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Large sulfur isotope fractionation during microbial sulfate reduction at low sulfate levels: Early life and environmental implications

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Dissimilatory sulfate reduction (DSR) is proposed among the earliest microbial metabolisms. However, pyrite depleted in the heavy sulfur isotopes is absent before ≈ 2.5 Ga, putatively due to small isotopic fractionation during sulfate-limited DSR. Here, we analyze microscale and bulk sulfur isotopes in pyrite from natural environments spanning sulfate concentrations from 28,000 to 3 μM , revealing large isotopic fractionation even under extreme sulfate scarcity (48‰ at 3 μM). Compiling microscale pyrite sulfur isotope data from rocks older than 2.2 Ga, we identify three samples that confidently indicate active DSR by ≈ 2.7 Ga with sulfate levels of a few to tens of μM . Narrower isotopic ranges in older rocks ($\leq 20\text{‰}$) likely reflect early emergence of sulfite reducers and emergence of complete DSR ≈ 2.7 Ga, and/or post-depositional overprinting.

Through its interaction with oxygen, carbon and iron, sulfur (S) occupies a central role in regulating Earth's surface (geo)chemistry and biology. Dissimilatory sulfate reduction (DSR), a microbial pathway in which sulfate is reduced to sulfide to gain the energy required for cellular function and growth, is thought to be one of the most ancient metabolic pathways, dated to the early Archean (3.6 to 3.2 billion years ago, Ga) [1, 2, 3]. Sulfide produced by DSR is depleted in the heavy isotopes of S, and while this sulfide is not directly preserved in sediments or sedimentary rocks, some of it reacts with Fe to form sedimentary pyrite with limited isotopic modification. Thus, the presence of ^{33}S - and ^{34}S -depleted pyrite is often considered evidence of DSR in environmental and geologic studies [4, 5, 6]. The absence of strongly ^{33}S - and ^{34}S -depleted pyrite prior to ≈ 2.5 Ga [6, 7] apparently contradicts an early Archean origin of DSR.

Laboratory cultures of sulfate reducers grown on different substrates display a wide range of net sulfate-sulfide S isotope fractionation (henceforth, the microbial fractionation, $^{34}\epsilon_{\text{mic}}$; Table 1), which depends on the cell-specific sulfate reduction rate (csSRR). Over a csSRR range spanning ≈ 1 to tens $\text{fmol cell}^{-1} \text{ day}^{-1}$, the microbial fractionation decreases from the equilibrium sulfate-sulfide S isotope fractionation of $\approx 68\text{‰}$ (at the experimental temperatures of 20-25 °C) to a kinetic fractionation of only a few ‰ [8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. This relationship can be extended to include ^{33}S , yielding metabolism-specific combinations of $^{34}\epsilon_{\text{mic}}$ and the mass exponent $^{33}\lambda_{\text{mic}}$ (Table 1) and allowing distinction of DSR from other S metabolisms, such as sulfide oxidation [18] and/or sulfur disproportionation [9]. Compared to laboratory cultures, the relatively low reactivity and/or availability of electron donors in most natural environments, as well as the limited capacity of natural microbial populations to utilize the available substrates, leads to much lower csSRR [19] and near-equilibrium $^{34}\epsilon_{\text{mic}} - ^{33}\lambda_{\text{mic}}$ values [20]. Indeed, grain-specific (hereafter, microscale) pyrite $\delta^{34}\text{S}$ analyses reveal isotopic offsets between the parent sulfate and the most ^{34}S -depleted pyrite grains in modern marine and lacustrine sediments that are consistent with large, and near-equilibrium $^{34}\epsilon_{\text{mic}}$ and $^{33}\lambda_{\text{mic}}$ values [7, 21, 22]. Thus, robust geologic records of microscale S isotopes in pyrite should allow identification of the activity of sulfate reducers through time.

To complicate matters, laboratory cultures and models of microbial metabolism and isotopic

fractionation show $^{34}\epsilon_{\text{mic}}-^{33}\lambda_{\text{mic}}$ values that decrease from their expected equilibrium values with decreasing sulfate concentration ($[\text{SO}_4^{2-}]$) [16, 23, 24]. This departure from equilibrium remains poorly understood, despite its importance for the identification of active DSR in the Precambrian oceans, where sulfate is thought to have been a trace constituent (a few to hundreds μM ; [25, 26, 27, 28]). Laboratory culture-based assessments of the departure from equilibrium at such low $[\text{SO}_4^{2-}]$ and at environmentally relevant csSRR (i.e., $<1 \text{ fmol cell}^{-1} \text{ day}^{-1}$) have not been performed. Natural calibrations of the departure from equilibrium are challenged by the scarcity of low-sulfate environments on the modern Earth’s surface due to ubiquitous sulfate delivery from oxidative continental weathering [29]. Additionally, the microbial fractionation is difficult to directly observe, and past studies in low-sulfate environments have often used the apparent aqueous sulfate-sulfide S isotope offset (Fig. 1.A) as a proxy for $^{34}\epsilon_{\text{mic}}$ [25, 30, 31]. A decrease in the apparent sulfate-sulfide S isotope offset with decreasing $[\text{SO}_4^{2-}]$ in euxinic lakes has been attributed to decreasing $^{34}\epsilon_{\text{mic}}$ [25]. However, observations and diagenetic models link this relationship to the effect of $[\text{SO}_4^{2-}]$ on the “openness” of the system with respect to sulfate [20, 7] and/or sulfide [22], rather than a direct control on $^{34}\epsilon_{\text{mic}}$.

A better understanding of the dependence of $^{34}\epsilon_{\text{mic}}-^{33}\lambda_{\text{mic}}$ values on $[\text{SO}_4^{2-}]$ is required before microscale S isotope analyses can be used to investigate the diversity, activity, and evolution of early S metabolisms. To this end, we performed both microscale and traditional bulk S isotope analyses on a suite of pyrite samples from 8 natural environments (figs. S1-S4), which span a $[\text{SO}_4^{2-}]$ range from 28000 (modern marine concentrations) to 3 μM . Isotopic compositions are given as the $\delta^{34}\text{S}$ and $\delta^{33}\text{S}$ offsets between pyrite (grains and/or bulk) and the local aqueous sulfate ($^{34}\Delta_{\text{pyr}}$; Table 1), and the maximum microscale offset is used to approximate the microbial fractionation ($^{34}\epsilon_{\text{pyr}}-^{33}\lambda_{\text{pyr}}$; Table 1). In high-sulfate environments ($>500 \mu\text{M}$), our reconstructed $^{34}\epsilon_{\text{pyr}}$ is close to the predicted sulfate-sulfide equilibrium fractionation over the range of ambient temperatures (≈ 66 to 78‰ ; Fig. 1.A) [23, 20]. Between 500 and 3 μM , we observe a systematic decrease of $^{34}\epsilon_{\text{pyr}}$ with decreasing $[\text{SO}_4^{2-}]$, relative to the predicted S isotope equilibrium (Fig. 1.A). For example, at $[\text{SO}_4^{2-}]$ of $\approx 500 \mu\text{M}$ $^{34}\epsilon_{\text{pyr}}$ is $\approx 64\text{‰}$, whereas at 3 μM its value decreases to 48‰ . Concurrently, $^{33}\lambda_{\text{pyr}}$ values depart from near-equilibrium (≈ 0.515 at high $[\text{SO}_4^{2-}]$) to values as low as 0.512 at 3 μM $[\text{SO}_4^{2-}]$. Taken together, our $^{34}\epsilon_{\text{pyr}}-^{33}\lambda_{\text{pyr}}$ values reproduce the $^{34}\epsilon_{\text{mic}}-^{33}\lambda_{\text{mic}}$ relationship

observed in culture experiments, and display a similar behavior to that predicted by a metabolic-isotopic model of DSR at low csSRR (at ambient temperatures; Fig. 1). This observation provides confidence that the largest $^{34}\epsilon_{\text{pyr}}$ values are a good approximation of $^{34}\epsilon_{\text{mic}}$, which is especially important in light of a recent suggestion that below $\approx 100 \mu\text{M}$ sulfate, most of the S in pyrite may originate from organic S compounds rather than DSR-sourced sulfide [32, 33]. Though such mechanisms could contribute to pyrite formation in low-sulfate environments, they are incapable of producing $^{34}\Delta_{\text{pyr}}$ ranges as large as those observed in our microscale analyses. An appreciable role for reaction of DSR-sourced sulfide with sedimentary iron is implied, and the persistence of a large apparent microbial fractionation ($^{34}\epsilon_{\text{pyr}} \approx 48\text{‰}$ and $^{33}\lambda_{\text{mic}} \approx 0.511$) under extreme sulfate limitation represents a pivotal constraint for reassessing the activity and evolution of S metabolisms.

The consistency of the $^{34}\epsilon_{\text{pyr}}-^{33}\lambda_{\text{pyr}}$ reconstructions with bio-isotopic predictions across sites, irrespective of the sample's depth below the sediment-water interface, argues against strong alteration of $^{34}\epsilon_{\text{pyr}}-^{33}\lambda_{\text{pyr}}$ during early burial (Fig. 2, fig. S2). Our bulk $^{34}\Delta_{\text{pyr}}$ values, in agreement with previous studies [30], confirm that local reaction-transport dynamics of porewater sulfate and sulfide play an important role in controlling bulk $^{34}\Delta_{\text{pyr}}$ values (7). The wide range of microscale $^{34}\Delta_{\text{pyr}}$ values, spanning the large $^{34}\epsilon_{\text{pyr}}$ to much lower values, reflect Rayleigh-type distillation of porewater sulfate at the site-specific microbial fractionation and reaction-transport dynamics [7]. Sulfidic environments (i.e., environments where aqueous sulfide accumulates in the porewater) give rise to much smaller ranges of $^{34}\Delta_{\text{pyr}}$ and, therefore, to lower reconstructed $^{34}\epsilon_{\text{pyr}}-^{33}\lambda_{\text{pyr}}$ values (see diamonds in Fig. 1). When microbial sulfide production exceeds its sequestration in pyrite (or by organic matter sulfurization), sulfide accumulates in the porewater. Mixing of the instantaneous sulfide produced by DSR then results in accumulated sulfide that is more isotopically homogeneous [22]. This results in a narrower distribution of microscale $^{34}\Delta_{\text{pyr}}$ centered around the bulk $^{34}\Delta_{\text{pyr}}$ value (Fig. 2), limiting our ability to extract a robust DSR isotopic fingerprint from sulfidic environments. Consequently, we expect that $^{34}\epsilon_{\text{pyr}}-^{33}\lambda_{\text{pyr}}$ from previous studies in euxinic environments have similarly underestimated the true microbial fractionation and, consequently, provide an erroneous picture of past seawater chemistry and/or microbial activity.

