



Sulfur K-edge XANES and acid volatile sulfide analyses of changes in chemical speciation of S and Fe during sequential extraction of trace metals in anoxic sludge from biogas reactors

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ABSTRACT

The effect of sequential extraction of trace metals on sulfur (S) speciation in anoxic sludge samples from two lab-scale biogas reactors augmented with Fe was investigated. Analyses of sulfur K-edge X-ray absorption near edge structure (S XANES) spectroscopy and acid volatile sulfide (AVS) were conducted on the residues from each step of the sequential extraction. The S speciation in sludge samples after AVS analysis was also determined by S XANES. Sulfur was mainly present as FeS (~60% of total S) and reduced organic S (~30% of total S), such as organic sulfide and thiol groups, in the anoxic solid phase. Sulfur XANES and AVS analyses showed that during first step of the extraction procedure (the removal of exchangeable cations), a part of the FeS fraction corresponding to 20% of total S was transformed to zero-valent S, whereas Fe was not released into the solution during this transformation. After the last extraction step (organic/sulfide fraction) a secondary Fe phase was formed. The change in chemical speciation of S and Fe occurring during sequential extraction procedure suggests indirect effects on trace metals associated to the FeS fraction that may lead to incorrect results. Furthermore, by S XANES it was verified that the AVS analysis effectively removed the FeS fraction. The present results identified critical limitations for the application of sequential extraction for trace metal speciation analysis outside the framework for which the methods were developed.

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1. Introduction

Sequential extraction methods are widely used for characterization of chemical forms or fractions of metals in samples from soil, sediment, and sludge. Reagents, such as solutions of neutral salts, weak and strong acids as well as reducing and oxidizing compounds, are selected to be as specific as possible toward solubilization of metal binding fractions. Thus, it is assumed that the reagents extract specific forms of metals in a mixture of metal species [1,2]. The reagents are applied in a successive manner with increasing degree of reactivity. Accordingly, metals released after the extraction steps are attributed to operationally defined fractions ranging from loosely bound to strongly bound forms [1–4]. Attribution of the specified fractions to certain chemical forms or

phases of metals has uncertainties associated with selectivity of the reagents and additive effects caused by reactions occurring in a mixture of metal forms and sequentially added reagents [5].

Trace metals are important micronutrients for growth and activity of microorganisms in biogas reactors in which organic content of the influent material is biologically converted to methane and carbon dioxide under anaerobic conditions [6–10]. Processes regulating the chemical speciation and bioavailability of trace metals are complex involving dynamic interaction of microorganisms with organic and inorganic compounds [11,12]. Laboratory and practical evidences have demonstrated that speciation and bioavailability of trace metals essential for the anaerobic digestion process are highly influenced by the chemistry of sulfur (S) [11–15]. The precipitation of metal sulfide phases and/or adsorption of trace metals to iron sulfide particles are considered to be the most important processes contributing to trace metal dynamics in biogas reactors. The sequential extraction methods are widely used for analysis of trace metal fractionations in sludge from biogas reactors e.g. [16–19], but possible changes in the S chemistry during sequential extractions, and how they may affect operationally defined trace metal fractions, have not been investigated previously for sludge samples from biogas reactors. In a parallel study, we observed that Co

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(but not Ni) was encountered in the residual fraction (after extraction of organic/sulfide bonded trace metals) of a biogas sludge together with Fe (Gustavsson et al., unpublished). The question arose whether or not this was due to an artifactual formation of an Fe-phase during the extraction procedure, to which Co was associated.

The objective of the present study was to determine the chemical speciation of S in sludge from biogas reactors, and to investigate possible changes in the chemical speciation of S and Fe during a sequential extraction procedure used for trace metal speciation. The feasibility of three different sequential extraction procedures used for analysis of trace metal fractionation in anaerobic granular sludge was compared by Hullebusch et al. [17]. They suggested that a modified version of the method developed by Tessier et al. [1] as described by Osuna et al. [4], has a better reproducibility and reagent selectivity compared to other sequential extraction methods. We used sulfur K-edge X-ray absorption near edge structure (S XANES) spectroscopy and acid volatile sulfide (AVS) extraction as analytical tools to quantify changes in S speciation during sequential extraction scheme of the modified Tessier method [1,4].

Sulfur K-edge XANES is a suitable method for identification and quantification of various S compounds, their oxidation states and binding forms in a sample [20,21], which allow us to follow the fate of S species during sequential extraction. Acid volatile sulfide analysis is a classical approach for measurement of the sulfide fraction reactive toward metals in anoxic samples, based on volatilization of reduced S bonded in mineral fractions, mainly FeS [12,22,23]. The method has in principal similar disadvantages as sequential extraction regarding the uncertainty in selectivity of the applied reagent for volatilization of reduced S compounds and simultaneously extracted metals [22]. To analyze possible changes in S forms during AVS extraction, we used S XANES to compare the S speciation before and after AVS analysis. To our knowledge, this is the first study quantifying changes in the S chemistry using S XANES, during sequential extraction of metals and before and after AVS extraction. Thus, this study will contribute to a better understanding of the accuracy of sequential extraction of trace metals, as well as AVS analysis, in general and their indirect effects on trace metal speciation in sludge samples from biogas reactors in particular.

