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## *Biocorrosion detection by sulphur isotopic fractionation measurements*

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### **Highlights**

- Iron sulphide are distributed at the micrometric scale in anoxic corrosion layers
- Sulphur isotopic fractionation determine the biotic/abiotic origin of iron sulphide
- $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  is negative for iron sulphide of bacterial origin
- $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  is close to zero for iron sulphide of inorganic origin
- NanoSIMS allows to measure the  $\delta^{34}\text{S}$  of sulphides at the submicrometric scale

### **Abstract**

This innovative study presents a local diagnosis approach based on nanoSIMS technique to determine the biotic/abiotic origin of iron sulphides of (sub)micron size heterogeneously located in the corrosion product layers of iron. For iron coupons corroded in controlled conditions, negative  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  were obtained in biotic conditions contrary to  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  obtained in abiotic ones. These results underline that nanoSIMS is a reliable local technique to detect the formation origin of (sub)micron iron sulphides. This successful methodology was then applied to a complex system representative of an iron corroded in the conditions similar to those of radioactive wastes storage in geological medium.

*Keywords: microbiological corrosion, iron sulphide, sulphur isotopic fractionation, NanoSIMS*

### **1. Introduction**

Resistance of metallic alloys to anoxic corrosion in soil or in marine and subaquatic water is a major issue whether for industrial facilities [1,2], geological nuclear waste storage [3,4] or cultural heritage [5]. Among the parameters influencing the corrosion in these environments and the possible threat encountered in these contexts, the presence of bacteria is particularly important to be taken into account for the preservation and maintenance of the solicited metals [6–13]. Thus, to better understand the role of bacteria in iron corrosion to predictive modeling of iron anoxic corrosion, it is crucial to describe accurately the systems. Previous studies have shown that the corrosion product layers (CPL) resulting from iron anoxic corrosion are mainly composed of iron carbonates, siderite ( $\text{Fe}^{\text{II}}\text{CO}_3$ ) [14–16] and sometimes chukanovite ( $\text{Fe}^{\text{II}}_2(\text{OH})_2\text{CO}_3$ ) [14,15], with discontinuous strips of magnetite ( $\text{Fe}^{\text{II,III}}_3\text{O}_4$ ) [14–16]. Moreover, several studies related to the iron anoxic corrosion show the formation of iron/sulphur-containing compounds, especially iron sulphides, among the corrosion products [5,17,18]. These iron sulphides present in the CPL of iron may lead to important changes in corrosion rates by inhibiting or accelerating corrosion processes [19–23]. Consequently, it is essential to determine the origin of these phases in order to achieve a more precise diagnosis on the formation of these corrosion products. This will allow to determine how far they endanger the mechanical sustainability of the concerned metals and to set-up adapted protection methods against corrosion [24,25].

In sulphate-rich environments, iron sulphides are suspected to result from the action of sulphate-reducing bacteria (SRB) that transform sulphates into sulphides [26–27]. However in sulphide-bearing environment as clay, iron sulphides may also result from totally abiotic reaction pathways [28–32]. For instance, dissolution/reprecipitation of pyrite may occur leading to the production of iron sulphides [33]. It was observed that whatever the formation origin of the iron sulphides (abiotic or biotic), the same crystalline phases are involved, going from mackinawite  $\text{FeS}$  up to pyrite  $\text{FeS}_2$  through the intermediate phase greigite  $\text{Fe}_7\text{S}_4$  [22,28,34,35]. Therefore the crystalline nature of the iron sulphides does not allow evidence the formation origin of iron sulphides. The usual way to diagnose the formation origin of these sulphides is to determine the sulphur isotopic ratio  $R^{34}\text{S}$  defined as:

$$R^{34}\text{S} = \frac{\text{Abundance of } ^{34}\text{S}}{\text{Abundance of } ^{32}\text{S}}$$

In the absence of SRB,  $R$  is known to be close to  $R^{34}\text{S}_{\text{natural}}$  of natural sulphur that is 0.04519. On the contrary, when sulphate-reduction phenomenon occurs through a bacterial metabolism, because of intra-cellular kinetic effects, SRB preferentially consume  $^{32}\text{S}$  of the sulphate. This phenomenon is explained by the fact that it is easier for bacteria to break the molecular bonds when sulphates are composed of the lighter sulphur isotope [36]. Therefore the residual sulphates of the medium become  $^{32}\text{S}$ -depleted and  $^{34}\text{S}$ -enriched and conversely the formed sulphides are  $^{34}\text{S}$ -depleted. In presence of ferrous ions, originated from any sources, among them corrosion, this leads to the precipitation of iron sulphides that are also  $^{34}\text{S}$ -depleted [36–39]. Therefore, when iron sulphides

come from a bacterial origin,  $R^{34}\text{S}$  is inferior to  $R^{34}\text{S}_{\text{natural}}$ . So, sulphur isotopic composition analyses could enable to distinguish the abiotic or biotic origin of the iron sulphides.

To compare the results issued from different publications, the isotopic fractionation in  $^{34}\text{S}$ , called  $\delta^{34}\text{S}$ , refers  $R^{34}\text{S}$  to a standard sample, the Canon Diablo Troilite (CDT), the international sulphur isotope standard [40].

$$\delta^{34}\text{S} \text{ ‰} = \frac{R^{34}\text{S}(\text{sample}) - R^{34}\text{S}(\text{standard})}{R^{34}\text{S}(\text{standard})} \times 1000$$

The  $\delta^{34}\text{S}$  can also refer to the isotopic composition of sulphate in the environment [40]. Indeed in the major part of the studies related to the sulphur isotopic fractionation,  $^{32}\text{S}$  and  $^{34}\text{S}$  isotopes are determined on bulk solution or sediments samples by global mass spectrometry on solutions [36,40–44] with the determination of  $\delta^{34}\text{S}$  in sulphides relatively to sulphates of the medium. The  $\delta^{34}\text{S}$  depletion values can be as high as 70 ‰ [40–42,45,46] for sulphides substrates when SRB are involved.

Very few studies are reported in the literature on micrometric samples. One of those conducted in the geoscience field by Lerouge et al [47], evidenced that, in the bottom part of the Callovo-Oxfordian argillite,  $\delta^{34}\text{S}$  measured by microSIMS goes from -38 ‰ to -22 ‰ in the pyrite suggesting its formation through the action of SRB. In the corrosion domain, the publications concerning the sulphur isotope fractionation are also rare. In the innovative study of Little *et al.* [48] the sulphur isotopic fractionation was measured by CF-IRMS (Continuous-Flow Isotopic Ratio Mass spectrometry) in corrosion products resulting from the activities of sulphate-reducing bacteria within biofilms on metallic copper surfaces:  $^{32}\text{S}$  were accumulated in the corrosion products while  $^{34}\text{S}$  was concentrated in the culture medium due to fractionation. But this innovative study in the corrosion field, was nevertheless carried out through the solely analyses of the corrosion solution, after the precipitation into  $\text{BaSO}_4$  of the sulphates, then combustion into  $\text{SO}_2$ , and detection by global mass spectrometry. The global scale analysis can be then conducted when a representative sample of the medium can be collected. Nevertheless it is not adapted to solid samples of corrosion layers constituted of metallic sulphides precipitated locally at the micrometer scale [49].