As noted above, a key goal of investigating $^{34}\epsilon_{\text{pyr}}-^{33}\lambda_{\text{pyr}}$ under low $[\text{SO}_4^{2-}]$ is to tap the potential

of the rock record to constrain the onset of DSR metabolism and its subsequent proliferation. To this end, we compiled and converted to $^{34}\Delta_{\text{pyr}}$ (fig. S5), all published early-Earth (>2.2 Ga), microscale S isotope data available at the time of submission (n=21,709 from $\approx$ 450 samples; Fig. 3). Several issues related to the long history of these rocks must be considered. The data collected are all preserved within Precambrian continental cratons, all of which have experienced complex post-depositional histories. To construct a robust record of the “original” environment and microbial activity we must clearly distinguish any data that do not reflect depositional or early diagenetic conditions (open circles in Fig. 3). For example, data from rocks with post-compaction features such as cross-cutting veins are suspected to reflect late-stage alteration. As another example, data coming from rocks with appreciable detrital pyrite are suspected to reflect contributions from abiotic processes to their isotopic compositions. We identified such data based on the information reported in original publications, which is often limited, and we cannot rule out the possibility that some altered samples have escaped curation. Similarly, some rock samples may have recorded multiple (discrete) episodes of mineralization during compaction and/or burial-exhumation cycles [34, 35, 36, 37, 38]. Yet, unless otherwise stated in the original publication (n=1), the ages assigned in this study reflect the age of the host rock as reported in the original manuscripts. Fortunately, the long-timescale analysis that is of interest here is robust to age inaccuracies of tens of millions of years.

Comparison of our findings in water bodies with varying $[\text{SO}_4^{2-}]$ with the resulting $^{34}\Delta_{\text{pyr}}$ record provides important insights into the diversity and evolution of early S metabolisms (Fig. 3). First, we found three samples with microscale S isotope data that meet the screening requirements to confidently identify past DSR (Figs. 2.B and 3). Those samples are preserved in the 2.7 Ga Tumbiana Formation [39], the 2.45 Ga Boolgeeda Iron Formation [40], and the 2.42 Ga Batatal Formation [41]. As discussed above, our criteria include (i) large $^{34}\epsilon_{\text{pyr}}$, as observed in modern low-sulfate lakes ($\approx$ 50‰) and (ii) large microscale $^{34}\Delta_{\text{pyr}}$ variability, reflecting microbial respiration and isotopic distillation of the aqueous sulfate reservoir. Given the observed dependence of microscale $^{34}\epsilon_{\text{pyr}}$ on $[\text{SO}_4^{2-}]$, our finding of maximal $^{34}\epsilon_{\text{pyr}} \approx$ 40‰ in the 2.7 Ga Tumbiana Formation is consistent with $[\text{SO}_4^{2-}]$ of a few μM . Maximal $^{34}\epsilon_{\text{pyr}}$ exceeding $\approx$ 50‰ in the younger rocks (2.45 and 2.42 Ga) is consistent with $[\text{SO}_4^{2-}]$ of a few tens of μM . The wide range of $^{34}\Delta_{\text{pyr}}$ in these rocks suggests the existence and activity of DSR in surface aquatic environments since $\approx$ 2.7 Ga, and

identification of additional similar arrays would provide confidence in this inference and timing. In contrast, the much narrower $^{34}\Delta_{\text{pyr}}$ range ($\leq \approx 20\text{‰}$) in older (mostly marine) sedimentary rocks may support the idea of seawater sulfate levels that were too low for DSR to flourish, except in spatially and temporally limited, semi-restricted, shallow marine and/or terrestrial basins [42, 43]. Such findings contrast with the suggestion that DSR is one of the oldest and most widespread energy conservation strategies [1, 5], and below we explore several scenarios that may explain the muted microscale $^{34}\Delta_{\text{pyr}}$ record.

All known sulfate reducers reduce sulfite using the Dsr protein and accessory proteins [44]. Recent large-scale phylogenetic and comparative genomic analyses of these proteins have identified the minimal protein set (DsrABCMK(N)) to have a primordial sulfite reduction function [45]. In the context of the early anoxic environment, sulfite was likely steadily supplied to the ocean through dissolution and aqueous speciation of volcanic SO_2 ($\text{SO}_2(\text{aq}) \rightleftharpoons \text{HSO}_3^- + \text{H}^+$ and $\text{HSO}_3^- \rightleftharpoons \text{SO}_3^{2-} + \text{H}^+$), possibly creating a reservoir of metastable species for sulfite reducers to utilize [46]. Hence, it is possible that the S isotope fractionation associated with dissimilatory sulfite reduction could be responsible for the muted microscale $^{34}\Delta_{\text{pyr}}$ observed, prior to the evolution and widespread proliferation of DSR metabolisms. A full metabolic-isotopic model of dissimilatory sulfite reduction is necessary to quantify the dependence of the microbial sulfite-sulfide S isotope fractionation on environmental concentrations of sulfite, sulfide and electron donors. In the absence of such a model, which is beyond the scope of this study, endmember values provide guidance. In a linear metabolic pathway, such as sulfite reduction, the net isotopic fractionation can vary between the equilibrium substrate-product isotopic fractionation and the kinetic isotope effect associated with the most upstream step in the pathway [23]. Hence, for sulfite reduction, the net microbial fractionation can vary between the equilibrium sulfite-sulfide S isotope fractionation ($\approx 43\text{‰}$ at an arbitrary environmental temperature of 10 °C ; [47]) and a near-zero kinetic isotope effect associated with sulfite uptake [17], assuming that sulfite and sulfate transporters are broadly similar. The thermodynamics of sulfite reduction to sulfide using a variety of electron donors is favorable enough over wide ranges of substrate and product concentrations (SM), that near-equilibrium sulfite-sulfide S isotope fractionations are unlikely (fig. S6). Under certain plausible concentrations, however, the microbial S isotope fractionation associated with sulfite reduction can be in the range of $\approx 15\text{-}20\text{‰}$,

consistent with culture experiments with mutants unable to reduce sulfate [48], possibly explaining the muted range in Paleo-Mesoarchean $^{34}\Delta_{\text{pyr}}$.

Rather than arising from an early emergence of sulfite reduction, the muted range of $^{34}\Delta_{\text{pyr}}$ may represent late metasomatic and/or metamorphic overprinting [35, 36, 37, 38]. To test this idea, we took advantage of our Precambrian data compilation to generate $^{34}\Delta_{\text{pyr}}$ distributions in metamorphic facies (Fig. 3.C, figs. S7 and S8). By dividing the microscale data according to their petrographic context, specifically identifying those that are clearly late diagenetic (i.e., post-compaction), we provide robust observations on the effect of metamorphism on the $^{34}\Delta_{\text{pyr}}$ variability preserved in sedimentary rocks. First, definitively post-compaction pyrite occupies a restricted range of $^{34}\Delta_{\text{pyr}}$ values ($\leq 10\text{‰}$), regardless of the metamorphic grade (Fig. 3.C black histograms). Second, there is a decrease in the overall preserved isotopic variability with increasing metamorphic grade (Fig. 3.C gray histograms), suggesting more severe isotopic homogenization and/or recrystallization at higher temperature, as observed in studies of coupled petrography and microscale $\delta^{34}\text{S}$ [35, 36, 37, 38]. We also find decreased isotopic variability in rocks containing a high diversity of sulfide minerals (fig. S8), which are expected to reflect high-temperature alteration. While it has long been known that late diagenetic fluids can induce new sulfide precipitation, including within and on preexisting sedimentary pyrite grains [35, 36, 37, 38], our observations suggest that as burial progresses, changes in pressure, temperature and/or the chemical environment promote isotopic homogenization within and among the sulfide minerals present in sedimentary successions. This metasomatic alteration minimizes the inherent isotopic variability associated with deposition of the original sediments, masking the diagnostic fingerprints associated with microbial and depositional processes (e.g., DSR and/or porewater distillation during accumulation of the overlying sediment).

Through their effect on the magnitude and the preservation of microbial fractionation of S isotopes in sediments and sedimentary rocks, the low sulfate levels typical of ancient environments challenge our ability to trace the lineage and evolution of early-Earth S metabolisms in the geologic record. We find that over a wide range of aqueous sulfate concentrations, from modern marine levels to the concentrations expected in the Archean ocean, large $^{34}\epsilon_{\text{pyr}} - ^{33}\lambda_{\text{pyr}}$ values persist even under extreme sulfate limitation (i.e., around 48‰ and 0.512 at 3 μM sulfate). When compared with a

new Archean-Paleoproterozoic microscale pyrite S isotope record (presented as $^{34}\Delta_{\text{pyr}}$ values), our results suggest the activity of DSR as early as ≈ 2.7 Ga in Neoarchean oceans with sulfate concentrations of a few to a few tens of μM . Prior to 2.7 Ga, a muted range of $^{34}\Delta_{\text{pyr}}$ values (0-20‰) most likely reflects an early emergence of dissimilatory sulfite reduction and later emergence (≈ 2.7 Ga) of a complete DSR pathway and/or post-depositional overprinting, the latter of which may prevent assessment of the earlier existence and proliferation of sulfate/sulfite reducers. Overall, this study highlights the value of investigations of modern environments and coupled microscale isotopic and petrographic analyses in providing robust interpretations of ancient S isotope records.

Table 1: Isotopic quantities reported and discussed in this study.

Symbol	Description
$\delta^{34}\text{S}$	$(^{3x}\text{R}_{\text{sam}} - ^{3x}\text{R}_{\text{ref}})/^{3x}\text{R}_{\text{ref}}$ in parts-per-thousand or permil (‰), where ^{3x}R is the $^{3x}\text{S}/^{32}\text{S}$ ratio, and ‘sam’ and ‘ref’ denote sample and reference.
$^{34}\epsilon_{\text{mic}} - ^{33}\lambda_{\text{mic}}$	The net microbial sulfate-sulfide ^{34}S fractionation and $^{33}\text{S}/^{34}\text{S}$ mass exponent, which relates the fractionation of ^{33}S and ^{34}S .
$^{34}\Delta_{\text{pyr}}$	The sulfate-pyrite $\delta^{34}\text{S}$ offset, measured in bulk or microscale pyrite.
$^{34}\epsilon_{\text{pyr}} - ^{33}\lambda_{\text{pyr}}$	The apparent sulfate-sulfide ^{34}S fractionation and $^{33}\text{S}/^{34}\text{S}$ mass exponent as constrained by pyrite and sulfate S isotope analyses, typically at the lowest $^{34}\Delta_{\text{pyr}}$ values.

