Can bioavailability of trace nutrients be measured in anaerobic digestion?

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HIGHLIGHTS

- Sequential extraction technique was successfully applied to biogas slurries.
- Sample pretreatment caused major shifts in element speciation.
- Recovery rates of elements were between 90% and 110%.
- Adapted method provides more reliable information about bioavailable fractions.

GRAPHICAL ABSTRACT
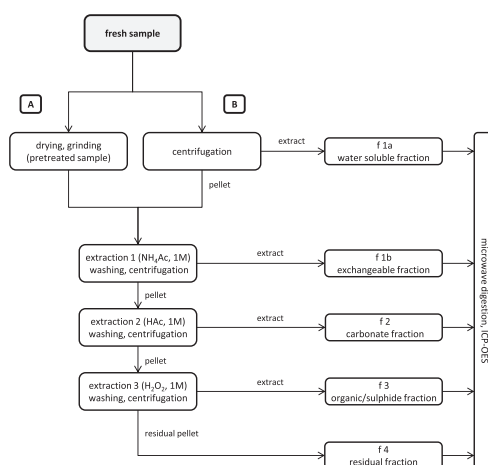
ABSTRACT

Trace nutrients significantly affect the microbial metabolic activity within anaerobic digestion processes but always imply the risk of overdosing of heavy metals. In this study the applicability of a sequential extraction scheme established for soil and sediment samples on biogas slurries with different compositions was tested and compared to an adapted version of this extraction method. The analytical results proved the successful applicability of the developed analytical technique for the speciation of trace nutrients in anaerobic digestion systems. The procedure fulfills the basic requirements of reproducible data, a time-saving analytical approach and economic feasibility. Recovery rates of 90–110% were obtained for the most important trace elements Fe, Co, Cu, Mo, Ni and Zn. However, it was demonstrated that the adapted method provides more reliable information about the bioavailable fractions and it is considered the more appropriate approach. Data on fractionation indicated that up to 76% of these essential trace nutrients might be present in an insoluble state. Depending on the specific trace element a significant fraction, from 30% to more than 70%, is not directly bioavailable. This important aspect should be

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considered to guarantee sufficient supply of the microbial consortium with trace elements and at the same time to avoid overdosage.

1. Introduction

The growing interest in biogas production by anaerobic digestion of biomass as a source of renewable energy leads to increasing research effort on optimization strategies of the bioconversion process within the anaerobic digesters [1–6]. Optimum supply of mineral nutrients needed for growth and enzyme activity in the digestion process, both in quantity and composition, is one of the key predominant strategies to increase the biomethanation rate. Microbial requirements for minerals often differ from their supply by the input material and consequently supplementation of those minerals is needed, which are not sufficiently provided.

To overcome nutrient deficits, a broad spectrum of commercial mineral supplementation products are available on the market, more or less addressing the specific needs for trace elements [7]. However, addition of trace nutrients also implies the risk of overdosing of heavy metals, in this way causing toxic effects on the microbial consortium of anaerobic digestion. Moreover, elevated heavy metal concentrations may limit the proper use of digestate as fertilizer and can cause environmental pollution.

Adequate supplementation requires not only substantial knowledge on trace nutrients content of the substrates but also bioavailability of the considered minerals. In particular about the latter aspect, bioavailability, little knowledge is available. Demirel and Scherer [8] reviewed the literature about requirements of macro- and micro-nutrients in anaerobic digestion of both agricultural substrates and the organic fraction of municipal waste (OFMSW). In this review chemical bonding state of the minerals was not considered, which may cause quite different degrees of bioavailability.

Some work has been done to investigate the effects of certain trace metal nutrients on methane production in anaerobic wastewater treatment including analysis of metal speciation. Most of them dealt with granular sludge in UASB-reactors [9–15]. Aquino and Stuckey [16] investigated the effect of copper complexation on bioavailability and toxicity in a laboratory CSTR.

Up to date there is no investigation on metal fractionation of digester slurry obtained from biogas plants utilizing agricultural substrates or organic wastes for renewable energy production. These kinds of processes differ from granular sludge processes by significantly higher concentrations of suspended solids in the digester. Therefore different patterns of metal fractionation can be expected, resulting in different bioavailabilities.