2. Material and methods

2.1. Sample sources

Samples were taken from two lab-scale biogas reactors semi-continuously fed with grain stillage. The reactors, designated as J2 and J4, were operated at 37 °C with a hydraulic retention time (HRT) of 20 days and organic loading rates (OLR) of 2.5 and 4.0 g L⁻¹ d⁻¹ volatile solid (VS), respectively. The only difference between J2 and J4 is their different OLRs. The reactors were supplemented with 0.5 mg Co L⁻¹ d⁻¹ (as CoCl₂), 0.2 mg Ni L⁻¹ d⁻¹ (as NiCl₂) and 0.5 g Fe L⁻¹ d⁻¹ (as a solution with Fe(III):Fe(II) ratio of 2:1). Trace metals were added in order to provide the microbial community with micronutrients. Iron was supplemented to precipitate inorganic sulfide and decrease the H₂S concentration in biogas. For a detailed description of the operational conditions and rationale for trace metal and Fe addition, the reader is referred to Gustavsson et al. [10].

2.2. Sequential extraction

Bulk sludge samples were transported to an anaerobic box (Vinyl Anaerobic Airlock Chamber type Coy, Coy Laboratories Inc., USA) containing gas composition of CO₂ (40%), N₂ (55%), and H₂ (5%). The CO₂ was introduced into the chamber in order to simulate

the headspace of biogas reactor and avoid degassing of dissolved CO₂ from sludge liquid when exposed to box atmosphere, which otherwise may result in a pH change of the sludge. The H₂ was used in order to remove traces of oxygen in contact with located palladium catalyst in the anaerobic box. Sub-samples were transferred into polypropylene tubes (Falcon, Sarstedt, Germany) and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and its Fe concentration was measured by atomic absorption spectroscopy (AAS, 1100 Atomic Absorption Spectrophotometer, Perkin Elmer, USA). Sub-samples corresponding to a dry mass of 0.18–0.30 g were used for sequential extraction of modified Tessier following the protocol by Osuna et al. [4]. Total solid (TS) content of the samples was measured according to Swedish Standard method (SS-028113) by drying the samples at 105 °C for 20 h.

A summary of the sequential extraction procedure is given in Table 1, beginning with a centrifugation of sludge and analysis of metals in the sludge liquid fraction. Results on trace metals such as Ni and Co will be reported in Gustavsson et al. [unpublished]. Here we only report Fe (as determined by AAS) after each sequential extraction step. The solid residue after each step was washed with deaerated Milli-Q water, centrifuged and the Fe concentration in the supernatant was measured in order to check possible Fe release from the solid phase during the washing step. After decanting the water, the reagent for extraction of the next fraction was added to the tubes. The same procedure was applied for all extraction steps. The two first fractions (i.e. exchangeable and carbonate) were extracted inside the anaerobic box to avoid exposure of the samples to air and oxidation of reduced S species. The residual fraction, i.e. last step of the sequential extraction, was extracted according to Swedish Standard method (SS-028311) for extraction of metals from sludge by 7 M nitric acid in an autoclave (at 120 °C for 30 min). The total S content of the samples was analyzed by Eurofins Environment Sweden AB (Lidköping, Sweden).

2.3. Acid volatile sulfide (AVS)

The AVS method presented by Hsieh and Yang [24] and modified by Brouwer and Murphy [25] and Leonard et al. [26] was applied in the present study. The AVS extraction was performed in the anaerobic box to avoid oxidation of sulfide due to exposure to air. In summary, 0.18–0.30 g TS of sludge was transferred into a 250 mL glass bottle with a vial attached to its inner wall. The vial was filled up with 10 mL of sulfide antioxidant buffer (SAOB) solution containing 2 M NaOH, 0.1 M ascorbic acid and 0.1 M EDTA. 25 mL of 1 M HCl acid was added to the glass bottles. The bottles were capped and the solid suspension was stirred by a magnetic stirrer for 1 h. The addition of HCl results in volatilization of reduced, inorganic S compounds of the solid phase which is trapped by the SAOB solution in the vial attached to the inner wall of the reaction bottle. After 1 h, the liquid fraction was centrifuged and its Fe concentration was measured using AAS. The concentration of sulfide in SAOB solution (AVS) was measured using a sulfide sensor (Ag/S 800 Wissenschaftlich-Technische Werkstätten, Germany). The sensor was calibrated with 0.1 M Na₂S·9H₂O diluted in SAOB solution to sulfide concentrations ranging from 10⁻⁵ to 10⁻² M, which covered all the measured AVS concentrations. For more information about the details of AVS experimental setup the reader is referred to Brouwer and Murphy [25] and Leonard et al. [26].

2.4. Quality control of extraction procedures

The extraction reagents were prepared using Milli-Q water purged with N₂ to degas dissolved oxygen. All the chemicals were of analytical reagent grade. Glassware was washed with acid (7 M nitric acid for sequential extraction and 10% hydrochloric acid for AVS analysis) for 48 h and rinsed with Milli-Q water at least three

Table 1
Summary of modified Tessier procedure for the sequential extraction of trace metals [1,4].