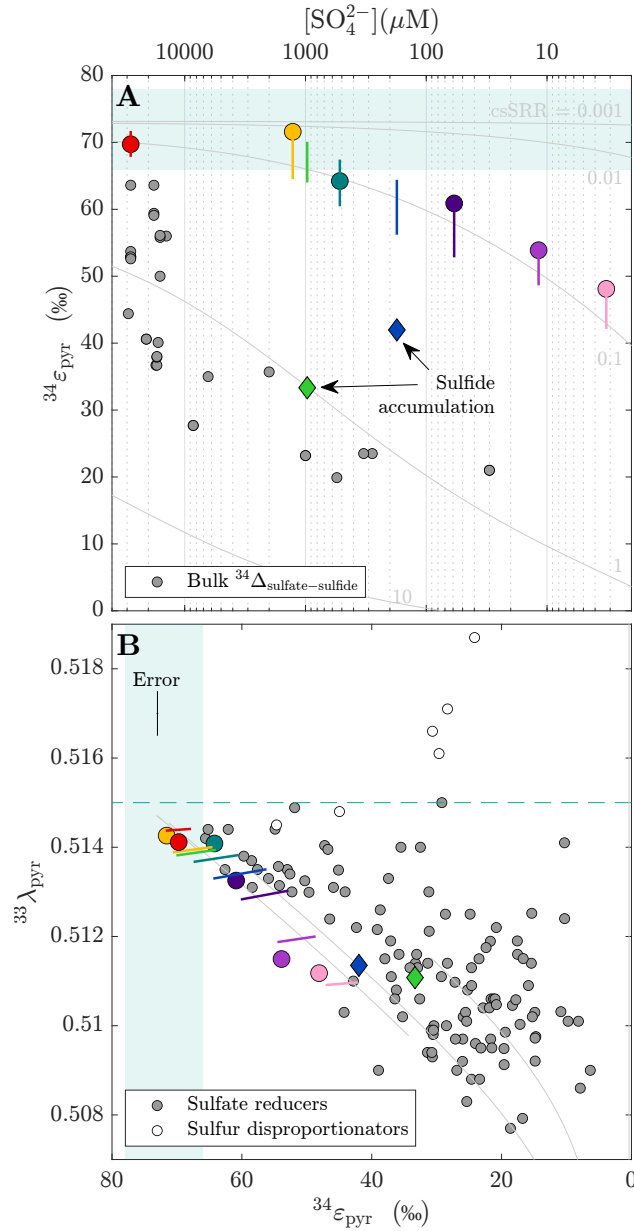


Figure 1: Environmental and laboratory constraints on S isotope fractionation during microbial sulfate reduction. (A) Reconstructed $^{34}\epsilon_{\text{pyr}}$ shown with reported environmental sulfate-sulfide $\delta^{34}\text{S}$ offsets ($\Delta^{34}\text{S}_{\text{sulfate-sulfide}}$ data from ref. [30]). (B) Reconstructed $^{34}\epsilon_{\text{pyr}} - ^{33}\lambda_{\text{pyr}}$ shown with laboratory experiments [8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. Diamonds and circles represent environments with and without porewater sulfide accumulation. Bio-isotopic model predictions are shown as grey lines (fixed csSRR in fmol cell⁻¹ day⁻¹, indicated by numbers at the right end of each line, varying $[\text{SO}_4^{2-}]$) and as coloured vertical lines (csSRR fixed at 0.1 fmol cell⁻¹ day⁻¹ and the specific lake $[\text{SO}_4^{2-}]$). The vertical range reflects varying temperature over the annual water-column temperature range. The teal boxes represent the range of the sulfate-sulfide equilibrium S isotope fractionation over the range of lake floor temperatures.

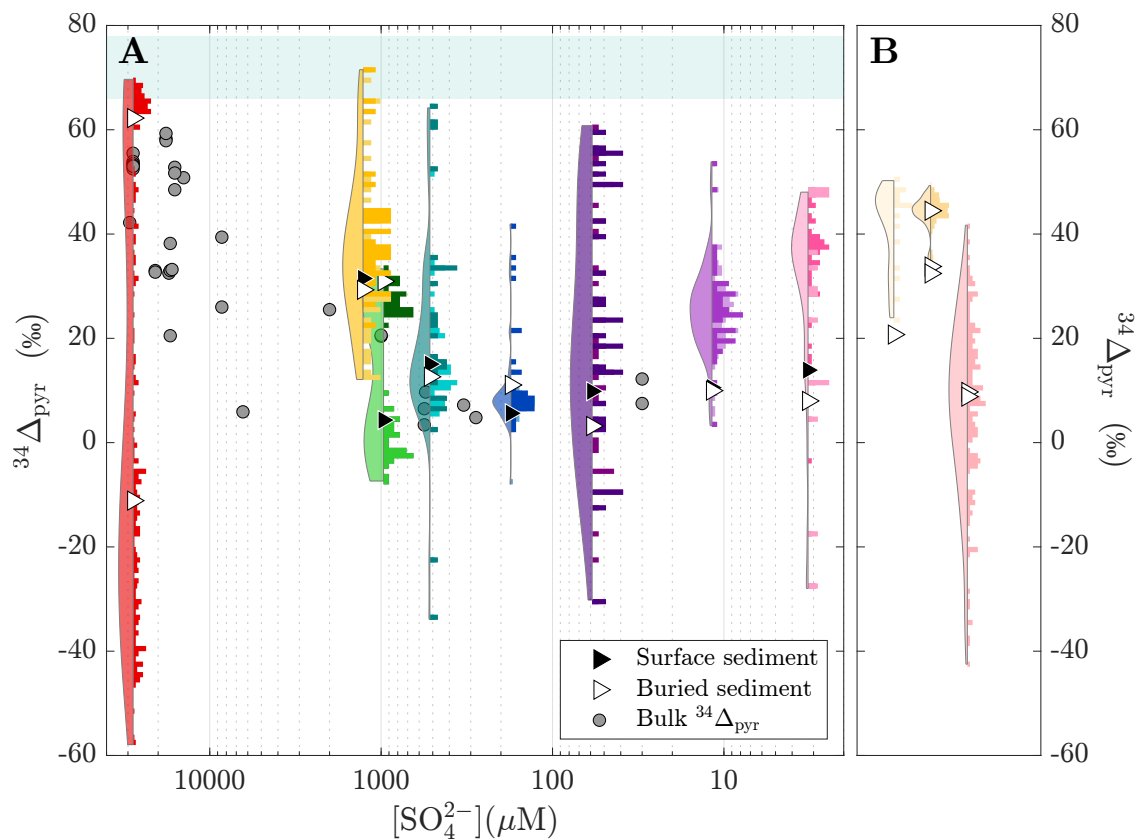


Figure 2: Observed $^{34}\Delta_{\text{pyr}}$. Histograms, probability density (of microscale data) and bulk (triangles) of $^{34}\Delta_{\text{pyr}}$ in (A) the studied environments and (B) Archean arrays consistent with active DSR. For modern samples, lighter colours reflect surface sediments (0–2 cm below the sediment-water interface), whereas darker colours represent buried samples (2–4 cm below the sediment-water interface). The teal boxes represent the range of the sulfate-sulfide equilibrium S isotope fractionation over the range of lake floor temperatures. Also shown (grey dots) are published $^{34}\Delta_{\text{pyr}}$ values in modern environments [30].

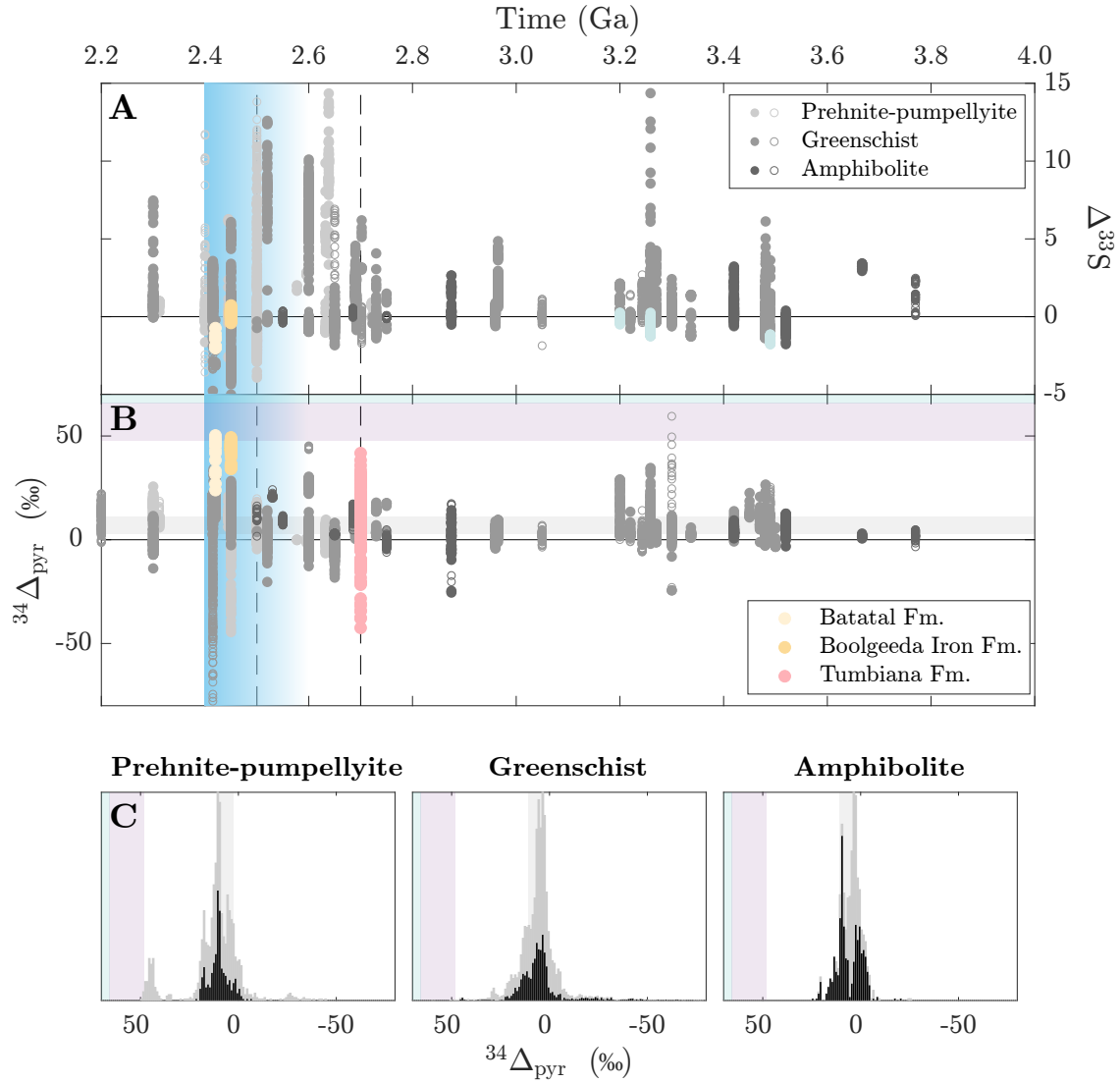


Figure 3: Precambrian compilation of microscale S isotope data. (A) $\Delta^{33}\text{S}$ values. (B) $^{34}\Delta_{\text{pyr}}$ values together with the range of $^{34}\Delta_{\text{pyr}}$ reconstructed in the modern low-sulfate lakes (horizontal purple box) and modelled sulfate-sulfide equilibrium S isotope fractionation (horizontal teal box). The shaded vertical gradient represents the time leading to the “Great Oxygenation Event”, when the oxidation state of the surface environment is thought to have increased. The grey horizontal box shows the 25th to 75th percentile of $^{34}\Delta_{\text{pyr}}$ values in post-compaction pyrite (open circles). (C) $^{34}\Delta_{\text{pyr}}$ histograms as function of metamorphic grade. Grey histograms represent all pyrite samples, and black histograms represent pyrite formed during late diagenesis after compaction and/or detrital pyrite. Coloured arrays in A and B show microscale data consistent with active DSR in the Precambrian record.