In soil bioremediation, bioavailability, has traditionally been an important issue been to provide information on potential transfer of sediment and soil bound potential toxic heavy metals to the ground water and the food chain [17]. Since many decades sequential extraction of soils is a very well established technique and numerous multi-step extraction schemes have been developed. Just recently such investigations have been applied to other materials such as sewage sludge and anaerobic sludge. However, in all cases the focus was to obtain information on the mobility of toxic metals in order to evaluate potential ecotoxicological risks [18].

The aim of this work was to examine the applicability of sequential extraction on anaerobic digestion slurries originating from different sources. The investigated biogas slurries were obtained from three biogas processes: an agricultural biogas plant utilizing a mixture of pig manure and maize silage as substrates, a plant using solely energy crops, and a plant processing animal byproducts.

For examination the modified Tessier scheme of sequential fractionation, a widely applied method in soil and sediment analyses was adapted [19]. This analytical procedure yields four fractions: an exchangeable fraction, a carbonate fraction, a fraction of organic matter and sulphides, and a residual fraction.

Moreover, an adapted procedure yielding an additional fraction was developed. Both, our adapted method and the original modified Tessier scheme were compared to see possible shifts in fractionation. Besides the evaluation of reproducibility of the analytical methods attention was given to the interpretability of the obtained results based on chemical characteristics of the samples and on thermodynamic considerations.

2. Materials and methods

2.1. Origin of the samples

Samples were taken from three different large scale industrial and agricultural AD plants. All of them used a single stage process with main and post fermenters with average hydraulic retention times (HRT) between 35 (plant III) and 50 days (plant I and II) for the main fermenter. AD plant I was an industrial biogas plant operated solely with slaughterhouse waste derived from the close-by pig abattoir. The biogas produced in the AD plant is purified by an external biological desulphurisation unit and combusted in a combined heat and power plant (CHP) with an electrical power of 525 kW. Plants II and III were agricultural AD plants using on the one hand grass and maize silage on the other hand a mixture of pig manure and maize silage. Again biogas was utilized in CHPs with an electrical power of 525 kW and 1050 kW, respectively.

Beside AD plant no. II, the two other plants received additional micro- and macronutrients (plant I trace element mixture, plant III ferrous chloride). All operating conditions (e.g. feeding, substrate composition stirring, organic loading rate) of all AD plants were kept relatively constant for at least 6 month before sampling. Samples were always taken from the main fermenter. General sample characteristics are provided in Table 1.

Immediately after sampling the pH was measured. All the other relevant parameters were analyzed in laboratory. Samples were stored at 4 °C for the time of 7 days maximum before further processing.

2.2. Analysis of standard parameter

TS (total solid content) and VSS (volatile suspended solid content) were analyzed by DIN methods [20,21], for NH_4-N (ammonium nitrogen) a slightly modified standard method [22] was used, where MgO was substituted by NaOH for pH adjustment.

2.3. Reagents and equipment

Double-distilled water was used for the preparation of all extracting agents and washing solutions. Glassware were rinsed with double-distilled water and dried at 105 °C.

All centrifugation steps were done with a Beckman GS-6 device at the rotation speed of 3000 rpm (1459 g) using polypropylene centrifuge tubes. The filtration steps were done with cellulose filters (Whatman 595½).

Table 1
Characterisation of the samples and the AD-plants.

AD-plant	Input material	Temp. [°C]	pH	OLR [kg m^{-3} d^{-1}]	HRT [d]	TS [%]	VS [%]	NH$_4$-N [g kg^{-1}]	Total sulfur [g kg^{-1}]	Additives
I	Slaughter-house waste (SH)	38	8.0	2.5	50	3.3	2.7	6.46	5.53	Trace element mixture
II	Grass silage/maize silage (GM)	48	7.6	3.0	50	12.6	12.6	1.86	4.06	–
III	Pig manure/maize silage (PM)	40	7.6	3.0	35	7.3	5.9	4.61	5.30	(Fe^{2+})

For the sequential extraction of both, pretreated and fresh sample, the modified Tessier method (A) according to van Hullebusch et al. [11] and an adapted modified Tessier scheme (B) was applied in duplicate. The different extraction steps are illustrated in Fig. 1. Using either method A or B, four or five fractions, respectively, were obtained.