Recent studies concerning the local determination of the sulphur isotopic fractionation in iron sulphides formed from abiotic or biotic way have been addressed [50,51]. These authors worked on solid samples and used a local secondary ion mass spectrometry technique, ToF-SIMS (Time-of-Flight Secondary Ion Mass Spectrometry), to determine the origin of the iron sulphide compounds [50]. Two types of iron sulphides were considered in their study: chemical iron sulphides as commercial pyrite and iron sulphide obtained by thermal sulphidation of a metallic pure iron foil, and biological iron sulphides prepared by anaerobic immersion of iron in a sulphide solution produced by reduction of sulphate by SRB. The authors evidenced a lower ratio  $^{34}\text{S}/^{32}\text{S}$  for the sulphide from biological origin than for those from chemical origin. This demonstrates the ability of the ToF-SIMS method to identify the biological origin of sulphide corrosion products on steel. However, they worked on laboratory

samples that were not representative of corrosion layers formed in anoxic soils. Indeed these latter are generally constituted of a mix of iron sulphide and iron carbonate at the micrometric scale and this can decrease the precision of the ToF-SIMS measurement due to the overlap of the  $O_2^-$  and  $^{32}S^-$  mass peaks. Moreover they worked on very short duration experiments (24 hours for the biotic samples) which don't authorize to determine the long term effect of the processes.

In specific cases,  $\delta^{34}S$  values can be positive even if iron sulphides come from bacterial origin: it is the case of closed or semi-closed or confined systems corresponding to the absence of the renewal of the sulphate source. Indeed, in this absence of sulphate source renewal, the residual sulphates of the medium are depleted in  $^{32}S$  and the bacteria have to consume  $^{34}S$  for their sulphate reduction, which leads to an increase of  $\delta^{34}S$ . This observation was reported by Lerouge et al on iron sulphides located in the confined upper part of the Callovo-Oxfordian where the values of  $\delta^{34}S$  ranges from 14 ‰ to 34.5 ‰ [47].

The aim of this paper is to develop a diagnosis method of the bio-origin of iron sulphides formed in iron CPLs thanks to the determination of the sulphur isotopic fractionation, by using the nanoSIMS technique, appropriate thanks to its excellent spatial and mass resolution, to the (sub)micrometer iron sulphide strips located in the CPLs. To test the relevance and the feasibility of the determination of iron sulphides origin by nanoSIMS, anoxic aqueous corrosion experiments in controlled conditions were carried out without or with SRB to determine  $\delta^{34}S$  values in each case and to compare to values reported in the literature. After the validation of nanoSIMS use on these model systems prepared in laboratory controlled condition the methodology was applied to a more representative samples of the nuclear waste storage in geological medium. For that an iron bar was corroded for 13 months in contact with clay and in presence of two bacterial strains, in an anoxic corrosion cell. In this paper, complementary techniques from micrometer to nanometer scales were used: SEM-EDS for the distribution of iron sulphides in the CPLs,  $\mu$ Raman for the crystalline nature of iron sulphides and nanoSIMS for the obtention of  $\delta^{34}S$  which permits the determination of the formation origin of iron sulphides.

## **1. Materials and methods:**

### **a. Corrosion in aqueous solutions**

For the laboratory anoxic corrosion experiments three set-ups were designed to corrode iron coupons in three different media with and without bacteria. The first one is fully abiotic and is named (A), the second one being fully biotic and named (B). The third one presents mixed conditions where the coupons were first corroded in presence of bacteria during two weeks. Then in a second step, the solution was filtered under anoxic conditions in order to eliminate the possible contamination of the

bacterial lyse on longer duration of corrosion. Thus, the coupons are first corroded in biotic conditions. Then they were corroded in abiotic conditions in the solution containing the remaining sulphide produced by the bacteria during the first step. These samples are therefore designated by the denomination (B-A) for biotic-abiotic experiments. All these experiments were conducted using Goodfellow® iron coupons (d = 10 mm; thickness = 1 mm; purity = 99.5 %) in a N<sub>2</sub>/CO<sub>2</sub> atmosphere (95 % / 5 %). All the elements required for these experiments were previously sterilised.

Two abiotic experiments (A<sub>1</sub> and A<sub>2</sub>) have been conducted at two durations (7 and 11 months). The experiments were conducted following the two stage protocol described by Sherar et al [52] to ensure the formation of iron sulphides of mackinawite type. So, two iron coupons per flask were first immersed into an anoxic carbonated solution without sulphides for one month. The composition of this medium was 25.2 g/L NaHCO<sub>3</sub>; 8.8 g/L NaCl and 21.3 g/L Na<sub>2</sub>SO<sub>4</sub>. Then, at the end of the first month, sodium disulphide salts (Na<sub>2</sub>S) were added to the flasks in order to reach a sulphide concentration of 0.2 g/L (2 mmol/L). Then the flasks were stored at ambient temperature during 6 and 10 months respectively, for a total duration experiment of 7 and 11 months (Figure 1).

For the biotic (B) experiments, two flasks containing the culture medium allowing an optimum growth of the bacterial strain were used [53]: a flask inoculated with the bacterial strain *Desulfotomaculum aquiferis* Bs105<sup>T</sup> (= DSM 24088<sup>T</sup>) [53] and a control flask without bacteria inoculation. The composition of this culture medium was 0.3 g/L KH<sub>2</sub>PO<sub>4</sub>; 0.3 g/L K<sub>2</sub>HPO<sub>4</sub>; 1.0 g/L NH<sub>4</sub>Cl; 1.0 g/L NaCl; 3.0 g/L Na<sub>2</sub>SO<sub>4</sub>; 0.1 g/L CaCl<sub>2</sub> · 2H<sub>2</sub>O; 0.5 g/L MgCl<sub>2</sub> · 6H<sub>2</sub>O; 2.2 g/L pyruvate; 10 g/L dried yeast extract; 0.1 % v/v solution of trace elements SL10; 0.05 % resazurin. In each flask, two iron coupons were immersed. The flasks were stored at the optimum growth temperature (35 °C) for 1 week. The (B) experiment ended at the end of this first week (Figure 1).

In the case of the (B-A) experiment the same solution than for biotic (B) experiments was prepared (see above). But in the (B-A) experiments the storage of the flasks at 35 °C lasted one more week so that the biotic period lasts 2 weeks in total. This period corresponds to the end of the stationary phase of the bacterial growth. At this stage the medium has been filtrated in anoxic conditions in order to remove the bacteria from the solution. This filtration process was performed to limit the contamination of the medium by sulphur-containing species coming from the lyse of the bacterial cells. Then to pursue the corrosion of the iron coupons on longer durations, the experiment was continued at 35°C in anoxic conditions to reach duration experiments ranging from 1 to 8 months (samples named of the (B-A<sub>i</sub>; i = 1 to 5, see Figure 1).