Methods

Materials

Push-core samples of shallow-water sediments (3–5 m below lake level) were collected in October 2022 and/or 2023. Each core, containing approximately 20–30 cm of sediment and overlying lake water, was transported to the laboratory and stored in the dark at 4°C. After two days, microelectrode measurements were conducted on sediments kept within the core liner, which remained covered by 4–5 cm of lake water (fig. S9).

Lake water samples for determining aqueous species concentrations were collected in HDPE Nalgene bottles. To recover sulfate, the lake water was acidified with a few drops of concentrated HCl to exsolve potential dissolved sulfide. A sub-sample was analyzed for sulfate concentration using ICP-OES, while the remaining water was evaporated to a volume of 10 mL. After evaporation, a solution of BaCl (10% w/w) was added in excess to precipitate dissolved SO_4^{2-} quantitatively as BaSO₄ for subsequent $\delta^{34}\text{S}$ determination.

Bulk $\delta^{13}\text{C}_{\text{org}}$ and Total Organic Content (TOC) determination

Prior to organic carbon analyses, the carbonate fraction in bulk samples was removed through a 48-hour reaction with an excess of 1.0 N HCl [49]. During digestion, centrifuge tubes were placed in an ultrasonic bath to enhance the mechanical separation of clay and calcium carbonates. After dissolution, residues were washed three times with distilled water, centrifuged, and dried at 50°C. The dried residues were homogenized, and 50 mg of the powder were loaded into tin capsules for analysis.

Analyses were conducted using an Elemental Analyzer (EA, Flash 2000 – ThermoScientific) coupled to an isotope ratio mass spectrometer (Thermo Delta V Plus EA-IRMS) at Washington University in St. Louis (WUSTL). Organic carbon isotope ratios are reported in delta (δ) notation as per mil deviations relative to the Pee Dee Belemnite (PDB) standard. The analytical error for organic carbon isotopes was $<0.5\text{‰}$ (2σ , based on replicate standards). TOC concentrations were measured using the thermal conductivity detector of the ThermoScientific Flash 2000 at WUSTL.

Bulk SF₆-GC-IRMS S-isotopes

SO₄²⁻ was reduced to sulfide by reacting 1 mL of concentrated lake water and/or BaSO₄ with 20 mL of Thode solution (32:15:53 ratio of HI:H₃PO₂:HCl) at 90°C (below the boiling point) for 60 minutes under a N₂ atmosphere. Pyrite sulfur from lake sediments was extracted using the chromium reduction method. In both cases, the liberated H₂S was carried by an N₂ flow through a silver nitrate solution, where it was captured as Ag₂S. The resulting Ag₂S was rinsed three times with Milli-Q water and dried overnight at 50°C.

For bulk multiple sulfur isotope analyses (i.e., ³³S/³²S, ³⁴S/³²S, and ³⁶S/³²S), approximately 3.0 mg of Ag₂S was converted to sulfur hexafluoride (SF₆) by reacting with a tenfold excess of F₂ at 300°C overnight in nickel reactors [50]. Excess F₂ was removed from the SF₆ through cryogenic and gas chromatographic purification. The multiple sulfur isotopes of the purified SF₆ were then analyzed using a Thermo Scientific MAT-253 isotope-ratio mass spectrometer at the Institut de Physique du Globe de Paris (IPGP).

The multiple sulfur isotope measurements were monitored using the international reference material IAEA-S2 and/or IAEA-S3 ($\delta^{34}\text{S}_{\text{IAEA-S2}}=22.091\text{‰}$ - $\Delta^{33}\text{S}_{\text{IAEA-S2}}=0.059$ and $\delta^{34}\text{S}_{\text{IAEA-S3}}=-32.51\text{‰}$ - $\Delta^{33}\text{S}_{\text{IAEA-S3}}=0.099$; [51]). The analytical precisions are estimated from the long-term reproducibility of IAEA standards (S1, S2, S3), expressed as 1 σ , is better than $\pm 0.15\text{‰}$ and ± 0.005 for $\delta^{34}\text{S}$, $\Delta^{33}\text{S}$, respectively.

Bulk MC-ICPMS S-isotopes

The bulk sulfate multiple sulfur isotopes of Lake Orny was (also) analyzed using MC-ICP-MS [52]. Briefly, 500 mL of filtered lake water (Nuclepore™ 0.2 μm , acidified with a few drops of 6 N HCl) was concentrated to 1 mL and subjected to aqua regia digestion (50:50 ratio of HNO₃:HCl) on a hot plate at 160°C for 24 hours in a PFA vial. Following this oxidizing step, the aqua regia was evaporated, and the residue was processed for subsequent anion-exchange column purification. Before purification, Biorad 10 mL disposable columns were prepared by loading 600 μL of clean AG1X8 anion-exchange resin (100–200 mesh) and conditioned according to the procedure detailed

in Paris [52].

Multiple sulfur isotopes (^{32}S , ^{33}S and ^{34}S) were measured at the CRPG (France) using a Thermo Fisher Scientific MC-ICP-MS Neptune Plus. Samples were introduced in $0.6 \text{ mM L}^{-1} \text{ HNO}_3$ using an ESI PFA-50 nebulizer coupled with a Cetac Aridus II, featuring a PTFE spray chamber heated to 110°C and a desolvating PTFE membrane maintained at 160°C . Measurements were conducted using a sample-standard bracketing approach to correct for instrumental drift, employing NaSO_4 solution calibrated against IAEA standards (S1, S2, S3). The standard solution was diluted at $20 \mu\text{mol L}^{-1}$ in $0.6 \text{ mM L}^{-1} \text{ HNO}_3$. Purified samples are prepared for the Neptune with an addition of NaOH so that the Na^+ concentration of the samples matches that of the isotope bracketing standard and that the sulfate concentration is equal or lower than that bracketing isotope standard solution [26, 52]. Internal errors follow counting statistics. A seawater purified and analyzed at the same time yielded values of $\delta^{34}\text{S}=21.17 \pm 0.05$ and $\Delta^{33}\text{S}=0.03 \pm 0.02$ (instrumental repeatability, $n=2$), consistent with previous results $\delta^{34}\text{S}=21.14 \pm 0.08$ and $\Delta^{33}\text{S}=0.05 \pm 0.03$ (intermediate repeatability, $n=4$, [52]). Here, the sample analytical precision was estimated by running the same purified sample 8 times on the Neptune and provided an instrumental repeatability consistent with our usual runs.

Pyrite extraction and preparation for microscale analyses

As SIMS analysis requires intact, non-oxidized pyrite, we adapted the physical isolation procedure from ref. [53] to minimize oxidation and dissolution of grains from marine sediment samples. Unlike the original method, carbonate minerals were not removed from bulk sediments to prevent potential small-grain oxidation or dissolution. Freeze-dried samples were gently crushed, by pressing the dry sample – protected in a weighting paper – between two fingers, then transferred to 50 mL centrifuge tubes. Each sample was mixed with 45 mL of lithium polytungstate (LST; density of 2.85 g cm^3), thoroughly vortexed, and deflocculated in an ultrasonic cleaner (35 kHz) for 30 minutes. The tubes were centrifuged at 3000 RPM for 38 minutes, and heavy mineral fractions were extracted using plastic micro-pipettes. To ensure complete recovery of pyrite grains, the procedure was repeated twice on the supernatants. Heavy extracts were rinsed three times with deionized water and placed in an ultrasonic cleaner (35 kHz) for 5 minutes. After settling for 10 minutes, the supernatants were removed with plastic micro-pipettes. The remaining solids were transferred to 3 mL centrifuge

tubes, frozen in liquid N₂, and subsequently freeze-dried.

For surface analyses (e.g., SIMS and SEM), the heavy mineral residues were transferred onto glass plates covered with double-sided tape and placed within 1-inch round plastic molds. The molds were filled with epoxy resin (Stuers® Epofix) and cured overnight in a pressure chamber (3 kbar) at room temperature. Once cured, the epoxy mounts were polished using 2000-grade abrasive paper, followed by 10, 6, 3, and 1 μm diamond pastes. To expose the pyrite grains and achieve a flat surface, all polishing steps were performed with a Mecatech® SPC 300 polishing machine. Pyrite standards, including Balmat and Ruttan [54, 55, 56], were prepared using the same procedure. Zones with high pyrite abundance were cut using a diamond wire saw (Well-dws) and pressed into 1-inch indium mounts along with pyrite standards (Ruttan, Balmat). To ensure an appropriate surface topography for SIMS analyses ($<5 \mu\text{m}$; [57]), the mounts were examined using a white light interferometric microscope (Bruker, Contour GT-K). Once prepared, the indium mounts were coated with a ≈ 50 nm-thick layer of gold to ensure conductivity for SIMS analysis.

Microscale S isotope

Microscale sulfur isotope measurements were performed using SIMS in spot mode with the Cameca 1280-HR instrument at the SwissSIMS facility, University of Lausanne.

Pyrite grains were pre-sputtered with a Cs⁺ primary beam for 60 seconds using a 1 nA current and a raster size of 10 μm . Multiple sulfur isotope ratio analyses were subsequently conducted on the same raster squares using Cs⁺ spot bombardment, following the procedure outlined by (10). Briefly, the isotopes ³²S⁻, ³³S⁻ and ³⁴S⁻ were simultaneously measured in multi-collection mode with three off-axis Faraday cups. Relative Faraday cup yields were calibrated at the start of the analytical session, while Faraday cup backgrounds were measured and corrected prior to each analysis during the pre-sputtering phase.

Measurements were conducted using a primary Cs⁺ ion beam with an intensity of 1.5 nA and a total impact energy of 20 kV, focused to a $\approx 7 \mu\text{m}$ diameter spot. Charge compensation was achieved using a normal incidence electron gun. NMR regulation was applied throughout, and the isotopes ³²S, ³³S and ³⁴S were measured with Faraday cup detectors (L2, C, and H1 for ³²S, ³³S and ³⁴S respectively). During the analytical session, the mass spectrometer was operated at a

nominal mass resolution ($M/\Delta M$) of approximately 4000 – sufficient to resolve ^{33}S from $^{32}\text{SH}^+$ – using an entrance slit of 81 μm and an exit slit of 243 μm on each multi-collector detector. The ^{33}S detector was slightly offset from the peak center to achieve the required higher mass resolution. Each analysis consisted of 40 seconds of pre-sputtering, 20 seconds for centering secondary ions in the field and contrast aperture (400 μm), and 125 seconds of measurement (five blocks of five cycles with 4.8-second integrations per cycle). An average count rate of ^{32}S at $\approx 1 \times 10^9$ counts per second was used to confirm the stability of the analyses.