The fractions were termed water soluble, exchangeable, carbonate, bound to organic/sulphides and residual.

2.4. Modified Tessier scheme (method A)

2.4.1. Sample pretreatment

Samples were dried for 48 h at 105 °C and stored in an exsiccator at room temperature. Dried samples were grinded with a swing mill (Retsch SM200) using ZiO$_2$ bakers and ZiO$_2$ bullets (Retsch) to a particle size of ⩽300 μm. The dried and grinded sample was used both, for the sequential extraction and for the determination of the total amount of elements.
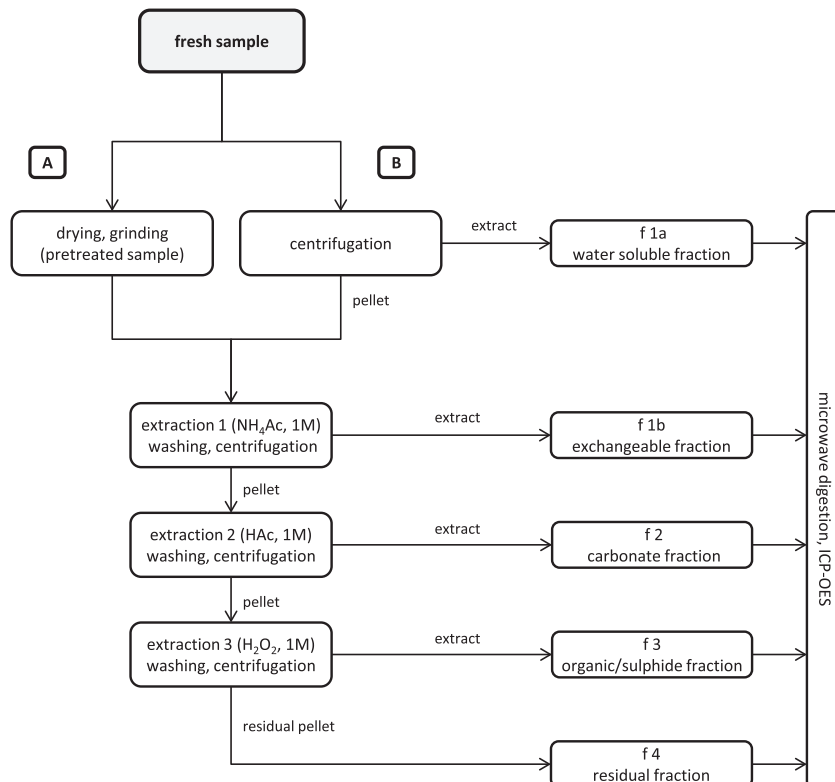
2.4.2. Sequential extraction

The principle of the modified Tessier method is the use of different extraction solvents solubilizing different fractions of relevant metals. Sequential extraction was done in triplicate, for each 1 g of sample was used.

Extraction step 1: 1 g of pretreated (dried and grinded) was extracted with 10 ml of ammonium acetate (1 M, pH 7) for 1 h at 25 °C with subsequent centrifugation for 10 min. The pellet was resuspended within 25 ml double-distilled water and centrifuged again. Both supernatants were combined and represented the water soluble and exchangeable components.

Extraction step 2: The pellet derived from the previous step was extracted with acetic acid (1 M, pH 5.5) for 1 h at 150 rpm at room temperature, centrifuged (10 min) and the supernatant was collected. Similar as above the pellet was washed with 25 ml double-distilled water and the two supernatants were combined representing elements bound as carbonates.

Extraction step 3: The pellet from extraction step 3 was extracted with 10 ml of H$_2$O$_2$ (30%; pH 2.0, adjusted with nitric acid) for 3 h at 150 rpm and 35 C°, centrifuged (10 min 3000 rpm). Like in the previous extraction steps the liquid phase was separated and the pellet was washed with 25 ml of double-distilled water. The received supernatants represent elements bound to organic matrix and sulphide precipitates.

Residual: The remaining pellet from the previous extraction step represented the fraction 4 and contained non-dissolved elements which precipitated as sulphides or silicates. Analysis of elements was made by means of micro-wave digestion with nitric acid (see Section 2.6).

Fig. 1. Sequential extraction scheme: modified Tessier scheme (A) and adapted modified Tessier scheme (B).