Extraction step	Reagent	Volume of reagent (ml)	Conditions
Sludge liquid phase	–	–	Centrifugation, 10,000 rpm, 10 min, inside the anaerobic box
Exchangeable fraction	1 M ammonium acetate	10	pH 7, shaken for 1 h, 150 rpm, 20 °C, inside the anaerobic box
Carbonate fraction	1 M sodium acetate	10	pH 5.5, shaken for 1 h, 150 rpm, 20 °C, inside the anaerobic box
Organic/sulfide fraction	30% hydrogen peroxide	5	pH 2 (adjusted by nitric acid), shaken for 3 h, 150 rpm, 37 °C
Residual fraction	7 M nitric acid	20	Autoclaved for 30 min, 120 °C

times. The tubes and glass bottles were stored in the anaerobic chamber two days before analysis to ensure strict anaerobic conditions. Sequential extraction and AVS was performed on triplicate samples. Furthermore, sulfide was spiked in Milli-Q water purged with N₂ and AVS analysis was performed in order to check the recovery of the analysis as well as the possible oxidizing effect of remaining traces of dissolved oxygen in reagents on AVS. Spiked and blank samples were analyzed to detect possible matrix interferences and contamination sources. The sulfide sensor was calibrated after every six measurements in order to correct for drift of the sensor signal.

2.5. Sulfur K-edge XANES data collection and analysis

The S XANES analysis was performed at the semi-conductive wiggler beamline I811 at MAX-lab, Lund University, Sweden. The monochromator consists of crystal pairs of Si(1 1 1) with a wavelength resolution of 0.25 eV at the S K-edge energy (2472 eV). Sludge samples were dried in an anaerobic box filled with Ar. After grinding with mortar and pestle, the dried sample powder was pressed on S-free tape to form a thin film in order to minimize self-absorption effects [27]. Samples were covered with S-free plastic foil to avoid exposure to air during transport from the anaerobic box to the sample chamber, which was continuously purged with He gas. The S XANES spectra were recorded in fluorescence mode using a PIPS detector at room temperature. Samples were typically scanned by incident X-ray energy over the range of 2450–2500 eV using a quick scan mode. Average spectra consist of 3–10 individual scans. From each averaged spectrum a polynomial pre-edge function was subtracted and data were normalized between 2460 and 2490 eV using software WinXAS97 [28]. The energy was reported relative to elemental S (2472.7 eV), setting the maximum of the Na₂S₂O₃ first peak at 2472.0 eV [29].

Microsoft Excel was used to deconvolute the S XANES spectrum for each sample into pseudo-components by a least-square fitting procedure. We used a modified version of the method reported by Xia et al. [20]. In short, s → p transitions of S atoms in FeS, FeS₂, elemental S, cysteine (RSH), methionine (RSR), cystine (RSSR), sulfonate and sulfate, occurring between 2470 and 2480 eV, were modeled by Gaussian peaks. Peak energy maxima and standard deviations for the Gaussian curves of the model compounds are reported as supplementary data (Table S1). One Gaussian peak was used to fit the sum of RSH and RSR due to the proximity of their energy maxima which are within the beam energy resolution of 0.25 eV. Two Gaussian peaks were used to fit the double cysteine (RSSR) peak. The increase in background caused by the transition of ejected photoelectrons to the continuum was modeled by arctangent step functions located at 0.8 eV above each peak maximum of FeS, FeS₂, elemental S, the sum of RSH and RSR, sulfonate and sulfate. The post-absorption-edge pattern of the reduced S species, FeS, FeS₂, elemental S, RSH, RSR and RSSR, occurring in the energy range of 4.0–8.0 eV relative to elemental S, was modeled using two Gaussian peaks, one representing the sum of RSH and RSR and one representing the sum of FeS, FeS₂, elemental S and RSSR. The absolute concentrations of the S components were calculated by multiplying the relative S composition with the total

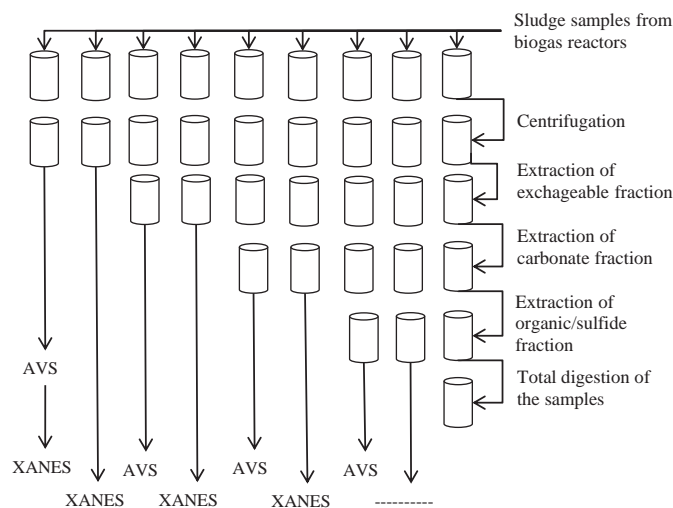


Fig. 1. Experimental protocol applied for S XANES and AVS analyses on sludge samples from J2 and J4 biogas reactors and on solid residues from each step of the sequential extraction. The arrows show the flow of sludge samples through the experimental procedure.

S concentration of the sample, after correction of the absorption cross-section for the oxidation state [20]. Because of peak broadening caused by self-absorption effects and the proximity of FeS₂ and elemental S peaks, the sum of these two compounds and possible polysulfides are reported as zero-valent S.