To account for instrumental mass fractionation, all data were normalized to the Balmat international standards. Alongside Balmat, several internal standards with known isotopic compositions were used [54, 58, 59, 39], including Ruttan, Maine, Son, and Pyr123 for sulfur isotopes, fig. S11.

Analyses were conducted across two instrumental sessions, one in September 2022 and the other in May 2024. In September 2022, the average and 2σ external precisions ($n=66$) for all Balmat pyrite samples analyzed were $\delta^{33}\text{S} = 8.12 \pm 0.14\text{‰}$, $\delta^{34}\text{S} = 15.81 \pm 0.16\text{‰}$ and $\Delta^{33}\text{S} = 0.01 \pm 0.1\text{‰}$. In May 2024, the corresponding values for the average and 2σ external precisions ($n=68$) for all Balmat pyrite samples were $\delta^{33}\text{S} = 8.13 \pm 0.13\text{‰}$, $\delta^{34}\text{S} = 15.81 \pm 0.12\text{‰}$ and $\Delta^{33}\text{S} = 0.00 \pm 0.09\text{‰}$. A detailed estimation of the external precision for standards can be found in fig. S11.

The calibration follows the procedure outlined in ref. [59]. Detector yield and background-corrected $^{33}\text{S}/^{32}\text{S}$ raw ratios (R_{raw}) were consistently drift-corrected using linear regression, even for drifts deemed “negligible”:

$$R_{\text{drift}} = R_{\text{raw}} - mx$$

where m is the slope of the regression, and x represents the number of analyses between bracketing standards, scaled to ensure the line passes through the origin ($x=y=0$). The drift ratios were then expressed as raw delta values (VCDT) using:

$$\delta^x X_{\text{drift}} = 1000 ((^xR_{\text{drift}} / ^xR_{\text{VCDT}}) - 1)$$

We used $^{33}R_{\text{VCDT}}$ of 0.00787725, $^{34}R_{\text{VCDT}}$ of 0.04416255.

Next, we calculated the instrumental mass fractionation (IMF) as the weighted average of all α_i

values of the $\delta^x X_{\text{drift}}$ for the bracketing standards using:

$\alpha_i = [1 + (\delta^x X_{\text{drift}}/1000)]/[1 + (\delta^x X_{\text{std}}/1000)]$, where $\delta^x X_{\text{std}}$ refers to the $\delta^{34}\text{S}$, $\delta^{33}\text{S}$ values of the working standard relative to VCDT.

The $\delta^{3x}\text{S}$ value relative to VCDT for each analyzed sample was calculated using:

$$\delta^{3x}\text{S}_{\text{sample}} = 1000 [((1 + (\delta^{3x}\text{S}_{\text{drift}}/1000))/\alpha) - 1].$$

The $\Delta^{33}\text{S}$ value for each sample was calculated using the exponential equation:

$$\Delta^{33}\text{S}_{\text{sample}} = (\delta^{33}\text{S}_{\text{sample}} - 1000 [(1 + \delta^{34}\text{S}_{\text{sample}}/1000)^{33\lambda_{\text{std}}} - 1]) - b,$$

where $^{33}\lambda_{\text{std}}$ represents the instrumental slope, and b is the y-intercept in the $\delta^{34}\text{S}'$ - $\delta^{33}\text{S}'$ space (see fig. S11).

Secondary electron microscopy

Backscattered electron images were utilized to identify pyrite grains in polished mounts and estimate their grain sizes. Imaging was performed using a Zeiss Gemini SEM 500 at the University of Lausanne (Switzerland), with a working distance of 6–9 mm and a primary beam voltage of 10 kV in all cases.

Based on the SEM images, pyrite grains were categorized as having framboidal, recrystallized framboidal (e.g., infilled or overgrown), or recrystallized morphologies (fig. S10.A). Grain diameters were measured using the SEM's operating software, with the longest axis used for size determination in cases of non-equant grains. While no correlation between grain morphology or size and $\delta^{34}\text{S}_{\text{PYR}}$ was observed (fig. S10), there is a notable tendency for smaller grains to occur with decreasing sulfate concentrations (fig. S10.B).

The energetics of sulfite reduction in the pre-Neoproterozoic

In a linear metabolic pathway, such as sulfite reduction, the net isotopic fractionation can vary between the equilibrium substrate-product isotopic fractionation and the kinetic isotope effect associated with the most upstream step in the pathway [23]. Hence, the net sulfite-sulfide S isotope fractionation associated with microbial sulfite reduction can vary between the equilibrium sulfite-sulfide S isotope fractionation ($\approx 43\text{‰}$ at 10 °C; [60] and a near-zero kinetic isotope effect

associated with sulfite uptake, assuming that sulfite and sulfate transporters are broadly similar [17]. To evaluate how close to thermodynamic equilibrium sulfite reduction may have been in the Paleoproterozoic and Mesoproterozoic marine environment, we calculated the Gibbs free energy of sulfite reduction to sulfide with a variety of electron donors (methane, hydrogen, formate, acetate, propionate) over concentration ranges relevant to the early oceans. Estimates of Archean sulfite levels are 10^{-3} to $10 \mu\text{M}$ (17, 18). To constrain sulfide levels, we consider an Fe-rich ocean (10 to $100\text{s } \mu\text{M}$) that is more acidic than present ($\text{pH} \approx 6\text{-}7$; Ref. [61]), from which mackinawite saturation allows up to $\approx 100 \mu\text{M}$ sulfide. The range of H_2 concentrations has been proposed to be 10^{-1} to $10^{+1} \mu\text{M}$ [62, 63], and guided by the ranges of formate, acetate and propionate concentrations in modern anoxic sedimentary environments [64, 65, 66, 67, 68, 69], we explore ranges of the concentrations of these compounds between 10^{-1} and $10^{+2} \mu\text{M}$. Over this range of concentrations, the Gibbs free energy of sulfite reduction to sulfide is no less negative than $\approx -80 \text{ kJ mol}^{-1}$ (fig. S6). The consequence of the high thermodynamic favorability of sulfite reduction is that the net sulfite-sulfide S isotope fractionation produced by microbial sulfite reduction was not close to equilibrium and likely within the range exhibited by sulfate-reducing mutants unable to reduce sulfate and grown on sulfite, of $\approx 15\text{-}20\text{‰}$ [70].

Table 2: Equilibrium and kinetic S isotope effects associated with sulfite uptake and reduction by Dsr.

Param.	Description	Value (units)	Ref
α_1^{eq}	Extracellular-intracellular sulfite S EIE	1.000	Est.
α_1^{kin}	Sulfite transport KIE	0.997	[23, 71]
α_2^{eq}	Sulfite-sulfide S EIE	1.0437 (at 10°C)	[60]
α_2^{kin}	Dsr KIE	1.015	[47]

* Assumed identical to sulfate transport, as constrained in experiments [72, 73]

End notes

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Competing interests:

The authors declare that they have no competing interests.

Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials and/or available at <https://www.pangaea.de/>. All geochemical and isotopic data on sediment lake samples included in this study are available in the main text and in the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Supplementary Information

Supplementary Information is available for this paper. Figs. S1 to S10
Data S1 to S3

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636 **Supplementary Information for**

637 **Large sulfur isotope fractionation during microbial sulfate**
638 **reduction at low sulfate levels: Early life and environmental**
639 **implications**

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642 **This PDF file includes:**

643 Figures S1 to S10

644 Captions for Data S1 to S3

645 **Other Supplementary Materials for this manuscript:**

646 Data S1 to S3

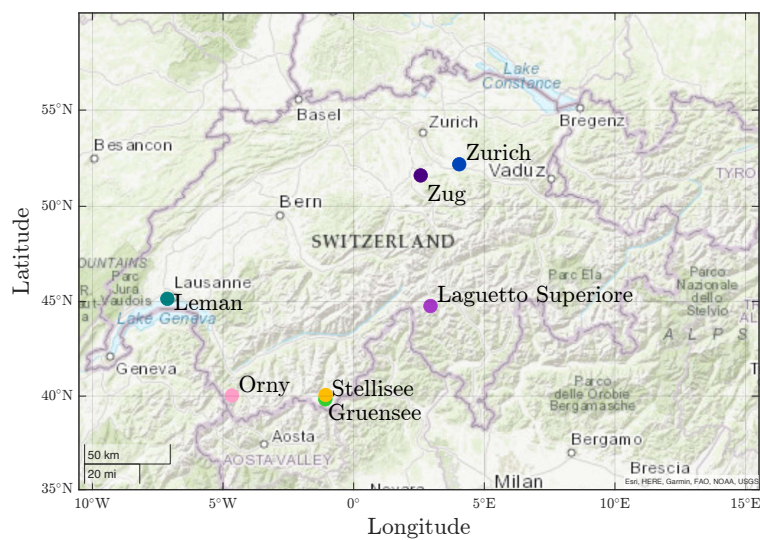


Figure S1: Studied modern environments.

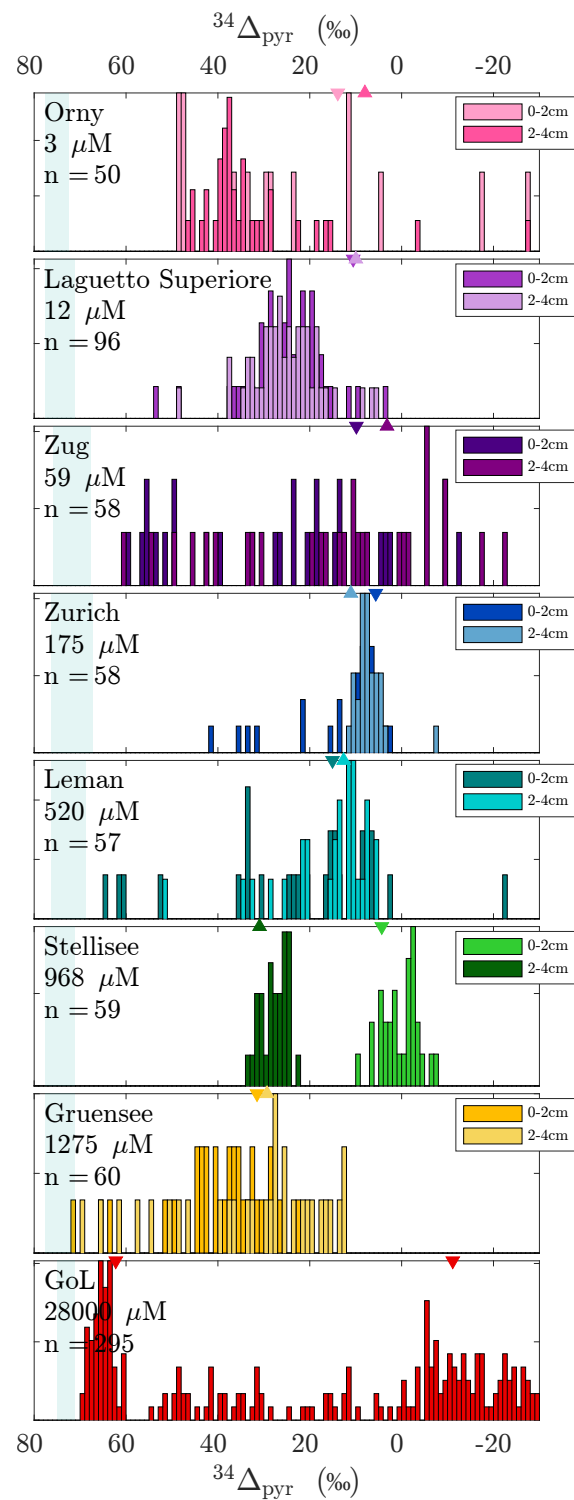


Figure S2: $^{34}\Delta_{\text{pyr}}$ distribution in modern environments. Colors represent the depth of the sample below the sediment-water interface. Teal boxes indicate the expected equilibrium sulfide-sulfate isotopic fraction over the annual temperature range of the lake.