## **b. Corrosion in clay**

The sample called “CBCC” results from a low-alloy steel corrosion tests in clay rock representative of the French High-Level Waste Disposal Concept and was briefly previously described [3]. This experiment was conducted during 13 months at 60 °C. It consisted of a cylindrical

percolation cell (see Figure 2) containing a vertical iron bar (diameter 3 mm; height 1 cm) inserted in a sample of Toarcian argillite of 42 mm diameter and 30 mm height [3]. The argillite was sampled at a depth of around 512 m in the IRSN (Institut de Radioprotection et de Sûreté Nucléaire) experimental station of Tournemire (Aveyron, France). At this altitude, the concentration of sulphates in the argillite porewater is estimated at 9.5 mmol/L [54,55]. The clay is mainly composed of kaolinite, illite and smectite mixed to quartz and present about 2 to 5 wt% of pyrite [54]. It contains also about 4% maximum of water. For this experiment all the materials were previously sterilised and the set-up was realised in an anoxic glovebox. During the experiment, a flow of about 0.7 mL/day of a synthetic solution was constantly injected from the bottom and evacuated at the top of the cell. The composition of this solution was chosen as close as possible to the pore water in equilibrium with the Tournemire clayey rock at 60 °C [54,56]: 0.395 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.041 g/L KH<sub>2</sub>PO<sub>4</sub>; 0.303 g/L NaHCO<sub>3</sub>; 1.155 g/L Na<sub>2</sub>SO<sub>4</sub>; 0.081 g/L NaCl; 0.054 g/L KCl; 0.161 g/L MgCl<sub>2</sub>.6H<sub>2</sub>O. A layer of compacted and calibrated iron powder ( $d_{\text{particule}} \leq 60 \mu\text{m}$ ,  $S_{\text{specific}} = 0.13 \pm 0.02 \text{ m}^2/\text{g}$ ) was placed at the bottom of the cell in order to ensure an iron reservoir releasing iron ions in the solution during the experiment. A difference in the concentration of the sulphate in the injected and evacuated solutions has been observed: this concentration is of 11.2 mmol/L in the injected one whereas it is of 3.5 mmol/L in the released one [3].

Lastly, two bacterial strains were inoculated in the system the SRB *Thermodesulfovibrio hydrogeniphilus* Hbr5<sup>T</sup> (= DSM 18151<sup>T</sup>) isolated from a hot spring water in Tunisia [57] and the iron-reducing bacteria *Thermotoga subterranea* strain SL<sup>T</sup> (= DSM 9912<sup>T</sup>) isolated from an oil reservoir in the east of the Paris basin [58]. Each of these strains was firstly cultivated in the medium recommended by the strain bank DSMZ namely media 641 and 688 for *T. hydrogeniphilus* HBr5 and *T. subterranea* SL1 respectively. Then both bacterial strains were cultivated simultaneously in the synthetic solution representative of the Tournemire clay's pore water to ensure that they grow in this medium, poor in nutritive and energetic elements but similar to the medium of the percolation cell. Finally they were inoculated in the percolation cell at a rate of 4.10<sup>8</sup> bacteria/mL.

### c. Sulphate solutions preparation for isotopic measurements

Sulphate isotopic ratios of the solutions were determined following the classical precipitation method developed in many papers (see for example Canfield 2001). Briefly, the sulphate was precipitated into BaSO<sub>4</sub> thanks to an addition of BaCl<sub>2</sub>.2H<sub>2</sub>O salts to the sulphate solution. Then the isotopic ratio in BaSO<sub>4</sub> is analysed by CF-IRMS (Continuous-Flow Isotopic Ratio Mass spectrometry). The sulphur isotopic composition of the sulphates in solution ( $R_{\text{exp}} = \frac{I^{34}\text{S}_{\text{exp}}}{I^{32}\text{S}_{\text{exp}}}$ ) was determined by CF-IRMS on BaSO<sub>4</sub> extracts. An elementary analyser EA3000 (EuroVector®) coupled with a mass spectrometer Horizon (Nu Instruments®) was used. Calibration was realised thanks to the international sulphur isotopic standard CDT (Cañon Diablo Troilite) [44,59].

#### **d. Sample preparation on transverse section**

Half of the iron coupons of the (B), (B-A), and (A) experiments were dedicated to surface analyses whereas the other half of the coupons as well as the CBCC sample were analysed on cross-section. For this purpose, these samples were embedded in an epoxy resin (Specifix-20, Struers®) and cut with a diamond saw. They were then ground with SiC paper (grad 320-4000) and diamond polished under ethanol down to 1  $\mu\text{m}$ . The whole preparation process was carried out in an anoxic glove box ( $\text{O}_2$  concentration < 100 ppm) whose relative humidity was controlled by silica gel to avoid the transformation of the anoxic phases.

#### **e. Analytical techniques**

For the sample characterisation, the following methodology was followed: coupons were first analysed by Scanning Electron Microscope (SEM) coupled to Energy Dispersive Spectroscopy (EDS) and Raman spectroscopy in order to determine respectively the chemical composition and the crystalline structure of the phases precipitated during the laboratory experiments. These techniques were also performed on the transverse sections of the coupons and of the CBCC sample in order to locate these phases in the corrosion layer prior to the isotopic analyses. Thus in order to study the sulphur isotopic composition of these thin iron sulphide layers, the high spatial resolution of the NanoSIMS technique was necessary. Another reason to use this technique is the presence of iron carbonates mixed with the sulphides at the submicrometric scale. Carbonates are oxygenated phases, and a high mass resolution ( $m/\Delta m$  of 10000) is required to separate the  $^{32}\text{S}$  from the  $^{16}\text{O}_2$  mass contribution (see section 2.5.3 for the acquisition strategy).

##### **i. Field-Emission Scanning Electron Microscopy (FESEM) and EDS**

FESEM images in secondary electron mode and EDS spectra were obtained with a microscope JEOL JSM-7001F. An acceleration voltage of 15 kV was used. The location of the phases, especially the sulphur-containing compounds, was determined by EDS thanks to a silicon drift detector. The surface of the samples was coated with a carbon layer before analysis to prevent sample charging.

##### **ii. $\mu$ Raman spectroscopy**

$\mu$ Raman measurements were carried out on the surface of the (A), (B) and (B-A) iron coupons and on the cross-section of the sample CBCC at room temperature via an Invia Reflex® spectrometer of Renishaw. Spectra were acquired through the glass window of an anoxic cell [60] enabling analyses of the samples in anoxic conditions. The Raman spectrum of the glass is presented in Figure 3. Spectra were acquired with an excitation wavelength of 532 nm. The laser power was filtered down to 0.1 mW and the spectra were recorded with a resolution of 2  $\text{cm}^{-1}$ . The laser beam diameter was of 1  $\mu\text{m}$ . The



spectrometer calibration was obtained from a silicon crystal which main peak is at  $520.5 \text{ cm}^{-1}$ . The acquisition time for each spectrum was between 30 and 120 seconds. The acquisition and treatment of the spectra were obtained with the software Wire 3.4<sup>®</sup>. Because of high fluorescence signal, the baseline was removed for all the Raman spectra.

In this study, five main corrosion products are expected: siderite ( $\text{Fe}^{\text{II}}\text{CO}_3$ ), greigite ( $\text{Fe}^{\text{II}}\text{Fe}^{\text{III}}_2\text{S}_4$ ), nanocrystalline mackinawite ( $\text{Fe}^{\text{II}}\text{S}$ ), crystalline mackinawite ( $\text{Fe}^{\text{II}}\text{S}$ ) and  $\text{Fe}^{\text{III}}$ -containing mackinawite ( $\text{Fe}^{\text{II,III}}\text{S}$ ). The detailed attributions of the Raman bands to liaison oscillation modes in iron sulphide phases have previously been detailed [49].