2.6. Experimental protocol

Fig. 1 shows scheme of the experimental procedure used in S XANES and AVS analyses. The bulk sludge samples taken from biogas reactors (J2 and J4) were divided into 20 tubes. The samples were centrifuged and the liquid fraction was decanted. Three tubes were selected for AVS analysis and one was kept sealed and stored at –80 °C for the S XANES analysis. The residue remaining after AVS extraction was immediately frozen and stored at –80 °C for S XANES analysis. After each step of the sequential extraction procedure the residue in three tubes was selected for AVS analysis, and one tube was immediately frozen at –80 °C for S XANES analysis. The variation in AVS concentration determined in the three replicate tubes after each step of sequential extraction was evaluated by Student's *t*-test. Due to coherent structure of the residues from organic/sulfide fraction, it was not possible to grind them with the mortar and the pestle in order to provide homogeneous powder for the S XANES analysis and therefore, their S XANES spectra were not recorded.

3. Results and discussion

3.1. Sulfur K-edge XANES

3.1.1. Sulfur speciation in the solid phase of the original sludge

Linear least-square fits of S XANES spectra from the sludge solid phases of the biogas reactors, as well as solid residues after each

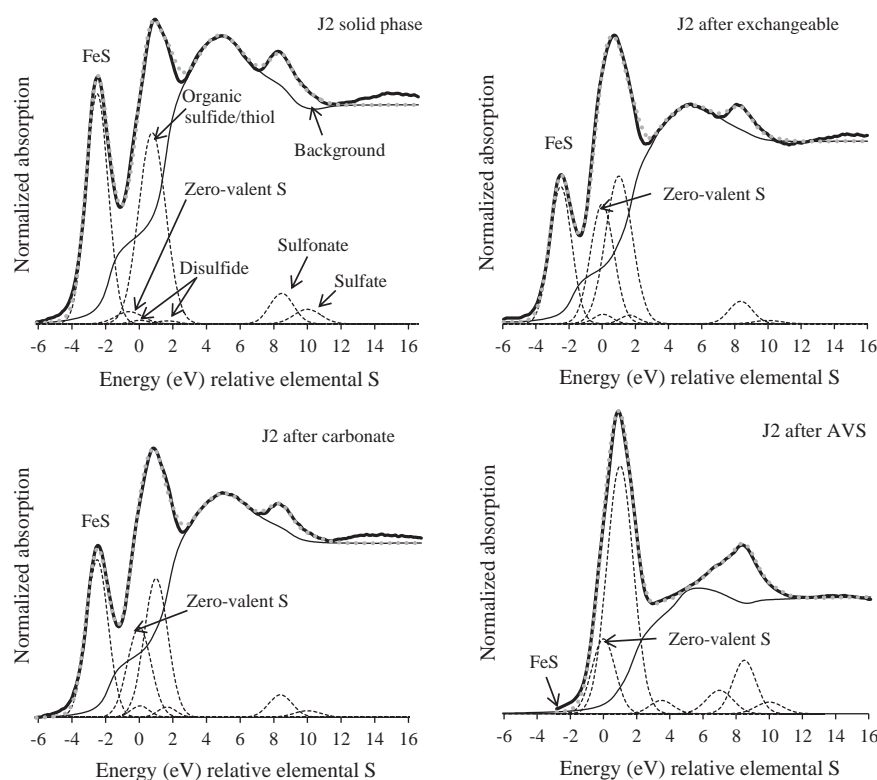


Fig. 2. Normalized S XANES spectra of the original solid sludge from the J2 reactor and solid residues after each step of sequential and AVS extraction (thick, solid line). Gaussian distribution functions, each representing a model compound, are shown by dashed lines. Overall model fits are represented by the grey dotted line and the thin, solid line represents the background.

step of sequential extraction and AVS analysis are shown in Fig. 2 (reactor J2) and Fig. S1 (reactor J4). The spectra were quite similar for both reactors, and are composed of several maxima in the XANES region, representing multiple oxidation states of S. The peak with a maximum at approximately -2.6 eV relative to elemental S is FeS, and the peak occurring at approximately 1 eV relative to elemental S represents reduced organic S (i.e. the sum of organic sulfides, organic disulfides and thiols). Two groups of oxidized S representing sulfonate and sulfate with peak energies at 8.3 and 10 eV relative to elemental S, respectively, are also observed. Sulfonate is an organic compound, and sulfate likely is represented by sulfate esters (carbon-bonded sulfate). The peak at approximately 4.5 eV is the post-edge background caused by the sum of reduced S species.

Seven Gaussian distribution functions were used to model the S XANES spectra of the solid fraction of sludge from J2 and J4 reactors. Energies for peak maxima and standard deviations of the model compound Gaussian curves are reported as supplementary data (Table S1). The percentage contribution from each model compound to total S content of the sludge solid phase of J2 and J4 are reported in (Table 2). Iron sulfide (FeS) accounted for 62% and 63% of total S in J2 and J4, respectively. The grain stillage used as reactor feed, was provided from downstream of a bioethanol production plant in which sulfuric acid is applied for pH adjustment during the process. Therefore, the feed material of the studied biogas reactors is initially rich in inorganic oxidized S. During the anaerobic digestion process, the oxidized S of the feed material is reduced, resulting in production of mainly sulfide [30]. The laboratory reactors were supplemented with Fe (as a solution with a Fe(III):Fe(II) ratio of 2:1 at a rate of $0.5 \text{ g Fe L}^{-1} \text{ d}^{-1}$) in order to decrease the $\text{H}_2\text{S}(\text{g})$ concentration in the biogas. Added Fe(III) is reduced (chemically or by iron reducing-bacteria), and FeS(s) is precipitated.