2.5. Adapted method (method B)

A disadvantage of the modified Tessier method is the initial drying step which may cause chemical alteration of the sample, e.g. the formation of metal oxides. Moreover the first step involves already the use of 1 M ammonium acetate and therefore does not allow determination of the dissolved metal ions in the untreated sample. To overcome that, a modification of the initial treatment was made. Drying of the sample was omitted; instead the liquid phase from the original sample was directly collected through centrifugation.

The amount of sample used varies depending on the total solid content. Fresh material with total TS of approx. 1 g was placed in a centrifugation tube (polypropylene) and filled up with double-distilled water to a total weight of 50 g. After centrifugation for 15 min the supernatant (water soluble fraction) was collected and stored at 4 °C until analysis.

The pellet moved further to the similar sequential extraction procedure as described for the modified Tessier method before.

All liquid fractions and pellets, received from the extraction process, were stored at 4 °C until further processing and analysis.

2.6. Analysis of elements

Analysis of the micro and macroelements was performed with an ICP-OE spectroscope (Jobin Yvon Horiba Ultima).

Microwave digestion: For determination of total amount of elements in the samples as well in the residual fraction, microwave digestion was performed with nitric acid in a Milestone ultraCLAVE III device at 240 °C with a final pressure of 100 bar for 20 min.

The following procedures were applied:

2.6.1. Liquid fractions

5 ml of concentrated nitric acid (puriss. 65% p.a. Sigma–Aldrich) was added to a 10 ml aliquot and transferred into Teflon digestion tubes. After digestion, the sample was filled up with double-distilled water to 50 ml and filtered through a cellulose filter in order to protect the injection nozzle of the ICP-OES device.

2.6.2. Residual fraction

20 ml of nitric acid was added to the remaining pellet and the same procedure was conducted as described above with the liquid fractions.

2.6.3. Total amount

10 ml of nitric acid was added to approximately 0.5 g of dried and grinded sample. The following steps were the same as described above.

2.7. ICP-OES analysis

The analysis comprises the determination of iron, nickel, cobalt, copper, molybdenum and zinc using the following wavelengths (in nanometers): 273.955 (Fe), 221.647 (Ni), 228.616 (Co), 327.396 (Cu), 202.030 (Mo) and 213,856 (Zn).

The result of the analytical determination is indicated in milligram per kilogram total solid content (mg/kg TS) including all the dilution steps done during the extraction and analysis process. The analysis was conducted in triplicate.

The recovery rate of each element was calculated as the sum of the element in all fractions related to the total element content in the original sample.

2.8. Measurement of sampling error

In order to get reliable information on the sampling error a comprehensive sampling campaign at two AD plants were conducted.

Samples (500 g) were taken hourly from AD plant II and III for the period of 2 days and were stored at 4 °C until analysis. A representative sample was guaranteed by opening the digester's exhaust valve for 5 min minimum. Thus an amount of approximately 100 l flushing the outlet tube was ensured.

The pretreatment and determination of the elements were done in triplicate in the same way as described in chapter 2.4 and 2.7, respectively.

2.9. Mathematic modeling – MINTEQA2

Mathematical modeling of species concentration and speciation at thermodynamic equilibrium conditions was performed by EPA computation software MINTEQA2 to check validity of the results obtained.

The program is a tool for simultaneous calculation of multi-component electrolytes based on a thermodynamic database. Depending on the concentration of the trace elements and the corresponding anions, such as phosphates as well as pH and temperature the equilibria can be calculated between dissolved, solid, adsorbed and gas phase species in environmental settings. For general aspects and basic understanding of chemical behavior in anaerobic systems dependent on pH, a simulation of 5 trace elements (Fe, Ni, Cu, Zn and Co) in presence of sulphides, phosphates and carbonates was performed.

To evaluate fractionation results, simulation including total concentration of trace elements obtained in the first 3 extracts (f1a, f1b, f2) and plant specific parameters such as alkalinity, phosphates, hydrogen sulphide (HS$^-$) and various macro elements (e.g. Na, K, Ca, Mg) was carried out. The HS$^-$ present in the liquid phase was calculated by using equilibrium between gaseous H$_2$S and HS$^-$ at the given pH and temperature. Fractions f3 and f4 were excluded from this model, because it can be assumed, that elements present in these solid phases are not significantly involved in rapid equilibration within a pH – range between 5.5 and 7.0. Instead, the reactions are much slower. Since MinteqA2 calculates ultimate equilibria, it does not include a kinetic term. Therefore, including f3 and f4 in the model would lead to irrelevant results for the real situation.

