammacerane contents causing a sharp disruption in the trophic status. The greater input of OM was possibly linked to increased human activity (change of land use and lake management) and a warmer and drier climate.

The stanol content showed continuous pollution derived from livestock and wild herbivores since the 15th century, as revealed by the ubiquitous presence of 24-ethylcoprostanol, a compound linked to animal faeces. This study shows the impact of faecal contamination in the Cantabrian Range since historical times and points out to the importance of paleoenvironmental reconstructions and monitoring in such high ecological value enclaves, like the Picos de Europa Regional Park.

## CRediT authorship contribution statement

**César Morales-Molino:** Investigation, Methodology. **Ignacio López-Cilla:** Investigation, Methodology. **Mario Morellón:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing. **Jon Gardoki:** Investigation, Methodology. **Yolanda Sánchez-**

**Palencia:** Investigation, Methodology. **José E. Ortiz:** Funding acquisition, Investigation, Writing – original draft.

## 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.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ancene.2024.100431.

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