The sum of organic sulfide and thiol was 32% and 29% of total S in the solid fraction of sludge of J2 and J4, respectively. Sulfur-containing amino acids and peptides are major compounds contributing to these S forms. Part of the organic sulfide/thiol may be the result of sulfurization processes occurring at high sulfide concentrations, when reactive sites of degraded organic particles are “quenched” by inorganic sulfide under the formation of thiol functional groups [31,32]. Thiol groups in turn react to form mono- and disulfidic ($-\text{S}-$ and $-\text{S}-\text{S}-$) bridges in organic compounds [33]. Despite daily supplementation of Fe to the reactors, the concentration of Fe in the liquid fraction was below the detection limit (precipitated as FeS). In contrast, inorganic sulfide was available in the liquid phase, as well as in the gaseous phase with concentrations of $\text{H}_2\text{S}(\text{g})$ reaching 0.3% and 0.4% in the headspace of J2 and J4 reactors, respectively. Therefore, sulfide was in excess and could take part in the sulfurization of labile organic compounds. It is noteworthy that the S XANES results also reflect the presence of biologically inherited S-containing organic compounds. Due to the small difference in absorption energy and possible peak broadening caused by self-absorption effects, it is not possible to distinguish between the contribution from small concentrations of pyritic S (FeS₂), elemental S and polysulfides. The fitting of a single Gaussian peak as representative for the zero-valent S demonstrated that approximately 2.6% and 4.4% of total S was present as pyrite, elemental S, polysulfides or a mixture of these compounds in J2 and J4, respectively. The overall contribution of organic, oxidized S (mainly sulfonate and ester-sulfate) was approximately 3% in J2 and 2% in J4.

3.1.2. Changes in sulfur speciation during sequential extraction

The recorded spectra for the sludge solid phase, and solid residues after extraction of exchangeable and carbonate fractions are stacked in Fig. 3 for comparative purposes and data of the model fits are reported as absolute concentrations (mg S g TS^{-1}) in Fig. 4.

Table 2
Distribution of different S-forms (% of total S) in the sludge solid phase of J2 and J4 and in solid residues after sequential extraction steps and AVS on untreated sludge samples, as determined by S XANES. Sulfate was likely dominated by sulfate esters. Zero-valent S may include some pyrite (FeS_2). n.d. means not-detected. Merit-of-fit represents percentage of the data which cannot be explained by the model.

	J2 solid phase	J2 after exchangeable	J2 after carbonate	J2 after AVS	J4 solid phase	J4 after exchangeable	J4 after carbonate	J4 after AVS
FeS	61.7	43.4	52	n.d.	63	45.5	41.4	6.7
Zero-valent S	2.6	23.8	18.4	20	4.4	23.8	25.7	24.4
Organic sulfide/thiol	31.9	28.2	24	66.8	28.7	22.3	28.1	55.8
Disulfide	<1.0	2.5	3.1	n.d.	2.8	7.5	3.6	n.d.
Sulfoxide	n.d.	n.d.	n.d.	2.4	n.d.	n.d.	n.d.	3.8
Sulfone	n.d.	n.d.	n.d.	3.4	n.d.	n.d.	n.d.	2.7
Sulfonate	2	1.8	1.9	6.1	<1.0	<1.0	1.2	5
Sulfate	<1.0	<1.0	<1.0	1.3	<1.0	n.d.	n.d.	1.6
Merit-of-fit ^a	4.3	3.9	4.4	4.5	8.9	4.2	3.5	3.0

$$^a \text{Merit-of-fit} = 100[\sum(\text{normalized absorption}_{\text{measured}} - \text{normalized absorption}_{\text{fit}})^2] / \sum(\text{normalized absorption}_{\text{measured}})^2.$$

For these calculations, it was assumed that the total S concentration of solid residue after the exchangeable and carbonate extraction steps remained unchanged within errors of measurements as compared to the total S concentration in the original solid phase of the sludge ($16 \text{ mg S g TS}^{-1}$ for J2 and $13 \text{ mg S g TS}^{-1}$ for J4). The S XANES results show that during the first step of the sequential extraction (i.e. for the exchangeable fraction) approximately 20% of total S is transformed to zero-valent S in samples from both reactors (increase in zero-valent S concentrations from 0.4 to $3.8 \text{ (mg S g TS}^{-1})$ for J2 and from 0.6 to $3.1 \text{ (mg S g TS}^{-1})$ for J4, Fig. 4). The increase in zero-valent S roughly corresponds to the decrease in FeS. Thus, considering a relative error of about $\pm 5\text{--}10\%$ in the quantification of the different S-forms by S XANES, it is concluded that the major change taking place during the extraction of the exchangeable fraction is an oxidation of FeS to zero-valent S (likely elemental or polysulfidic S). Pyrite is slowly formed by partial oxidation of FeS in natural environments such as sediments [34], but it is highly unlikely that the short time used in the sequential extraction procedure would be enough for formation of pyrite.

Because Fe was not detected in the solution, neither after the exchangeable nor after the carbonate extraction step, the transformation of FeS to zero-valent S did not result in a mobilization of Fe from the solid phase. Thus, Fe released upon the oxidation of FeS can be assumed to have reacted with the remaining sludge solid matrix e.g. organic compounds [35]. The change in speciation of Fe caused

by the exchangeable extraction step, will directly affect the results of Fe fractionation, and indirectly the fractionation of other trace metals during the following sequential extraction steps. In both J2 and J4 samples, the concentration of reduced organic S forms decreased slightly after extraction of the exchangeable fraction. Interestingly, the changes in the concentration of FeS, elemental S and organic forms of S caused by the exchangeable extraction step, remained largely unaffected during the subsequent carbonate extraction step (Fig. 4).