2.10. Relevance of the investigated elements

The considered elements are essential for the anaerobic digestion process and therefore should be provided in sufficient amount [25].

Iron is defined as the most important trace element in anaerobic digestion and is acting in various methanogenic key enzymes as certain species such as Fe–S clusters. The concentration of iron can vary very strong between biogas plants due to the fact that some plants add ferrous compounds for internal desulphurization purposes.

Copper and zinc are essentially required as coenzyme of hydrogenases in facultative anaerobes, archaea and clostridia [15,26].

Nickel is also an essential cofactor in various methanogenic enzymes such as hydrogenase, CODH or co-factor F$_{430}$ [8,25,27].

Molybdenum is part of formyl-MF dehydrogenase, a major enzyme in methanogenesis [28,29].

For both hydrogenotrophic and aceticlastic methanogenesis the membrane bound methyltransferase is an essential key enzyme. Since cobalt is incorporated in the enzyme's corrinoid co-factor, a

depletion of cobalt can be directly linked to a decrease of methane yields [25,30].

3. Results

3.1. Characterization of the AD-sludges

The operating conditions and types of input materials of the AD plants as well as the main physico-chemical properties of the investigated sludge samples are presented in Table 1.

The differences in composition can be related to the origins of the substrates, measured parameters were within the typical range of the considered plant type [23].

The pH value varied between 7.6 (plant II and III) and 8.0 (plant I). As it can be seen, there were also large differences in total solid contents (TS). The values ranged between 3.3% and 12.6% and represented typical values for the applied substrates and should guarantee a wide applicability of the method [2,23].

3.2. Measurement of sampling error

Sampling of anaerobic sludge is often a major source of uncertainty in analytical results. Compared to sample treatment and analysis, sampling has the highest impact on the final result [24].

Very little is known about the influence of the sampling error of anaerobic sludge on trace element speciation. To estimate potential impact on the results obtained in this study, total element concentration of six selected elements in two sampling campaigns was measured.

In Table 2 basic statistical parameters from two nonstop 48 h sampling campaigns from two AD plants are shown. The indices "PM" and "GM" refer to the input material and represent the mixture of pig manure/maize silage and grass silage/maize silage, respectively.

The overall number of samples was 41. In general the coefficient of variation (CV) was between 6% and 12% for almost all samples. However, for certain elements, i.e. copper GM (24.59%) and molybdenum PM (316.7%) high variations were observed, although good sampling practice was applied. With regard to molybdenum this higher CV can obviously be attributed to the very low concentration measured, in the case of copper the reason remained unclear.

It is supposed that the variation of the results is mainly caused by inhomogeneities originated from low mixing efficiency in the digesters, which is a typical phenomenon of biogas plants. Despite different total solid contents (5.9% for PM; 12.6% for GM) there was no significant difference between the coefficients of variation of both plants.

These results clearly showed that for evaluation and interpretation of analytical results of micronutrients, the sampling error has

Table 2
Sampling error on total element content in a 2 days sampling campaign (sampling hourly).

Element	Mean [mg/kg TS]	CV [%]	Min [mg/kg TS]	Max [mg/kg TS]
Co$_{PM}$	1.65	8.13	1.06	1.82
Co$_{GM}$	1.45	8.53	1.09	1.60
Cu$_{PM}$	70.78	6.27	61.50	79.91
Cu$_{GM}$	25.71	24.59	16.33	45.69
Fe$_{PM}$	2131.89	8.36	1803.27	2435.10
Fe$_{GM}$	3598.29	7.01	2675.08	3950.00
Mn$_{PM}$	201.12	9.46	162.81	226.03
Mn$_{GM}$	247.16	6.66	187.66	270.10
Mo$_{PM}$	4.04	12.18	3.30	6.50
Mo$_{GM}$	0.0058	361.7	0.0000	0.0876
Ni$_{PM}$	4.51	7.62	3.70	5.07
Ni$_{GM}$	9.03	8.24	6.64	10.11

to be kept in mind. Additional to the analytical error a CV of 6 to 12% at minimum derived from the sampling has to be considered.