### iii. nanoSIMS

The S-isotopic composition analyses of the iron sulphide were conducted on the nanoSIMS N50 (CAMECA<sup>®</sup>). The analyses were carried out on polished resin-embedded cross-sections coated with a golden layer (~20 nm) to prevent charge accumulation. The area of interest was firstly presputtered for ~30 minutes to remove the gold coating from the area of interest, clean the surface and reach the steady-state sputtering equilibrium. Presputtering was realised with high-current (18 pA) primary  $\text{Cs}^+$  beam using D1-1 aperture diaphragm  $\text{Cs}^+$ . After this preparation the aperture diaphragm was then set at  $150 \mu\text{m}$  (D1-4) to reduce the current to about 1 pA for the analysis. This allowed reaching a measured beam size of about 70 nm.

During the analysis the secondary ions  $^{32}\text{S}^-$ ,  $\text{S}^-$ , and  $^{16}\text{O}^-$  were detected simultaneously to abrasion in a multicollection mode using three consecutive electron multipliers. Analytical conditions (ES3, AS3, EnS-20%) were set to achieve high mass resolution, e.g. of about 10,000 (Cameca definition) on  $^{32}\text{S}^-$ .

The isotopic fractionation in  $^{34}\text{S}$ , called  $\delta^{34}\text{S}$ , refers  $R^{34}\text{S}$  to a standard sample, the Canon Diablo Troilite (CDT), the international sulphur isotope standard, or to the sulphate of the environment. For samples extracted from natural systems, not only the sulphur isotopic fractionation resulting from the corrosion processes but also the sulphur isotopic fractionation accumulated by the sulphur species before the beginning of corrosion processes contribute to the value of  $\delta^{34}\text{S}$  sulphides-CDT. On the contrary, the value of  $\delta^{34}\text{S}$  sulphides-sulphates only quantifies the sulphur isotopic fractionation occurring during the corrosion processes. So, the use of  $\delta^{34}\text{S}$  sulphides-sulphates is more adapted to corrosion studies. Therefore, only the values of  $\delta^{34}\text{S}$  sulphides-sulphates are discussed in this article.

For standards and references, areas of  $5 \times 5 \mu\text{m}^2$  were presputtered and central zones of  $3 \times 3 \mu\text{m}^2$ , 64 x 64 pixels, were analysed. For the samples, a  $12 \times 12 \mu\text{m}^2$  area was presputtered, and  $9 \times 9 \mu\text{m}^2$  zones, 256 x 256 pixels, were analyzed. Each image is a stack of 26 to 170 scans. The experimental  $^{34}\text{S}/^{32}\text{S}$  ratios are deduced from the stack image processing realized with the L'IMAGE software package developed by L. Nittler (Carnegie Institution of Washington) (see Figure 4).

Due to the high ionization efficiency of S the QSA (quasi-simultaneous arrival) effect was taken into account for the correction of the obtained isotopic ratios [61,62]. To quantify the QSA effect and to correct it was proceeded as follows: the entrance slit was set to 30  $\mu\text{m}$  (ES3) and the other aperture and energy slit widths were varied. This results in the measurement of the secondary ion count rates which varies from 5,000 cps to 330,000 cps for  $^{32}\text{S}$  depending on the local matrix and topography. In this study the mass fractionation due to QSA effect ranges from 0.6 ‰ ( $K_{\text{cor}} = 0.001$ ,  $I^{32}\text{S} = 5300$  cps, B-A<sub>1</sub> sample) to 39 ‰ ( $K_{\text{cor}} = 0.045$ ,  $I^{32}\text{S} = 330000$  cps, CBCC sample point 3). Consequently, the QSA effect was corrected based on methods previously detailed in the literature [63,64]. In our case, three different sulphide phases matrix have been used to correct the  $^{34}\text{S}/^{32}\text{S}$  ratios from the QSA: the CDT [44,59], a hydrothermal pyrite (PLV) and a greigite  $\text{Fe}_3\text{S}_4$  reference. Pure greigite mineral was unavailable and the synthesis of homogeneous greigite samples was very difficult because of its high reactivity to oxygen. That's why an archaeological nail (RH1202) containing pure greigite as located by Raman spectroscopy ( $S \approx 2000 \mu\text{m}^2$ ) and coming from the terrestrial site of Rådhuspladsen (Copenhagen, Denmark) [49] was used as greigite matrix for the QSA correction

## 2. Results

### a. Corrosion in aqueous solution

The surfaces of the (A), (B-A) and (B) coupons were first analysed in the anoxic cell by  $\mu$ -Raman spectroscopy. Examples of spectra obtained for each kind of experiment are presented on Figure 5. In all cases a mix of crystalline mackinawite ( $\text{FeS}$ , main peak at 294-297  $\text{cm}^{-1}$ ), and greigite ( $\text{Fe}_3\text{S}_4$ , peak at 340-370  $\text{cm}^{-1}$ ), is identified [49]. On the (A) coupon, nanocrystalline mackinawite (main peak at 280  $\text{cm}^{-1}$ ) and iron carbonates ( $\text{FeCO}_3$ , sharp band at 1083  $\text{cm}^{-1}$ ) are also observed [49]. Thus, similar iron sulphides are identified in both biotic and abiotic systems.

FESEM-EDS analyses realised on cross-sections enabled to precisely locate the sulphur-containing phases. An example of the iron and sulphur EDS maps obtained on a (A) coupon is presented on Figure 6. It shows that the sulphur layer presents a thickness of few micrometers. The EDS maps obtained on the cross-section of other (A), (B-A) or (B) coupons led to the same observation.

The sulphur isotopic fractionation  $\delta^{34}\text{S}$  of the sulphate powders used for the preparation of the solutions was measured by CF-IRMS relative to the CDT standard. The  $\delta^{34}\text{S}_{\text{sulphates-CDT}}$  obtained is of  $0.7 \pm 0.3 \text{ ‰}$ . As it was not possible to measure the  $\delta^{34}\text{S}$  of  $\text{Na}_2\text{S}$  due to the deliquescent state of the compound and considering that both sulphur containing compounds, were acquired from the same supplier, the hypothesis that the  $\delta^{34}\text{S}_{\text{sulphides-CDT}}$  is close for both  $\text{Na}_2\text{SO}_4$  and  $\text{Na}_2\text{S}$  compounds is reasonable. So in the following the  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  fractionations measured on the experimental

samples are expressed relative to the  $\delta^{34}\text{S}_{\text{sulphates-CDT}}$  of  $0.7 \pm 0.3 \text{ ‰}$ .

The sulphur isotopic fractionation measured on the sulphides for each coupon were calculated from the measured and corrected ratio  $^{34}\text{S}/^{32}\text{S}$  (see methodology section) and are displayed in Table 1 relative to the CDT ( $\delta^{34}\text{S}_{\text{sulphides-CDT}}$ ) and relative to the sulphates of the experimental solution ( $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$ ).

For the abiotic coupons (A),  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  ranged between  $4.8 \pm 6.2 \text{ ‰}$  and  $14.5 \pm 5.7 \text{ ‰}$ , namely between  $-1.4 \text{ ‰}$  and  $20.2 \text{ ‰}$  when taking the error into account. On the biotic (B) coupon,  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  are very close and yield from  $-24.7 \pm 5.2 \text{ ‰}$  to  $-25.5 \pm 6.4 \text{ ‰}$ , namely between  $-31.9 \text{ ‰}$  and  $-19.5 \text{ ‰}$  when taking the error into account. Finally, for the biotic and then abiotic (B-A) coupons  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  ranges from  $-29.6 \pm 6.5 \text{ ‰}$  to  $-7.5 \pm 6.2 \text{ ‰}$ , namely between  $-36.1 \text{ ‰}$  and  $-1.3 \text{ ‰}$  when error is included.

