Sulfur K-edge XANES spectroscopy as a tool for understanding sulfur chemical state in anaerobic granular sludge

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Abstract. Sulfur is an essential biological element, yet its biochemistry in anaerobic biofilm is poorly understood because there are few tools for studying this element in biological systems. X-ray absorption spectroscopy provides a unique approach to determining the chemical speciation of sulfur in intact biological samples. When treating sulfate containing wastewaters in full scale up-flow anaerobic sludge bed bioreactors, microbial activity forms biofilms, consisting of a complex mixture of cells and associated extracellular substances as well as undefined inorganic precipitates. In addition to the anaerobic sludges, a large variety of model compounds of S (esp. sulfides) were investigated to find consistencies in the XANES that were used to model each “valence state” of S. The results confirmed that attributing a specific valence to most sulfides is impossible as we measured a continuum of edge shifts from sulfur “–2” to “–1”, depending on the electronic structure of S in the probed sulfides. In the sludges, various sulfur hot spots were probed for speciation, despite photo-reduction was sometimes a problem. First, we index the main features of complex K-edge XANES spectra for S²⁻-type units and sulfate units. Organic sulfur compounds were also shown to contribute significantly to the sulfur species present in some anaerobic granular sludge.

1. Introduction
Sulfur plays a central role in the metabolism of many anaerobic microorganisms. For instance sulfate acts as electron acceptor for the sulfate-reducing bacteria harboured in anaerobic granular sludges [1-2]. Anaerobic granular sludges are specific anaerobic biofilms, which are dense conglomerates of microorganisms, minerals and microbial produced compounds like extracellular polymeric substances (EPS). The microbiological and chemical composition of the granules depends on the seed sludge and

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the chemical composition of the wastewater treated [3-4]. It is well known that such type of biofilm harbour poorly crystallised iron sulfide precipitates, but the overall sulfur (bio)chemistry in such type of biofilm is inadequately described in the literature.

A priori, the near-edge region of the X-ray absorption spectrum is dominated by dipole-allowed bound-state transition of the 1s electron (for a K-edge- to vacant molecular orbital with substantial p-character). The near-edge spectrum thus provides a sensitive probe of electronic structure and hence of the chemical form [5]. Sulfur exhibits particularly rich K-near-edge spectra due to relatively sharp line widths and large chemical shift range and is thus a very useful tool –although difficult- for speciating S in complex biological samples [6]. XANES rather probes the electronic structure around S that is known to vary extensively, in a way that conventional “redox states” cannot describe well (see [6]).

In this study we compare the speciation of sulfur in several anaerobic granular sludges samples. In addition, S K-edge XANES spectra were collected for a large variety of model compounds to find consistencies in the XANES that will be used to model each valence state of S in the sludge samples. We studied a number of sulfate and sulfite compounds but also many sulfides that have not been studied systematically in the past. In the present work, we explore the utility of sulfur K-edge X-ray absorption spectroscopy as a probe of sulfur chemistry in intact anaerobic biofilms, and show that it can, with appropriate analysis, be used to qualitatively identify sulfur metabolites in biofilm without any chemical handling of the sample.

2. Samples and methods

We studied the speciation of sulfur in samples of 3 different types of anaerobic granular biofilms treating synthetic wastewaters, contaminated with traces of selenate (see Figure 2 left). One sample was obtained from a methanogenic bioreator [4], another one from a sulfate-reducing bioreator [4], and the last from a nitrate-reducing bioreator [7]. Because of the complexity of S speciation in the studied biofilm, XANES data from several synthetic and natural model compounds were used to help interpret XANES data from biofilm. Special care was taken to select unoxidized sulfide models. For this, we studied 3-4 samples (from the MNHN) for each sulfide species (more than 120 samples studied) and made sure the spectra were free of sulfate absorption at 2482 eV. X-ray absorption near edge structure (XANES) data were recorded at the LUCIA undulator beamline [8] of the SOLEIL facility located at the Swiss Light Source (SLS, Paul Scherer Institut, Switzerland). A Si(111) double-crystal monochromator was used to produce monochromated x-ray beam that was focused down to 5 x 10 µm² by two Kirkpatrick-Beaz type mirrors. Harmonics were rejected using Ti-coated mirrors. Flux on the sample is 2 x 10¹² ph/s at 400 mA injected currents at 2 keV. The samples were placed

Figure 1: (left) S K-edge X-ray absorption peak energy vs. the “oxidation state” of S for model compounds used; (middle) S K-edge spectra of some selected models; (right) same for sulfides.
inside a chamber under primary vacuum and oriented normal to the x-ray beam. The spectra were collected in fluorescence mode using a dispersive Si-drift (SDD) chamber solid-state detector. The monochromator energy was calibrated at the sulfur K-edge, using elemental sulfur ("white line" maximum set at 2472 eV). µXRF maps were collected at 2.5 keV with 1 sec/pt and down to 5x5 µm pixels. µXANES spectra were collected in the 2400-3200 eV energy range, with 0.2 eV steps. Energetic resolution is 2500 at 2 keV while lateral resolution was about 3x3 µm. XANES spectra were normalized following standard procedures, using the XAFS 3.1 package [9].