3.3. Application of sequential extraction schemes A and B

Fig. 2a–f shows the results obtained by application of the modified Tessier sequential extraction scheme (A) in comparison to the results of the newly introduced adapted modified Tessier scheme (B) for six relevant micro and macroelements (iron, copper, zinc, nickel, molybdenum and cobalt).

It can be seen that pretreatment in terms of drying and milling exhibited a major influence on the distribution pattern of the elements.

Fraction no. 4 (f4) represented the residual, nondissolvable and nonextractable fraction. Using sequential extraction scheme A the determined amount of elements was significantly higher in all cases compared to extraction scheme B, where no pretreatment was performed. The influence of pretreatment has been observed in other fractions as well. The concentration of elements determined in the mobile fraction f1b (method A) was in all cases lower compared to the mobile fractions (f1a + f1b) in method B.

4. Discussion

4.1. Comparison of methods

The difference of the two methods was the pretreatment of the samples. Using the modified Tessier extraction scheme (A) the sample was dried and ground before applying the different extraction agents. In contrast to method A, method B required no pretreatment and generated an additional fraction, (water soluble, f1a) which represents the actual trace element concentration in the dissolved phase at the prevailing conditions in the biogas digester.

By definition of ISO 11074 (2005) bioavailability is the degree to which elements are available for interaction with biological systems [31]. As reported by Harmsen [32] bioavailability may be assessed by applying sequential extraction techniques, in which the bioavailability of the elements decreases in each subsequent fraction. Fuentes et al. [33] described that not only the water soluble but also the exchangeable fraction is highly bioavailable.

Therefore in the investigated case, for method A the first (f1b) whereas in method B the first and the second extract (f1a and f1b) can be assigned as highly bioavailable to the microbial consortium.
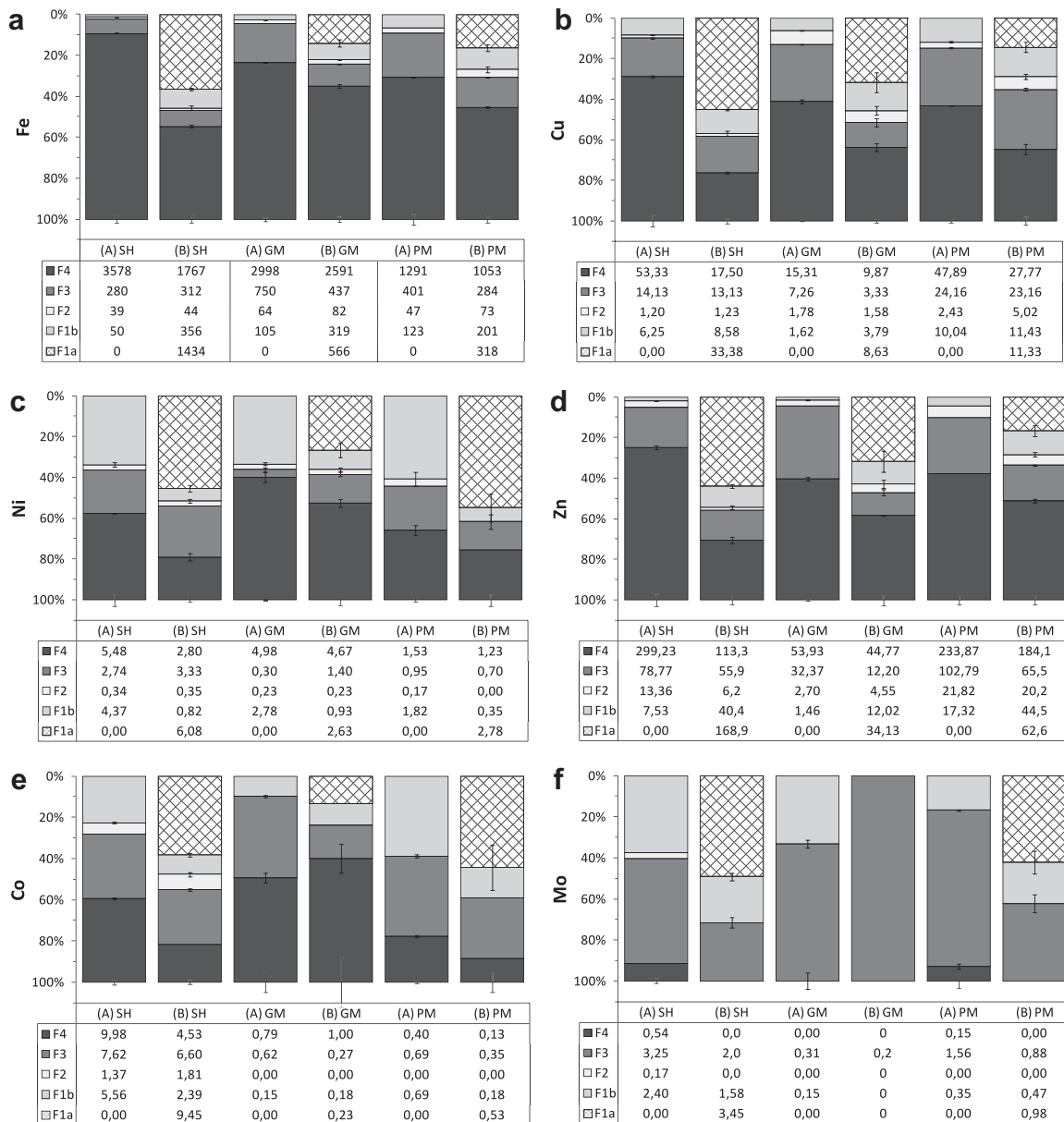
All other fractions are either bound to particular matter or precipitates. Their mobilization depends on aqueous solubility which is usually very poor in the cases of sulphides, phosphates, hydroxides and carbonates. In terms of bioavailability of metals bound as sulphides Gustavsson et al. [34] stated in their study that Nickel- and Cobalt might act as a metal source to methanogens although they are bounded in sulphide precipitates.