### **b. Corrosion in clay**

Previous observations have shown that the main corrosion profile observed is constituted of siderite and chukanovite characteristic of corrosion in anoxic conditions in presence of a carbonated solution [3]. But locally the observation on transverse section of the CBCC sample by optical microscopy reveals the presence of a brilliant strip of several micrometers to several tens of micrometers thick. It is located in contact with the metallic matrix (Figure 7) and is mainly composed of sulphur and iron: 29.4 w% and 54.2 w% respectively (Figure 7 - red point). The  $\mu$ -Raman spectrum obtained on this strip (Figure 8) presents a peak at  $292 \text{ cm}^{-1}$  characteristic of the crystalline mackinawite and a large band between  $335$  and  $365 \text{ cm}^{-1}$  that could be attributed to greigite [53]. Thus, this strip is composed of a mixture of iron sulphides similar to those observed in aqueous solution.

In this set-up the  $\delta^{34}\text{S}_{\text{sulphates-CDT}}$  of the sulphate of the solution injected in the cell was not measured but as the compound was from the same supplier as the one used for the coupons experiment the same  $\delta^{34}\text{S}_{\text{sulphates-CDT}}$  value of  $0.7 \pm 0.3 \text{ ‰}$  was taken as a reference for the calculation of the  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  in this experiment. Three analyses were realised by nanoSIMS on the iron sulphides detected within the corrosion layer (Figure 7 and Figure 9). The values of  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  obtained on this sample are comprised between  $-4.0 \pm 5.0 \text{ ‰}$  and  $-3.1 \pm 4.7 \text{ ‰}$  namely between  $-9 \text{ ‰}$  and  $+1.6 \text{ ‰}$  when error is included (Table 2).

## **3. Discussion**

The graph of Figure 9 gathers the values of  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  of all the samples studied in this paper. The results are commented and discussed in the paragraphs below.

### a. Corrosion in aqueous solution

For each experiment in aqueous solution, the iron sulphides formed on the surface of the iron coupons could result from a single process: either abiotic (A) or biotic (B and B-A). In the following paragraphs, the possibility to determine the origin from the sulphur fractionation  $\delta^{34}\text{S}$  measurement will be discussed.

For the (A) experiments, two measurements were obtained after 7 months and four after 11 months of corrosion. The average value is of 12.6 for 7 months and 9.5 for 11 months. Thus values vary inside each sample but they remain close for the two durations of the experiment. The range of sulphur isotopic fractionation obtained ( $\Delta = 19 \text{ ‰}$ ) is similar to what is given in the literature ( $\Delta \approx 20 \text{ ‰}$ ) for iron sulphides collected from an abiotic reaction [65]. Yet, the values obtained in this work are slightly superior to what was expected for abiotic sulphides, namely a range between -10 and +10 ‰ even since the reference used is the source of sulphates with a measured  $\delta^{34}\text{S}_{\text{sulphates-CD}}$  value of  $0.7 \pm 0.3 \text{ ‰}$ . This could be explained by the slight oxidation of the (nano)-crystalline malachite into greigite when experiments are stopped, samples are dried in the glovebox and transported in the raman cell. Indeed, sulphur isotopic fractionations up to +10 ‰ during inorganic oxidation processes have been previously reported in the literature when sulphur oxidation degree varies. For example, Oana *et al.* [66] conducted laboratory experiments and have shown that disproportionation reaction of sulphurous acid ( $\text{H}_2\text{S}^{\text{IV}}\text{O}_3$ ) into sulphuric acid ( $\text{H}_2\text{S}^{\text{VI}}\text{O}_4$ ) and sulphur ( $\text{S}^0$ ) induced an enrichment of up to +10.4 ‰ in  $^{34}\text{S}$  for the  $\text{H}_2\text{S}^{\text{VI}}\text{O}_4$  compound and a depletion in  $^{34}\text{S}$  of -13.5 ‰ for the  $\text{S}^0$ .

Thus, the slight oxidation of the iron sulphides produced during the abiotic experiments could explain the slight enrichment in  $^{34}\text{S}$  while no fractionation was expected since the sulphides are from inorganic origin.

Concerning the variation of the duration of the abiotic experiments, no influence of this parameter has been evidenced on the sulphur isotopic fractionation values obtained. Thus for the abiotic experiments it can be concluded that the sulphur isotopic fractionation values obtained by nanoSIMS are close to 0 ‰ and are therefore consistent with the abiotic origin of the iron sulphides.

For the biotic experiments, two protocols were applied. In the first one, called the biotic (B) experiments one, coupons were exposed no more than one week to the direct contact with the solution containing bacteria. For the second protocol called biotic-abiotic (B-A), durations of experiments were extended from one week to 8 months but coupons were exposed to the filtered solution obtained from the biotic experiments. This protocol was chosen to reduce the impact of the bacterial lysis which could induce the presence of dead cells inside the corrosion layers formed and disrupt the final fractionation measurements due to the presence of intra-cellular sulphur.

First, prior to the comment of the results, we may assume that the same phenomenon of oxidation of the iron sulphides from abiotic origin can impact the biotic experiments and shifts their fractionation towards higher values as well.

Apart of that, it has been observed that results are very homogeneous for the biotic coupons,

between, -24.7‰ and -25.5‰ for the  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$ . Moreover while considering the first short duration of one month for the B-A<sub>1</sub> coupon, the  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  is very close as well as the biotic one at -29.6‰. In these experiments (B and B-A<sub>1</sub>) the iron sulphides are depleted in <sup>34</sup>S relative to the sulphates which shows that the results obtained by nanoSIMS are consistent with the fact that the source of sulphides in this system was the bacterial metabolism.

Concerning the  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  values measured for the B-A<sub>2</sub> to B-A<sub>5</sub> coupons corroded on periods from 2 to 8 months the  $\delta^{34}\text{S}$  results obtained are more dispersed. They extend from -28.1‰ to -9.7 for the highest. It can be first noted that there is no correlation of these variations with the duration of the experiments. Moreover the slight oxidation of the sulphur inside the sulphide compounds can't explain such strong variations as discussed previously.

As obtained for the biotic coupons, the negative values obtained on the (B-A) coupon tend to show that the iron sulphides are depleted in <sup>34</sup>S and therefore that they would be due to biotic origin. Nevertheless the inhomogeneity of the results compared to the strictly biotic preparation raise questions about the influence of the long term duration process. In the literature, the case of an evolution of the  $\delta^{34}\text{S}$  fractionation towards higher value has been observed in geological environments [44,47]. It is explained by the confinement occurring in such environments due to the sedimentation phenomenon. Thus, in sediments the renewal of a solution can be weak because controlled by diffusion processes only. The SRB metabolism in the conditions adapts to the available sulphate. Thus if the concentration of <sup>32</sup>SO<sub>4</sub><sup>2-</sup> ions decreases due to their consumption by the biological processes, bacteria will increase their consumption of <sup>34</sup>SO<sub>4</sub><sup>2-</sup> ions. Therefore the fractionation  $\delta^{34}\text{S}$  of the sulphide produced by the bacterial metabolism will increase according to a Rayleigh modelling as it has been shown in the literature [47].