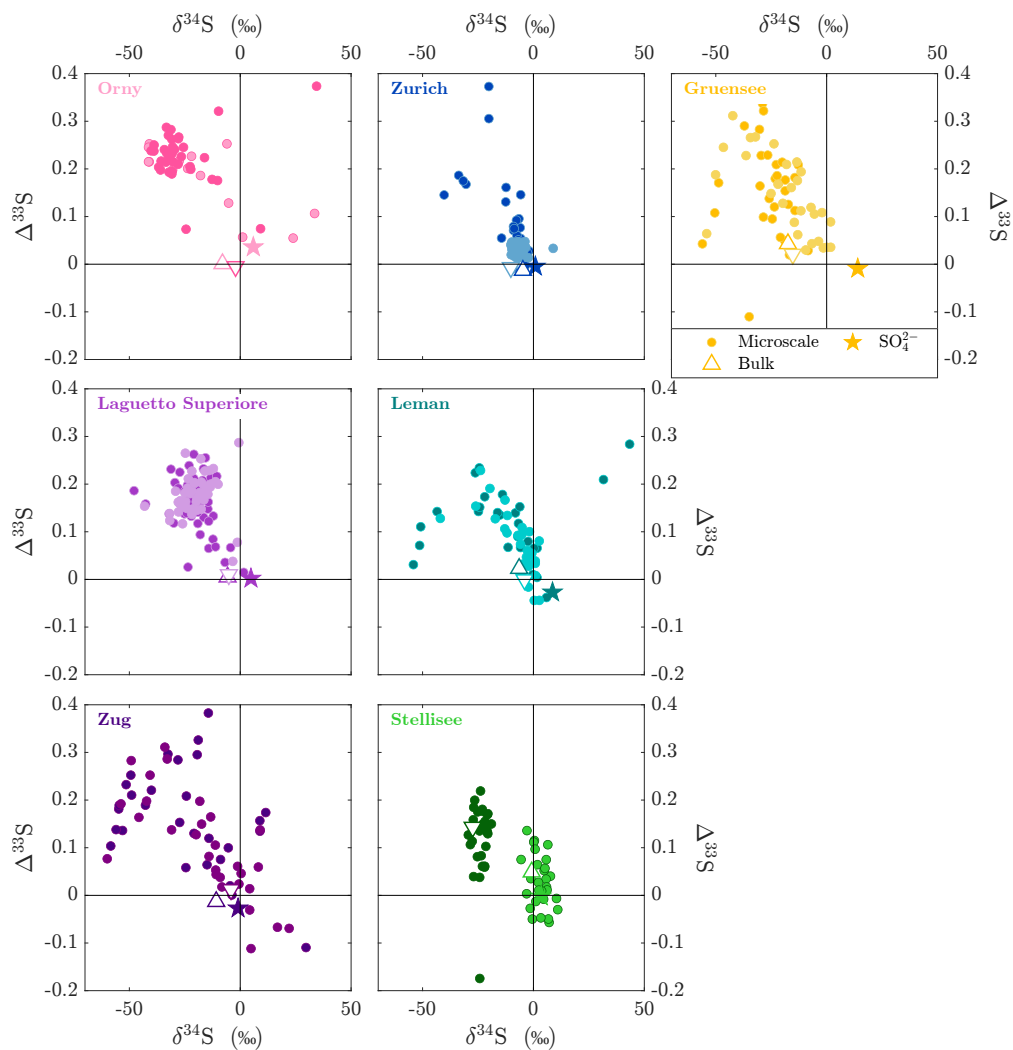


Figure S3: Microscale and bulk S isotope in modern environments. Colors represent the depth of the sample below the sediment-water interface where brighter colours reflect surface sediments (0–2 cm below the sediment-water interface), whereas darker colours represent buried samples (2–4 cm below the sediment-water interface). Dots, triangles and stars represent microscale pyrite, bulk pyrite and lake water sulfate S isotopic composition, respectively.

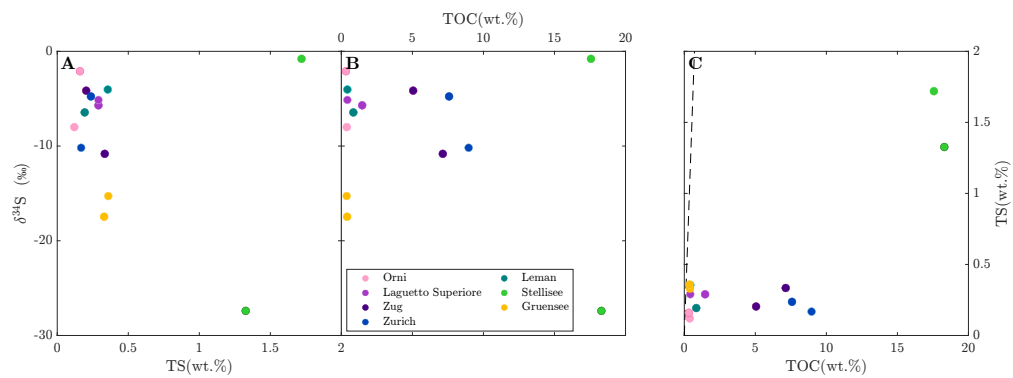


Figure S4: Auxiliary data. Total sulfur (TS) and total organic carbon (TOC) vs. $\delta^{34}\text{S}$ are shown in the panels (A) and (B), respectively. (C) TS vs. TOC. Also shown in panel C is the marine organic matter line 50:1 TOC:TS from ref. [48].

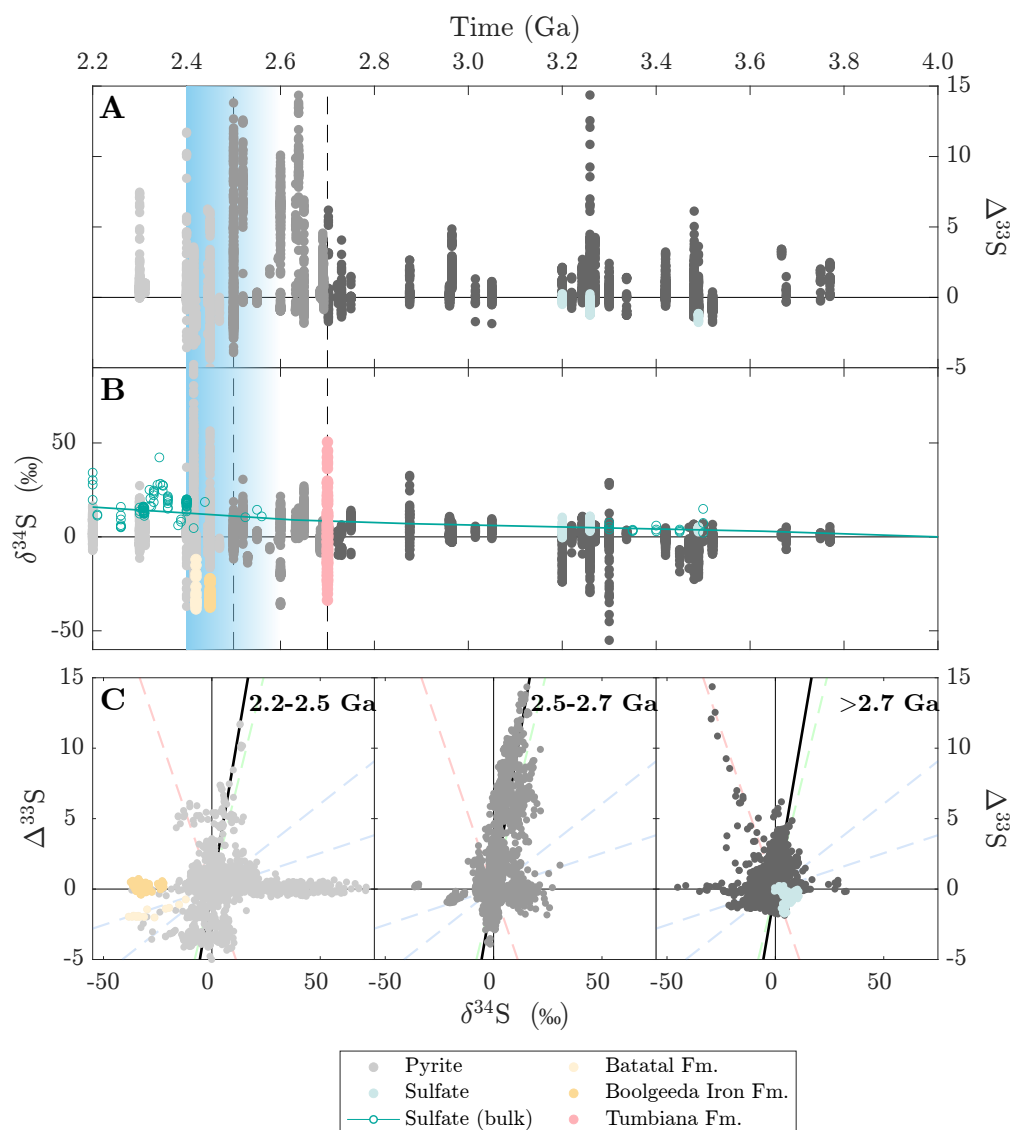


Figure S5: Precambrian compilation of microscale S isotope data. (A) $\Delta^{33}\text{S}$ values. (B) Microscale $\delta^{34}\text{S}$ values of pyrite (grey circles) and sulfate minerals (light blue circles). Also shown are bulk sulfate $\delta^{34}\text{S}$ (open teal circles; ref. [74]) and the $\delta^{34}\text{S}$ interpolation line used to calculate $^{34}\Delta_{\text{pyr}}$ values [6]. (C) Microscale $\delta^{34}\text{S}_{\text{pyr}} - \Delta^{33}\text{S}$ crossplot. Geologic pyrite samples are color-coded by time interval [75] as shown in panel C, whereas sulfate minerals (barite and anhydrite) are shown in blue. In panel C, the black line represents the Archean reference array, the dashed lines show photochemical experiments (blue and green, refs. [76, 77]) and the felsic volcanic array (red, ref. [58]).