3.1.3. Changes in sulfur speciation after AVS

The relative concentrations of S components in the solid phase of the sludge and in the residue after AVS extraction are compared in Table 2, and the absolute concentrations in Table 3. The total S concentration in residues after the AVS extractions was calculated by subtracting the measured AVS from the total S concentration in J2 and J4. During the AVS extraction, addition of 1 M HCl to the sludge samples volatilized reduced inorganic S. No FeS remained in J2 samples after AVS extraction, as shown by removal of the first peak (at approximately -2.6 eV relative elemental S representing FeS) in the S XANES spectrum (Fig. 3). However, in the J4 sample a small shoulder remained at approximately -2.6 eV relative elemental S, corresponding to $0.3 \text{ mg S g TS}^{-1}$, suggesting that the added HCl was not enough to reach a complete volatilization of FeS in this sample (Fig. 3). It is also noted in Table 3 that the AVS extraction step resulted in a partial transformation of S to

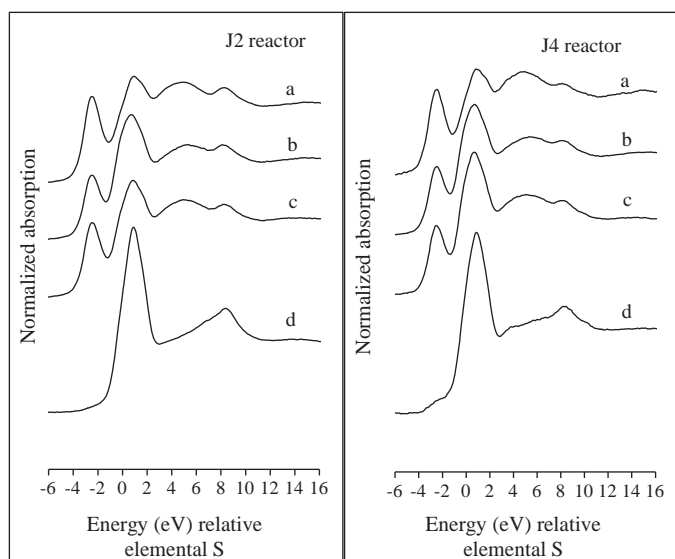


Fig. 3. Comparison of normalized S XANES spectra of the solid sludge (a), solid residue after the extraction of exchangeable fraction (b), carbonate fraction (c), and AVS (d) for the J2 and J4 reactors. For clarity the spectra are shifted along the y-axis (arbitrary units).

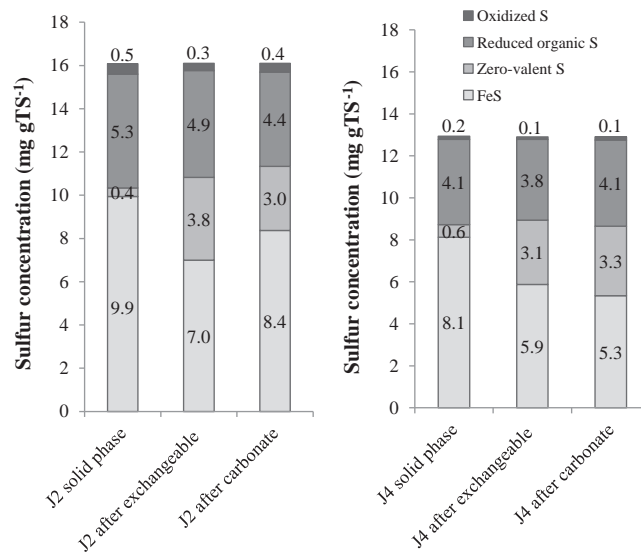


Fig. 4. Concentration of major sulfur forms in the original sludge from the J2 and J4 biogas reactors, as well as in the residues after exchangeable and carbonate extraction steps as determined by S XANES.

Table 3

Absolute concentrations of S components (mg S g TS^{-1}) in the sludge solid phase of samples from the J2 and J4 biogas reactors, and in residues after AVS extraction as determined by S XANES.

	J2 solid phase	J2 after AVS	J4 solid phase	J4 after AVS
FeS	9.9	–	8.1	0.3
Zero-valent S	0.4	1.6	0.6	1.1
Organic sulfide/thiol	5.1	5.3	3.7	2.5
Disulfide	0.2	–	0.4	–
Sulfoxide	–	0.2	–	0.2
Sulfone	–	0.3	–	0.1
Sulfonate	0.3	0.5	0.1	0.2
Sulfate	0.2	0.1	0.1	0.1

Table 4

Acid volatile sulfide (AVS) and simultaneously extracted Fe (AVS-Fe) concentrations (mg g TS^{-1}) in residues after each sequential extraction step (mean \pm standard deviation, $n=3$).