In some cases, when a long-term confinement occurs (at a geological timescale), positive  $\delta^{34}\text{S}$  can be measured. But in the B-A experiments, the conditions of a decrease of the <sup>32</sup>SO<sub>4</sub><sup>2-</sup> ions is not relevant because the bacteria are suppressed from the solution before starting the second step of these experiments (namely the abiotic step). The solution of the experiment contains sulphide ions produced by bacterial processes at the beginning of the B-A experiments with a certain  $\delta^{34}\text{S}$  value probably close to the one measured on the biotic coupons. That is why even for the short term experiment B-A1 of one week, the sulphur isotope fractionation  $\delta^{34}\text{S}$  of the sulphides precipitated in the corrosion layer are close to the biotic experiments. That conducts to the precipitation of depleted sulphide on the corroded coupons (between -29.6 and -24.7‰). But when the duration of exposure of the coupons to the biotic sulphide in a sealed beaker in anoxic conditions is longer, this provokes an increase of  $\delta^{34}\text{S}$ . Thus in this experiment, the increase of the  $\delta^{34}\text{S}$  can be explained by the production of sulphide from the sulphate containing solution maintained in anoxic conditions. As the corrosion processes in anoxic

conditions lead to the production of  $H_2$ , the solution is maintained anoxic. One can assume that the equilibrium in solution between sulphate and sulphide is shifted to the sulphide side in reducing conditions. This induces a production of sulphide ions that present  $\delta^{34}S$  close to the one of the original sulphate used to prepare the solution ( $\delta^{34}S_{\text{sulphates-CDT}}$  of  $0.7 \pm 0.3 \text{ ‰}$ ). Therefore the precipitation of sulphide phases on the corroded coupons integrate a mix of depleted and less depleted  $\delta^{34}S$  sulphides so that the results obtained presents more extended and higher  $\delta^{34}S$  values on the B-A<sub>2</sub> to B-A<sub>5</sub> coupons.

### **b. Corrosion in clay**

Contrary to the iron coupons corroded in aqueous solution, the iron sulphides in the sample CBCC could result from two possible processes: a biotic and an abiotic one. Indeed, abiotic iron sulphides could result either from the production of anoxic sulphide during the experiments or from the dissolution and re-precipitation of the pyrite contained in the argillite. Besides biotic iron sulphides could result from the SRB strain, *Thermodesulfovibrio hydrogeniphillus* [57] that was inoculated in the percolation cell in presence of a renewed solution containing sulphate.

To conclude on the biotic or abiotic origin of the iron sulphides observed in this sample, the methodology previously tested on the iron coupons corroded in controlled conditions was applied. Thus, the sulphur isotopic composition of the iron sulphides was compared with the one of the sulphates of the surrounding medium. These sulphates are composed of sulphates from the synthetic solution injected in the cell and sulphates from the natural porewater contained in the cylindrical Toarcian argillite sample. Their respective quantities have been therefore estimated to evaluate their influence on the precipitation of sulphides. The quantity of sulphates provided by the Toarcian argillite sample is estimated at 43  $\mu\text{mol}$ . This is calculated by taking into account the volume of the argillite sample (42 mm diameter and 30 mm height) [3], its density ( $2700 \text{ kg/m}^3$ ), a percentage by mass of water of 4% [55], and a concentration of sulphates in the porewater of 9.5 mmol/L [54]. The quantity of sulphates provided by the synthetic solution retained inside the cell is estimated to be 2200  $\mu\text{mol}$ . This is calculated thanks to the following experiment parameters: the injection solution flux (0.7 mL/day) [3], the difference between the sulphate concentration in the synthetic solution injected in the cell (11.2 mmol/L) and the sulphate concentration in the solution released from the cell (3.5 mmol/L) [3]. This demonstrates that the porewater of the argillite clay sample only contributes up to 2% of the total sulphates. Consequently, as a first approximation, the sulphur isotopic fraction of the sulphides is calculated relative to the sulphur isotopic fractionation of the sulphate powders used to make the synthetic porous solution. The sulphur isotopic fractionation of this sulphate powder relative to the international standard CDT is  $-0.7 \pm 0.3 \text{ ‰}$ . Three analyses were realised on the iron sulphides within the corrosion layer (Figure 7 and Figure 9). The  $\delta^{34}S_{\text{sulphides-sulphates}}$  measurements are close regardless of the area analysed: between  $-4.0 \pm 5.0 \text{ ‰}$  and  $-3.1 \pm 4.7 \text{ ‰}$ . These negative values support the hypothesis of a biotic origin of the iron sulphides formed in the sample CBCC. However, the iron

sulphides are only slightly depleted in  $^{34}\text{S}$  relative to the sulphate's source in the medium compared to the isotopic fractionations obtained on the coupons from the (B) and (B-A) solution experiments.

The  $\delta^{34}\text{S}$  of the precipitated sulphides measured on this sample result from a complex set of parameters occurring during the whole duration of the experiment. The slight negative  $\delta^{34}\text{S}$  values proceed from the processes occurred during the 13 months it lasted. But the period of the precipitation of the sulphide is not known. It could have occurred during the first period of the experiment. In this case, as measured on the coupons they should have been clearly depleted in  $^{34}\text{S}$  as observed on the B or B-A<sub>1</sub> coupons. As it is not the case, these sulphides may have precipitated later during the corrosion process. The same hypothesis than for the biotic-abiotic experiments can be made to explain the slight depleted fractionation obtained on CBCC experiment. In the bottom of the cell of the CBCC experiment, iron powder was filled up in order to ensure a low redox and the maintenance of anoxic conditions throughout the experiment. When the cell was dismantled, iron powder was entirely corroded and a mix of magnetite and siderite phases were identified [67]. It shows that the cell could have been under a  $\text{H}_2$  atmosphere during a long period of the experiment. Thus the reduction of the sulphate ions into sulphide ones was occurring during the experiment. The precipitated iron sulphides observed on the iron bar have probably precipitated from these sulphide ions that had a  $\delta^{34}\text{S}$  fractionation very close to the one of the original synthetic sulphate solution. The slight depletion could be explained by a mix of these anoxic sulphides with a  $\delta^{34}\text{S}$  close to 0 with few sulphides produced through bacterial metabolism although when taking the error bar into account this hypothesis can't be fully verified.

However several other explanations can conduct to understand the slight depletion of  $\delta^{34}\text{S}$  in the observed sulphides. According to the literature, the difference of sulphur isotopic fractionation can be explained by a difference of medium (state, temperature and composition) [46], by a difference of oxydation state of the sulphur compounds [66] and/or by the use of a different SRB strain [68] and various growing media [40]. Besides, the contribution of the sulphates from the porewater of the argillite through bacterial processes could have led to the precipitation of these iron sulphides. The sulphur isotopic fractionation of sulphates from the argillite porewater ranges indeed from 0.6 to 45.6 ‰ relative to the CDT due to the long-term confinement of the argillite during the sedimentation processes [56] whereas the sulphur isotopic fractionation of the commercial sulphate powder measured by CF-IRMS is of -0.7 ‰ relative to the CDT. If the sulphides are the result of a bacterial transformation of the sulphate of the argillite alone enriched in  $^{34}\text{S}$ , the  $\delta^{34}\text{S}$  of the precipitate sulphide can be less depleted and could then approach values close to 0 as it was measured. The sulphides could be therefore from a biotic origin. This hypothesis is nevertheless not very comforted by the fact that the sulphates from the argillite are 50 times less concentrated than the one of the solution. As bacteria metabolize preferentially the  $^{32}\text{SO}_4^{2-}$  ions, the precipitated sulphides should be then depleted in  $^{34}\text{S}$  in this case.