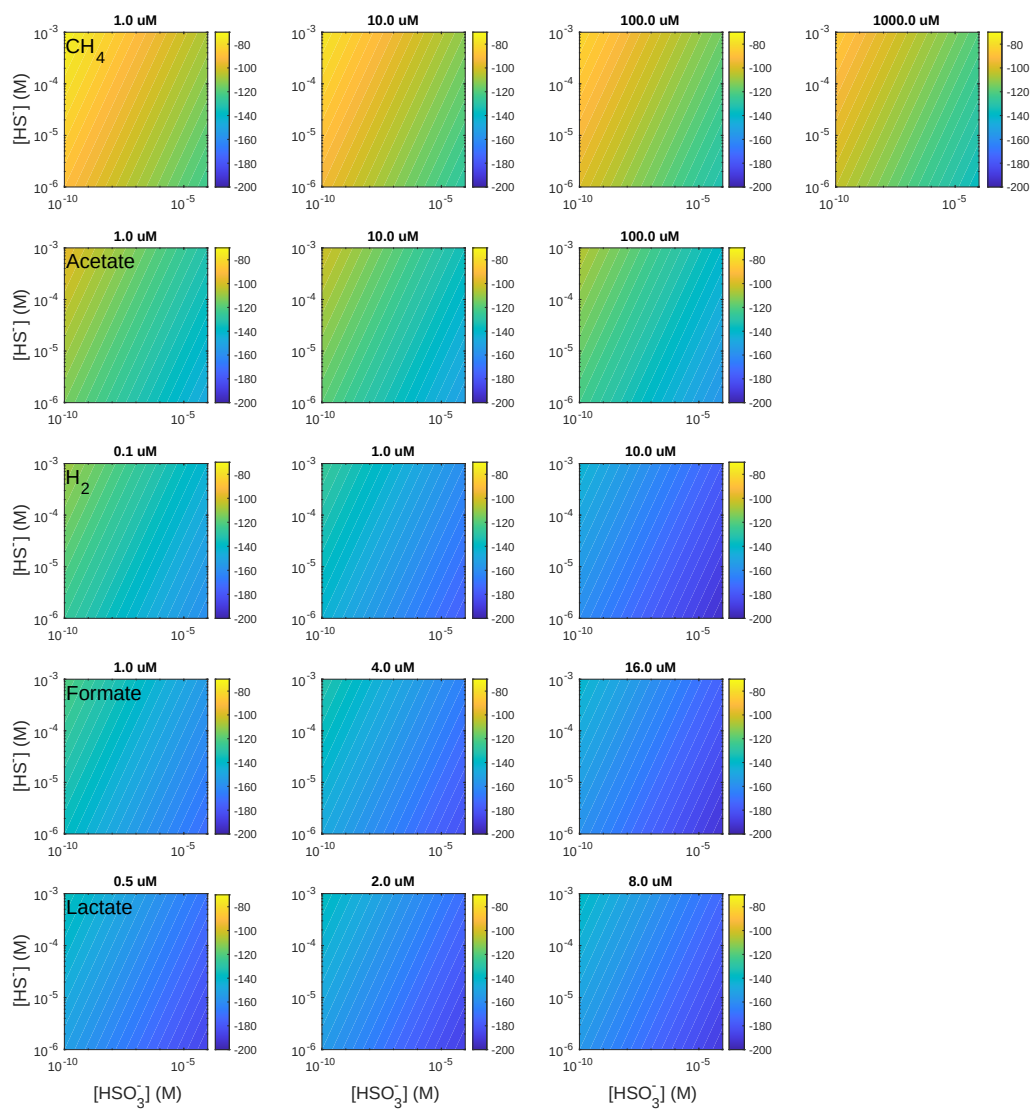


Figure S6: Contour plot of the catabolic energy (ΔG_r) available as a function of environmental (extracellular) $[\text{SO}_3^{2-}]$ and $[\text{HS}^-]$ for a variety of electron donors.

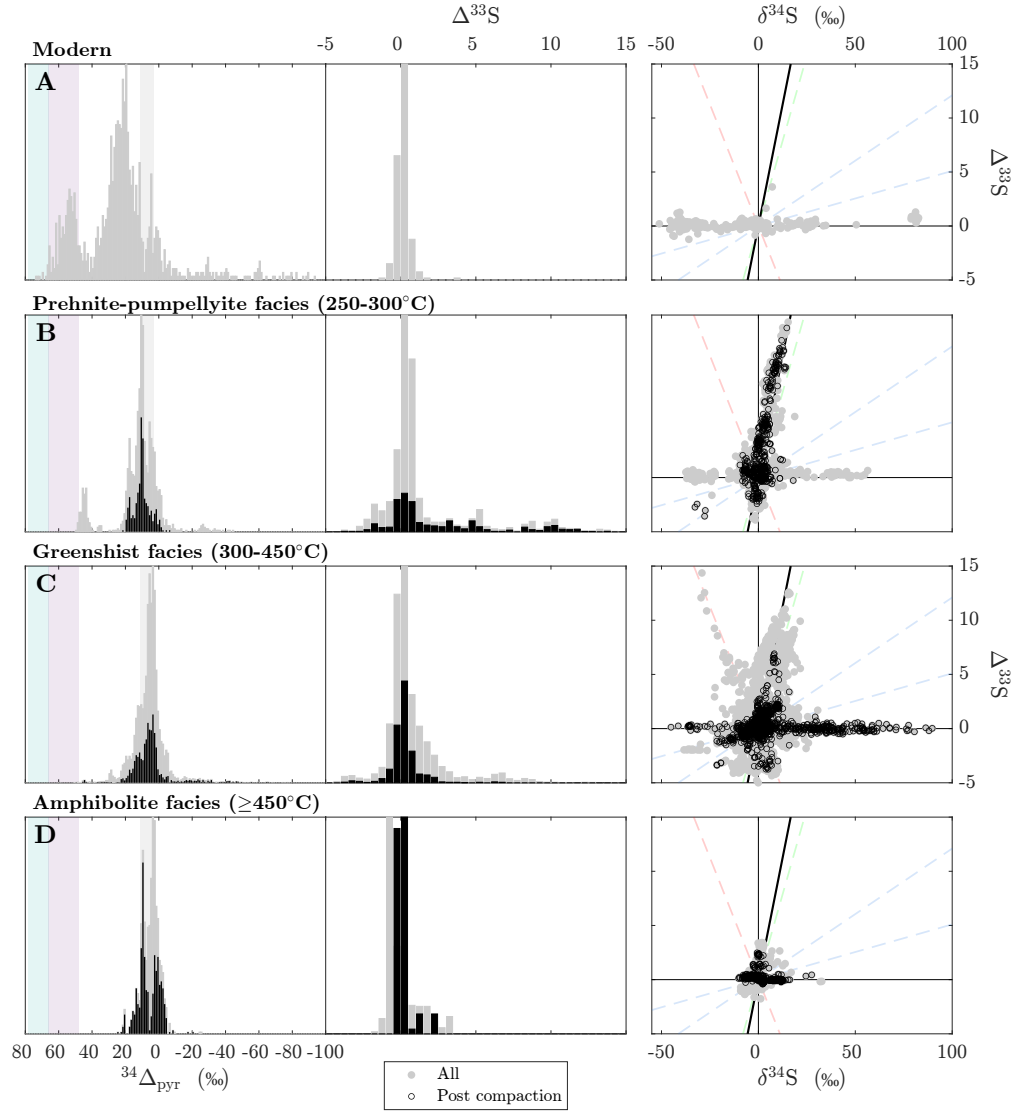


Figure S7: The isotopic impact of burial and metamorphism. The left panels show histograms of $^{34}\Delta_{\text{pyr}}$ and $\Delta^{33}\text{S}$, and the right panels show the data as $\delta^{34}\text{S}_{\text{pyr}}-\Delta^{33}\text{S}$ cross plots classified by the metamorphic grade of the host rock. The grey symbols and histograms represent the entire pyrite population, whereas the black symbols and histograms represent the post-compaction pyrite (out of the total population). In the left panels, the horizontal purple and teal boxes show the range of $^{34}\epsilon_{\text{pyr}}$ reconstructed and modelled sulfate-sulfide equilibrium S isotope fractionation in the modern low-sulfate lakes, respectively. In the right panels, the black line represents the Archean reference array, the dashed lines show photochemical experiments (blue and green, refs. [76, 77] and the felsic volcanic array (red, ref. [58])

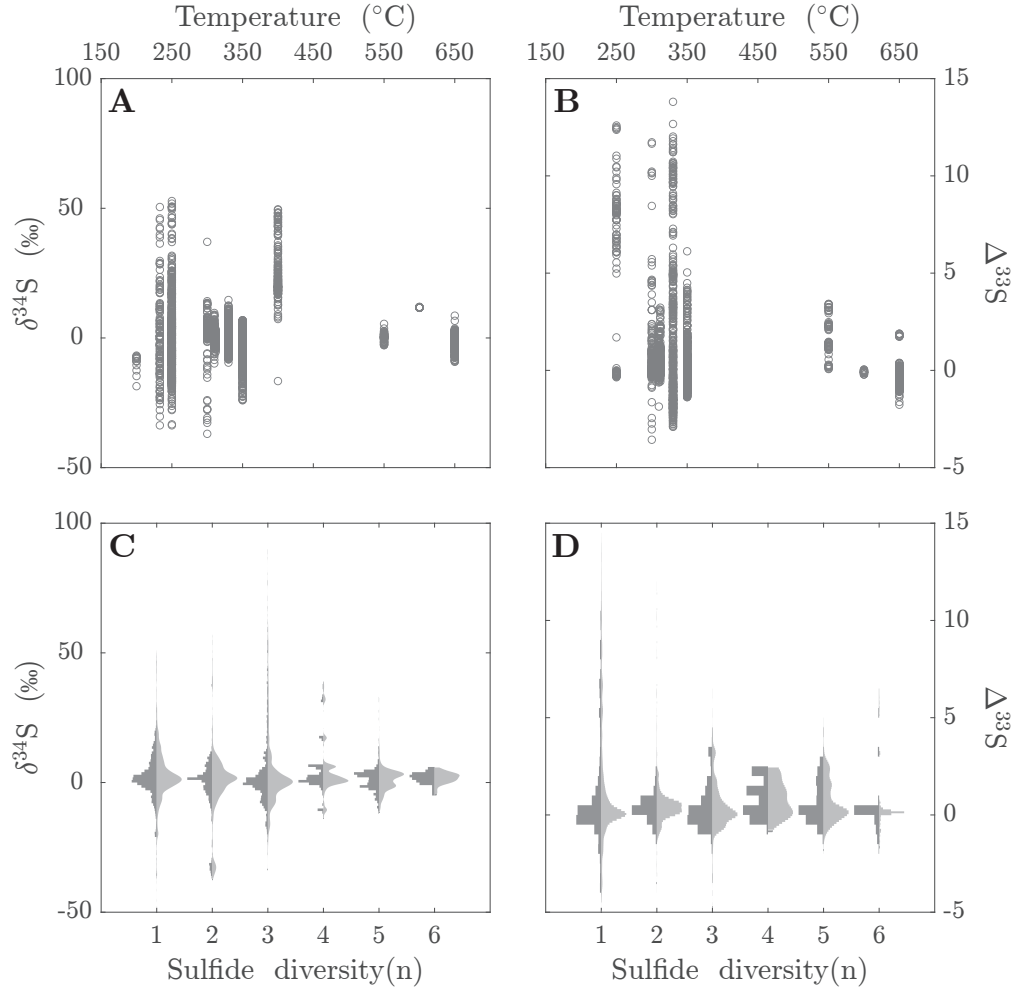


Figure S8: The influence of temperature and sulfide diversity on preserved S isotopes. Upper panels show $\delta^{34}\text{S}$ (A) and $\Delta^{33}\text{S}$ (B) versus the maximum metamorphic temperature of the host rock. Bottom panels show the isotopic variability ($\delta^{34}\text{S}$ on panel C, $\Delta^{33}\text{S}$ on panel D) as a function of the sulfide diversity in the host rock, defined as the number of distinct metal-sulfide minerals.