	J2-AVS-Fe	J4-AVS-Fe	J2-AVS	J4-AVS
Sludge solid phase	11 \pm 0.2	12 \pm 0.4	7.9 \pm 0.0	8.4 \pm 0.7
After exchangeable	11 \pm 0.1	12 \pm 0.4	6.8 \pm 0.3	7.2 \pm 0.0
After carbonate	11 \pm 0.3	11 \pm 0.1	7.0 \pm 0.3	6.7 \pm 0.3
After organic/sulfide	2.6 \pm 1.3	3.2 \pm 0.7	0.0 \pm 0.0	0.0 \pm 0.0

zero-valent S (an increase from 0.4 to 1.6 mg S g TS^{-1} in J2 and from 0.6 to 1.1 mg S g TS^{-1} in J4). In the J4, the 1 M HCl in the AVS extraction seems to have attacked some thiols or organic sulfides (decrease from 3.7 to 2.5 mg S g TS^{-1}). Small increases in sulfoxide and sulfonate is insignificant, and within the error of the method used to deconvolute the S XANES spectrum.

In the J4 sample, the concentration of FeS determined by S XANES (8.1 mg S g TS^{-1}) was well in agreement with the AVS determinations (8.4 mg S g TS^{-1}) in the original sludge. However, for the J2 sample there was a discrepancy between FeS, as determined by S XANES (9.9 mg S g TS^{-1}) and AVS (7.9 mg S g TS^{-1}). The reason for this is unclear. The FeS fraction as determined by S XANES comprises a consortium of possible iron monosulfides with slightly different ordering (microcrystallinity) and stoichiometry. Mackinawite (microcrystalline FeS), pyrrhotite (Fe_{1-x}S) and greigite (Fe_3S_4 , an intermediate in the formation of pyrite from mackinawite) are possible phases, which may occur in the sludge. While these forms may be included in the model peak for FeS in the XANES spectrum, AVS analysis is not equally efficient as an extractant of these FeS minerals. Rickard and Morse [22] demonstrated that the extraction efficiency of 1 M HCl for synthetic mackinawite, and synthetic greigite was 92% and 40–67%, respectively.

3.2. Changes in AVS and simultaneously extracted Fe during sequential extraction

Despite some differences in absolute concentrations, the relative changes in S speciation observed after the exchangeable extraction step by S XANES (Fig. 4) were also noted in the AVS analysis of both J2 and J4 samples (Table 4). Results of *t*-tests on the measured AVS for every step of sequential extraction showed that the AVS fraction decreased significantly ($p < 0.05$) after the exchangeable step during the extraction of exchangeable fraction. Thus, the AVS analysis also confirmed that during the extraction of the exchangeable fraction some of the iron monosulfides were oxidized (to elemental S or other forms of zero-valent S), and were not volatilized as AVS. No statistically significant change in the AVS concentration did occur during the carbonate extraction. Because no Fe was released into solution during oxidation of FeS to zero-valent S in the exchangeable step, it is suggested that Fe was adsorbed to the sludge matrix and/or a secondary phase of Fe was formed. In contrast to AVS, the concentration of simultaneously extracted Fe

(AVS-Fe) did not change after neither the extraction steps of the exchangeable nor carbonate fractions (Table 4). Thus, the AVS-Fe extraction by 1 M HCl was powerful enough to release not only FeS, but also secondary phases of Fe formed during this step (as well as all other extraction steps).

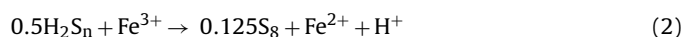
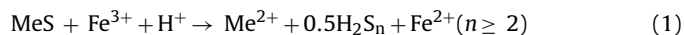
After the extraction of the organic/sulfide fraction by 30% hydrogen peroxide at pH 2.0, no AVS was detected, which was expected due to oxidation of sulfides by the hydrogen peroxide. However, still 2.6 and 3.2 mg Fe g TS^{-1} was released in the AVS extraction from the solid residues of J2 and J4, respectively (Table 4). This suggests a formation of a secondary Fe phase during extraction of organic/sulfide fraction by the hydrogen peroxide at low pH. Considering the low pH (2.0) and high concentrations of sulfate (by oxidation of sulfide), jarosite ($\text{H, K, Na} \text{Fe}_3(\text{OH})_6(\text{SO}_4)_2$ (s)) is a much possible secondary precipitate, but also schwertmannite $\text{Fe}_8\text{O}_8(\text{OH})_6\text{SO}_4$ (s) may form if pH is raised above 2.5 during reaction between the extraction solution and the sludge [36].

3.3. Possible Fe-involving reactions during the extraction step of the exchangeable fraction

The results of the S XANES analysis (Fig. 4) and the AVS (Table 4) extractions revealed a partial oxidation of iron monosulfides to zero-valent S during the sequential extraction of the exchangeable fraction. Two main mechanisms have been proposed for oxidation of metal sulfides depending on the structure of the sulfide minerals and environmental conditions [37]. Disulfide minerals such as pyrite are oxidized via the “thiosulfate mechanism” by attack of an oxidizing agent resulting in a sequence of reactions with production of sulfate as the end product. Acid soluble sulfide minerals such as FeS are oxidized through a “polysulfide mechanism” by attack of an oxidizing agent and protons on the metal sulfide lattice. This results in an oxidation of FeS to polysulfide as an intermediate compound and further formation of elemental S [37,38].