Another hypothesis connected to the medium could support an abiotic origin of the sulphide precipitated since the pyrites from the argillite could themselves present a sulphur isotopic fractionation of around -4 ‰ relative to the source of sulphates. Unfortunately, no isotopic analysis has been done on the pyrites of the argillite. Nevertheless, the only case where the iron sulphides could result from abiotic processes is if pH and redox conditions are suitable for pyrite dissolution. Yet, the measured pH is 7.4 and the redox potential obtained by modeling ranges from -200 to -700 mV/ESH [69] depending on the progress of the experiment. These conditions are unfavorable to pyrite dissolution [28,70].

To conclude on this corrosion experiment in presence of argillite and bacteria, it seems that the local precipitation of iron sulphide on the corroded bar could be related both to the intervention of a bacterial metabolism and to the anoxic conditions of the experiment. These phenomena could respectively explain the negative  $\delta^{34}\text{S}$  fractionation obtained; and that this depletion in  $^{34}\text{S}$  is slight. The higher medium temperature, the different bacterial strain and growing media used may also explain the slight depletion in  $^{34}\text{S}$  observed relative to those obtained in the B and B-A<sub>1</sub> experiments. These observations are nevertheless very instructive on the possibility to determine the biotic or abiotic origin of iron sulphides. It shows that an application to field measurement must integrate a good knowledge of the corrosive conditions before concluding only from the  $\delta^{34}\text{S}$  values measured.

#### 4. Conclusion

It was successfully demonstrated in this paper that sulphur isotopic analyses by nanoSIMS is adapted to determine the biotic or abiotic origin of localized (sub)micrometric iron sulphides in the corrosion product layers of iron coupon. The methodology developed in this study has been tested in the case of iron coupons corroded in solution in abiotic and biotic solutions to demonstrate the feasibility of the diagnosis method. Then it has been applied to an iron bar corroded in a laboratory cell in a clay environment to test it in a more applied experiment. The  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  values obtained for iron sulphides formed through a bacterial pathway in fully biotic conditions, range between -25.5 and 24.7‰ and denote a depletion of  $^{34}\text{S}$  content in the precipitated iron sulphides. On the contrary, the  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  values obtained on iron sulphides formed by an abiotic pathway are close to 0 ‰ which means that they are not depleted neither enriched in  $^{34}\text{S}$ . Thus, the values obtained for both biotic and abiotic experiments are consistent with what has been previously stated in the literature. To apprehend better the phenomenon occurring on longer periods, a mixed experiment combining a short biotic period to an abiotic one in presence of the filtered biotic solution to avoid the bacterial lysis have been also conducted. It shows that after a longer period in absence of living bacterial activity, the  $\delta^{34}\text{S}_{\text{sulphides-sulphates}}$  is still depleted but can increase due to the precipitation of abiotic sulphides generated through the equilibrium reaction between the sulphate and the sulphide of



the medium in anoxic reducing conditions inducing the formation of sulphides ions not depleted in  $^{34}\text{S}$ . This process can be accelerated at the vicinity of the corroding piece of iron that produces  $\text{H}_2$  during the corrosion process.

To conclude, the method developed in this study is especially useful to identify a biotic iron sulphide formation process when the iron sulphides are present as (sub)micrometric layers and/or when the surrounding dissolved sulphates can't be sampled to determine their sulphur isotopic ratio as it is usually performed in geological environments. Furthermore, the study of the spatial distribution of the sulphur isotopic fractionation in forthcoming works could allow to distinguish the successive steps involved in the formation of the corrosion facies observed in anoxic conditions thanks to the coupling of the characterization of the origin of the sulphur phases (nanoSIMS) and the nature of the sulphur compounds mixed at the submicrometric scale.

### **Author statement**

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**Data availability**

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal or ethical reasons.

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**Table 1 : Sulphur isotopic fractionation of iron sulphides in (A), (B) and (B-A) (NanoSIMS analyses) expressed relative to the CDT,  $\delta^{34}\text{S}_{\text{Sulphides-CDT}}$  (‰), and relative to the sulphur isotopic composition of the sulphates of the medium,  $\delta^{34}\text{S}_{\text{Sulphides-sulphates}}$  (‰).**

Coupon	A1 - a	A1 - b	A2 - a	A2 - b	A2 - c	A2 - d		
$\delta^{34}\text{S}_{\text{Sulphides-CDT}}$ (‰)	10.3 ± 6.4	14.2 ± 5.7	7.7 ± 6.1	4.8 ± 6.2	12.3 ± 6.4	13.0 ± 6.4		
$\delta^{34}\text{S}_{\text{Sulphides-sulphates}}$ (‰)	10.6 ± 6.4	14.5 ± 5.7	7.7 ± 6.1	4.8 ± 6.2	12.3 ± 6.4	13.0 ± 6.4		
Coupon	B - a	B - b						
$\delta^{34}\text{S}_{\text{Sulphides-CDT}}$ (‰)	-24.6 ± 5.2	-25.5 ± 6.4						
$\delta^{34}\text{S}_{\text{Sulphides-sulphates}}$ (‰)	-24.7 ± 5.2	-25.5 ± 6.4						
Coupon	B-A1	B-A2	B-A3	B-A4 - a	B-A4 - b	B-A5		
$\delta^{34}\text{S}_{\text{Sulphides-CDT}}$ (‰)	-29.5 ± 6.5	-8.7 ± 6.6	-26.0 ± 4.9	-12.4 ± 4.9	-25.0 ± 5.0	-7.2 ± 6.2		
$\delta^{34}\text{S}_{\text{Sulphides-sulphates}}$ (‰)	-29.6 ± 6.5	-9.7 ± 6.6	-28.1 ± 4.9	-9.8 ± 4.9	-22.5 ± 5.0	-7.5 ± 6.2		

**Table 2 : Sulphur isotopic fractionation of iron sulphides in CBCC experiment (NanoSIMS analyses) expressed relative to the CDT,  $\delta^{34}\text{S}_{\text{Sulphides-CDT}}$  (‰), and relative to the sulphur isotopic composition of the sulphates of the medium,  $\delta^{34}\text{S}_{\text{Sulphides-sulphates}}$  (‰).**

Coupon	CBCC1	CBCC2	CBCC3
$\delta^{34}\text{S}_{\text{Sulphides-CDT}}$ (‰)	-4.9 ± 5.0	-4.4 ± 4.5	-4.0 ± 4.7
$\delta^{34}\text{S}_{\text{Sulphides-sulphates}}$ (‰)	-4.0 ± 5.0	-3.5 ± 4.5	-3.1 ± 4.7

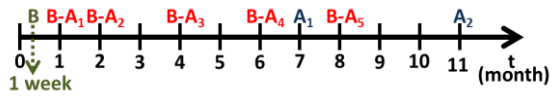


Figure 1: Schematic diagram of the duration for abiotic (A), biotic (B) and biotic-abiotic (B-A) experiments.