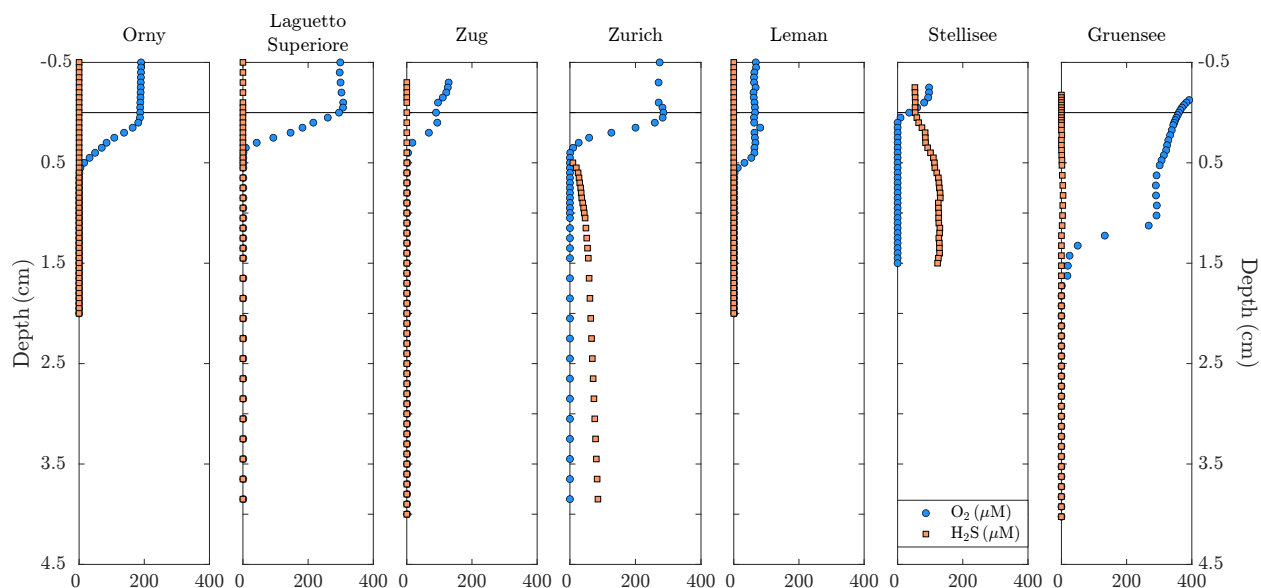


Figure S9: Microsensor profiles of O₂ and H₂S in lake sediments. The black horizontal line shows the sediment-water interface.

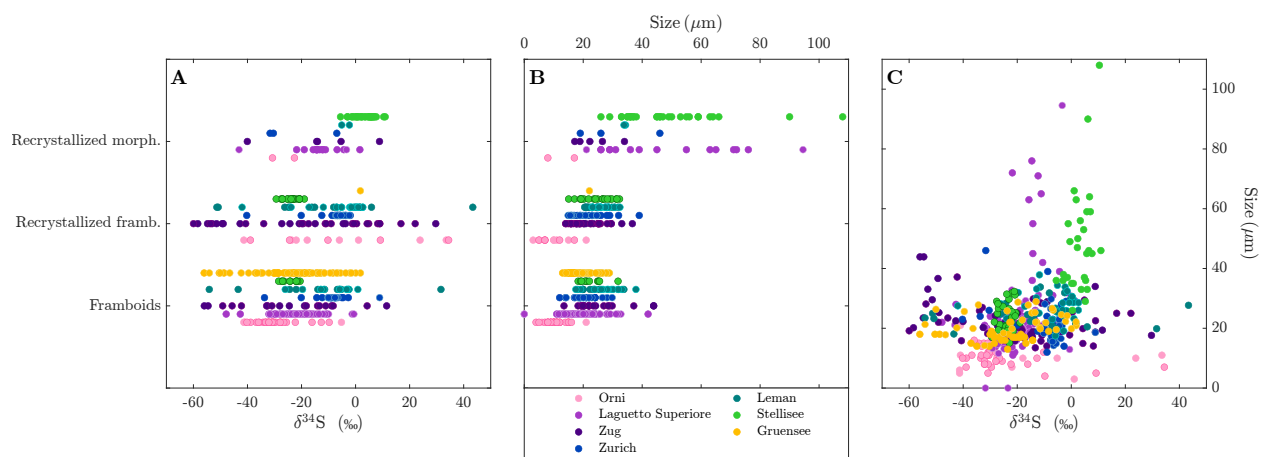


Figure S10: Relationships between the S isotope composition of pyrite, grain size and morphology. (A) Pyrite $\delta^{34}\text{S}$ of pyrite with different grain morphologies. (B) Grain size ranges of the different grain morphologies. (C) Crossplot of pyrite $\delta^{34}\text{S}$ -grain size. Different circle colours represent the different lakes studied.

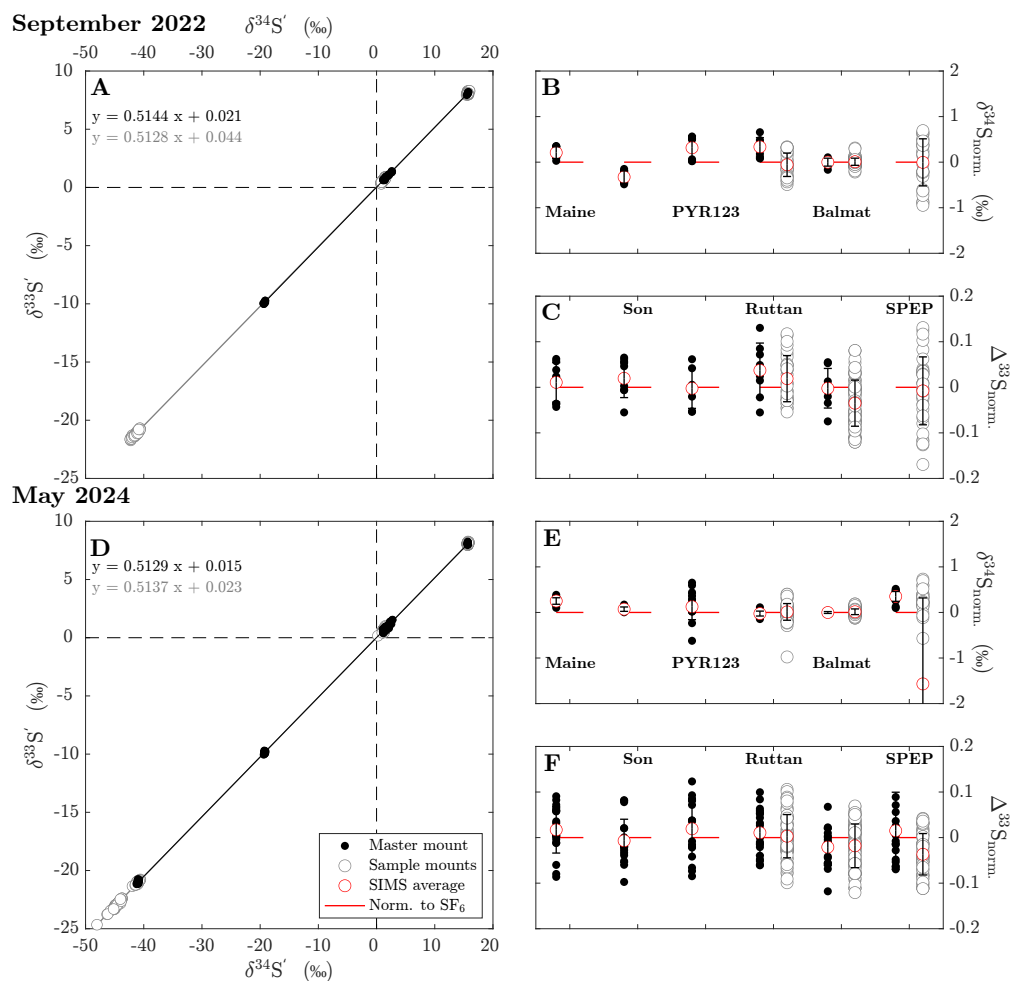


Figure S11: SIMS S isotope internal standard results. (A-D) $\delta^{34}\text{S}'$ vs. $\delta^{33}\text{S}'$ values. Black and grey lines and equations represent regression lines obtained in the SWISSIMS master mount and in our sample mounts, respectively. The right two panels show the $\delta^{34}\text{S}$ (B-E) and $\Delta^{33}\text{S}$ (C-F) values normalized to the bulk isotopic values obtained on our internal standards (Maine, Son, PYR123, Ruttan, Balmat and SPEP) in both the master and sample mounts. Microscale S isotope were acquired during two analytical sessions: in September 2022 (A, B and C) and in May 2024 (D, E and F).

Caption for Data S1. Modern environment data. $\delta^{33}\text{S}$, $\delta^{34}\text{S}$ and $\Delta^{33}\text{S}$ data collected by microscale (SIMS) and bulk on sedimentary pyrite and bulk sulfate water body from individual modern environments, reported against VCDT. Also included are the depth of the analyses, the size and morphology of the pyrite grain, and the μsensor profiles of O_2 and H_2S . The raw SIMS data are available on request.

Caption for Data S2. SEM images of individual pyrite grain analysed for microscale S isotope. Also shown are the analytical name, $\delta^{34}\text{S}$ and $\Delta^{33}\text{S}$ values in red (lower left corner) and the yield of the analyses with respect to the Balmat standard (yellow, lower right corner).

Caption for Data S3. List of references included in the Precambrian microscale S isotope compilation (Fig. 3). Data available on request.