3. Results and discussion
The edge energy (±0.2 eV) for the most intense transition for the present set of model compounds was plotted as a function of the apparent “oxidation state” of sulfur (Fig. 1a). XANES spectra of the standard compounds are shown in Fig 1b. As previously reported by many previous authors [5-6, 10-11], there is a 12 eV shift between the absorption edges of the “–2” and “+6” oxidation states. In addition, there is also a shift towards higher energy with decreasing metallic/covalent bonding within the S₂⁻ compounds. The position of the S K-edge for sulfides varies extensively (Fig. 1c), from 2469.5 eV for bornite (Cu₅FeS₄) to 2473.2 eV to sphalerite (cubic-ZnS). Based on the present extensive set of mineral sulfides probed in this study, we confirm that the edge energy is not just a simple function of valence state alone (see also [6] for organic sulfides). Thus, we identified 3 main categories of sulfides: (1) the ones with an apparent “–2 oxidation state” (such as bornite and chalcopyrite, CuFeS₂); (2) those with an apparent “–1 redox state” of S (as in pyrite, FeS₂), while proustite (semiconductor, Ag₃AsS₃) and sphalerite show much higher edge energies (2471-2473 eV). Because of that variance, it was difficult to choose a peculiar model compound of S that is representative of those bio-inorganic samples. Therefore, no quantitative models were attempted.

Figure 2: (left) scanning electronic pictures of a sludge (top) and a detail (bottom); (middle) S K-edge spectra for a methanogenic biofilm, showing the extent of photoreduction through 4 successive scans; (right) S K-edge for three different anaerobic biofilms cultivated in different microbial conditions.

Figure 2 (middle) shows the extent of the photoreduction processes that can affect the speciation of sulfur species in our organic samples. This effect was obvious as the samples were darkening on the x-ray spot impact. Accordingly, the relative intensities of the peak related to sulfates decreased with time. We observed this phenomenon in details in a companion paper (Farges and Keppler, this issue). Figure 2 was used to constraint the data collection within 10 minutes to minimize photoreduction artefacts. Figure 2 (right) shows the normalized S K-edge spectra obtained for various biofilms. Each spectra is the average of 2 scans, with minimal photoreduction. When a sample displays sulfur moieties with several oxidation states, it is challenging to determine the quantitative speciation of sulfur by least linear square fitting. This is due to the extent of S-speciation that S can adopt (resulting, hence, in highly variable S K-edge XANES spectra). This is why we will present only qualitative sulfur moieties assignments, performed based on the peak position compared to model compounds of sulfur. Figure 3 shows a typical S Kα XRF map collected for a sulfate reducing biofilm. We compared
this peculiar biofilm to the relevant compounds such as chalcopyrite and troillite (sulfide, $S^2$), methionine and cysteine (organic sulfide, $S_{\text{org}}$) and calcium sulfate (oxidized sulfur compounds, $S^{6+}$). For each “redox state” of $S$, each of these species seem to be equivalent, in the lack of more resolved XANES spectra. The same strategy was applied to a biofilm exposed to nitrate-reducing conditions. The speciation of $S$ in the nitrate-reducing biofilm is clearly dominated by sulfide-type moieties, whereas the extent of bioreduction is less advanced in the sulfate-reducing biofilm. In both biofilms, organic sulfides are never the dominant $S$-species: they are even nearly absent in the nitrate-fed sludges. This result, despite qualitative, cannot be obtained with any other method at this $\mu$-scale.

4. Conclusion
The present study confirms that attributing a specific valence to most sulfides is challenging as we measured a continuum of edge shifts from sulfur $"-2"$ to $"-1"$, depending on the electronic structure of $S$ in the probed sulfides. In the sludges, various sulfur hot spots were probed, despite photo-reduction is a problem that was monitored carefully. We « indexed » the main features of those complex S K-edge XANES spectra as compared to sulfide and sulfate moieties. Organic sulfur compounds were also shown to contribute significantly to the S K-edge XANES spectra in methanogenic and sulfate-reducing biofilms but their exact identification still needs a more comprehensive S K-edge XANES study. However, the preset $\mu$XANES information provides major information: some sludges have clearly most of their sulfur content as insoluble sulfides (such as nitrate-reducing biofilms) whereas others (sulfate-reducing biofilms) are have other reduced sulfur compounds that could not yet be identified under our current experimental set-up.

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Figure 3: (left) S-K$\alpha$ $\mu$XRF map for a sulfate reducing biofilm and example of spots probed by $\mu$XANES (middle) with a qualitative sulfur species assignment for a sulfate-reducing biofilm, based on model compounds; (right) same as previously, for a nitrate-reducing biofilm.