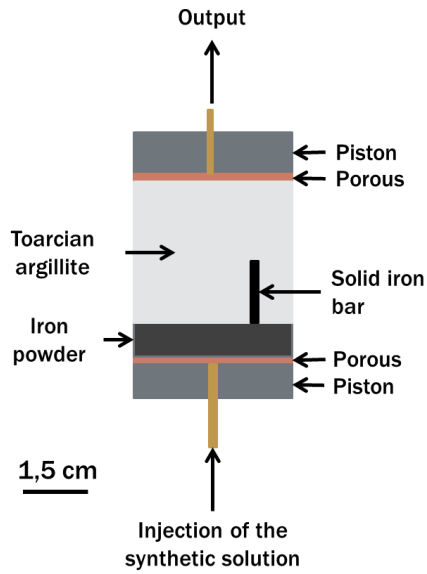


Figure 2: Biotic percolation cell 3.

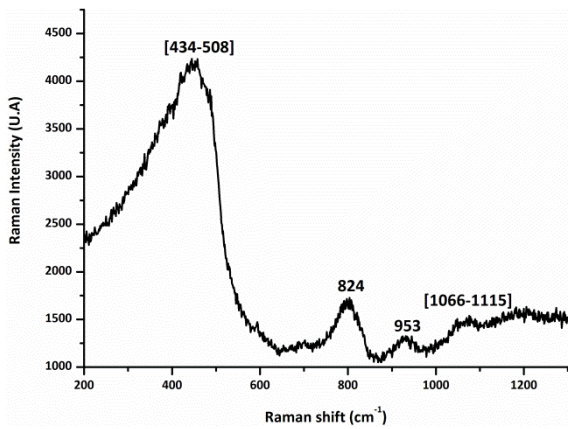


Figure 3: Raman spectrum of the anoxic cell glass.



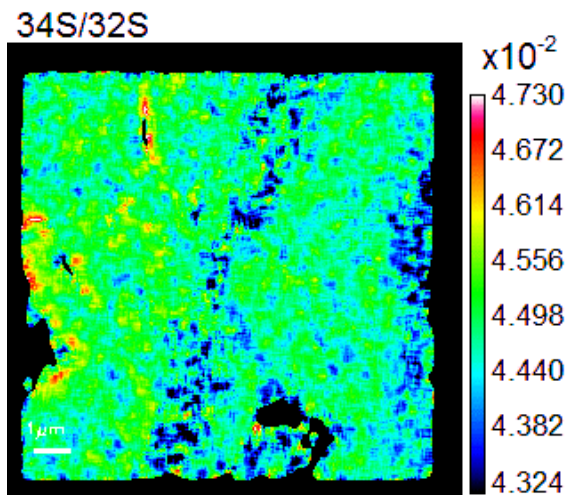


Figure 4: Example of a collected NanoSIMS map showing the 34S/32S ratio on the CBCC sample.

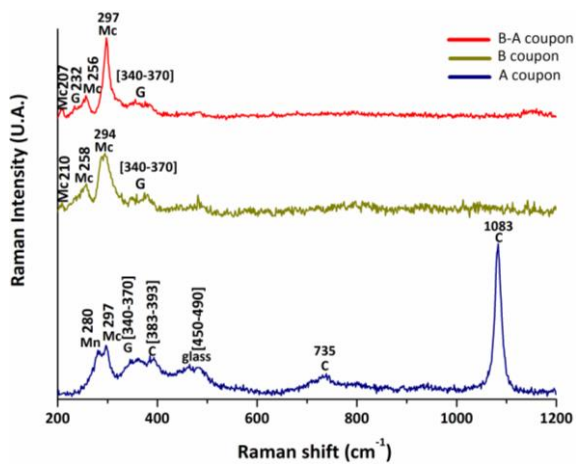


Figure 5: Raman spectrum obtained in an anoxic cell set-up on a (A) coupon (11 months), a (B) coupon (7 days) and a (B-A) coupon (8 months). Mn: non-crystalline mackinawite; Mc: crystalline mackinawite; G: greigite; C: carbonates.

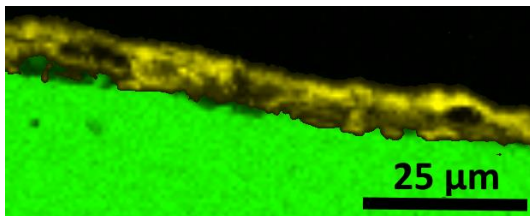


Figure 6: Superposition of Fe  $K_{\alpha}$  (green) and S  $K_{\alpha}$  (yellow) EDS maps (15 keV) obtained on the cross-section of an A coupon (11 months).

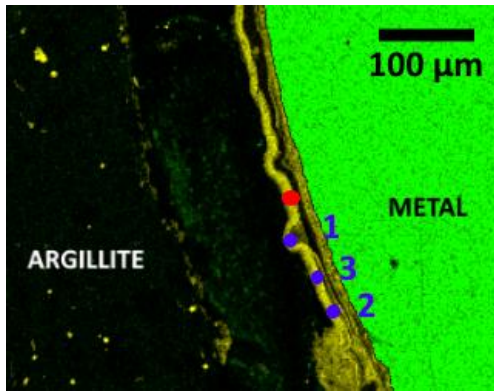


Figure 7: Superposition of Fe  $K_{\alpha}$  (green), S  $K_{\alpha}$  (yellow) EDS maps obtained on CBCC's cross-section. Points 1, 2 and 3 represent the location of the sulphur isotopic analyses 1, 2 and 3 of the CBCC sample in the Figure 9.

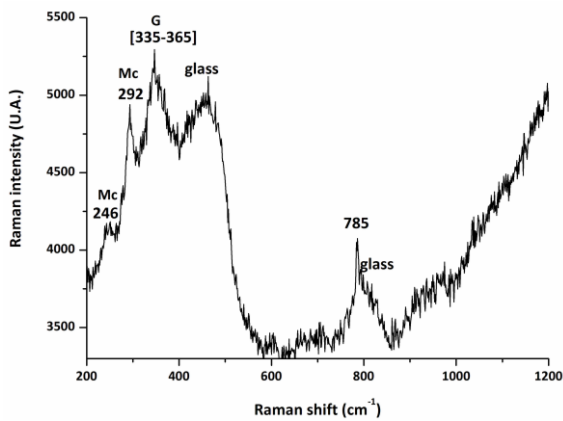


Figure 8: Raman spectrum obtained on cross-section of CBCC sample. Mc: crystalline mackinawite; G: greigite (anoxic cell set-up).

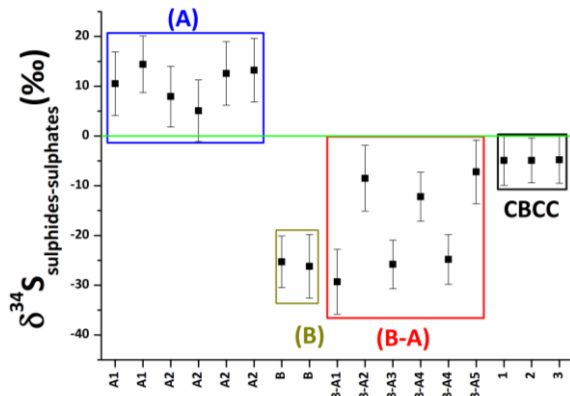


Figure 9: Sulphur isotopic fractionation of iron sulphides in (A), (B), (B-A) and CBCC experiments (NanoSIMS analyses) expressed relative to the sulphur isotopic composition of the sulphates in their respective medium (CF-IRMS analyses).