A prerequisite for oxidation of FeS is the availability of potential electron acceptors. In environmental samples, major electron acceptors thermodynamically capable of oxidizing FeS are O_2 , NO_3^- , Mn(IV)oxides, and Fe(III) oxides [39]. In this study, proper measures were undertaken to avoid exposure of the samples to O_2 during the extraction procedures by flushing the reagents water with N_2 and performing extraction procedures (i.e. centrifugation, decanting of supernatant, and shaking, see Table 1) in an anaerobic box. Thus, it is unlikely that the transformation of FeS during extraction of the exchangeable fraction was due to an oxidation by O_2 . Furthermore, if the S oxidation was due to a contamination of samples with O_2 during the extraction procedure, the solid residue after carbonate extraction should also be more oxidized compared to the previous step.

Manganese (IV) has been proposed to be an oxidation agent for FeS to zero-valent S [39]. However, the low concentration of Mn in the samples (data not shown) makes such a transition unlikely. Fe(III) ions are known to act as oxidizing agents for metal sulfides at circumneutral and acidic conditions [37] and Fe(III) is assumed to be the main oxidizing agent responsible for FeS oxidation in the absence of MnO_2 [39]. It has also been demonstrated that Fe(III) ions are more efficient oxidizers of metal sulfides than oxygen [40,41]. Sand et al. [37] proposed the following reaction scheme for oxidation of metal sulfides (MeS) via a “polysulfide mechanism” in the presence of Fe(III):



Rapin et al. [40] applied a sequential extraction method, which included the extraction of a “Fe and Mn oxide fraction” for sediment samples and performed AVS analysis for every step. They

showed that, under anaerobic condition, AVS was preserved during exchangeable and carbonate extraction, but it was reduced by 5% after “Fe and Mn oxide” extraction. The same observations were reported by Ngim and Lim [41] by measurements of the redox potential after each step in a sequential extraction scheme. It was suggested that released Fe(III) ions concurrent with reduction of Fe oxides during sequential extraction acted as the oxidizing agent for FeS oxidation.

The presence of oxide minerals in sludge samples is unlikely, due to overall anoxic conditions in biogas reactors. However, daily supplementation of 0.5 g Fe L⁻¹ d⁻¹ using a solution with Fe(III):Fe(II) ratio of 2:1 might be a source of short term Fe(III) accumulation in the system. In general, Fe(III) is reduced to Fe(II) chemically and/or biologically by iron-reducing bacteria, during the anaerobic digestion process [42]. The rate of Fe(III) reduction might be limited by the availability of Fe(III) through processes like; complexation of Fe(III) with organic and/or inorganic compounds, particle aggregation, Fe(III)–cell membrane contact, as well as a passivation of cells and mineral active sites by other available ions [43]. Thus, it is possible that the daily addition of Fe(III) to the sludge may have resulted in a short-term build-up of less available Fe(III) forms in the solid phase of the reactors. The extraction step with 1.0 M ammonium acetate (Table 1), as well as addition of 1 M HCl during the AVS extraction, might dissolve Fe(III) from the sludge matrix, which in turn may act as a FeS-oxidizing agent and promote oxidation of FeS to elemental S during the extraction procedures. According to (Eq. (1)) and (Eq. (2)) the end products of these reactions are Fe(II) and elemental S. The Fe(II) formed upon oxidation may have been incorporated as organic and inorganic complexes or precipitates (FeS) in the sludge solid matrix.

4. Conclusions

In this study we have used S XANES spectroscopy and AVS extraction to (1) determine the chemical speciation of sulfur and (2) evaluate a method used to sequentially extract trace metals in anoxic sludge from two lab-scale biogas reactors fed with grain stillage and augmented with Fe(II,III). The results demonstrated that S is mainly present as FeS (62% and 63% in J2 and J4, respectively) and reduced, organic S (32% and 29% in J2 and J4, respectively) in the anoxic solid phase of the sludge samples. A small fraction of the S was identified as zero-valent S (or possibly pyrite) in the original sludge. The first step of the sequential extraction procedure (the exchangeable fraction) resulted in partial oxidation of the FeS fraction to zero-valent S. Iron was not dissolved upon this FeS oxidation implying a transformation of Fe from a sulfide mineral to another fraction or secondary formed phase in the solid matrix. These results were confirmed qualitatively by the AVS analysis.

Furthermore, during the organic/sulfide extraction step (by 30% H₂O₂ at pH 2.0), 24–27% of Fe remained unextracted but it was dissolved by the AVS extraction step, despite the fact that the AVS concentration was zero. This suggests that the organic/sulfide extraction step dissolved FeS, but a secondary Fe phase (likely jarosite) was formed. The change in Fe and S speciation during sequential extraction might lead to inaccurate results for the speciation of trace metals like e.g. Co and Ni, known to associate to FeS by co-precipitation and/or adsorption. Results from a parallel study on trace metal speciation in sludge samples from J2 and J4 by Gustavsson et al. [unpublished] demonstrated that Co was associated with Fe in the residual fraction of the sludge samples, whereas Ni was 100% associated with the organic/sulfide fraction. The finding of the present study which showed a secondary phase of Fe was formed after extraction of organic/sulfide fraction may suggest that Co remaining in the residual fraction likely was caused by an association to this Fe phase, possibly jarosite. It is known that

Co and Ni can be incorporated by a substitution for Fe(III) in the jarosite structure [44,45]. Accordingly, the results from the present study revealed critical limitations for the application of sequential extraction of trace metal speciation analysis outside the framework for which the methods were developed particularly for samples from biogas reactors augmented with Fe(II) and Fe(III) for removal of sulfide from biogas product.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.12.065.

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