Fig. 2a–f clearly showed a significant difference in elements considered as bioavailable comparing the two approaches. It is presumed that in method A the drying step induced the conversion of hydroxides in highly insoluble metal oxides. As a consequence, these elements shifted into the residual fraction (f4). Fig. 3 proposes a general scheme of main element fluxes during anaerobic digestion. Exchange between f1a/f1b and f3 is considered to be most important for microbial activity. Fractions f1b and f2 are only indirectly linked to uptake by microorganism through fraction f1a, dependent on concentration equilibria. Fraction 4 represents a sink for nutrients, as they are not available at all.

Generally, applying method B, 24–62% of the elements were credited to the bioavailable fraction whereas method A delivered

Fig. 2. (a–f) Fractionation pattern of Fe, Cu, Zn, Ni, Mo and Co: Comparison of 3 different biogas slurries (slaughterhouse waste (SH), grass/maize silage (GM) and pig manure/maize silage (PM)) by application of sequential extraction method A and B. The units of the fractions in table are [mg/kg TS].

only 1–40%. In relation to the type of element, in B the increase of bioavailable elements was 308–3498% for iron, 143–632% for copper, 8–53% for nickel, 517–2781% for zinc, 90–269% for molybdenum and 52–143% for cobalt.

These figures underline the drastic impact of the applied analytical procedure. Iron was the most sensitive element to pretreatment and obviously oxidation of ferrous ions occurred readily in the presence of air-oxygen at 105 °C during the drying process. The main part of iron in f4 was ferric oxide, which is characterized by low solubility. Due to the fact that the sample was ground to an average particle size of 27.3 μm (median value) the oxidation was even amplified as consequence of the enlargement of reactive particle surface. Also Li et al. [35] showed that chemical oxidation of metal particles is directly dependent from particle size and that lower particle sizes led to higher oxidation rates. Iron oxide is known to adsorb or occlude trace elements such as copper or zinc [36,37] and thus may influence the general distribution pattern. These results are confirmed by Zehl and Einax [38] who observed

that the presence of oxygen leads to a significant decrease of metal content (Fe, Ni, Co) in the bioavailable fractions.

As expected, the residual fraction showed also significant differences between the extraction methods. Method A delivered values for the residual fraction f4 of 22–91% of total element content, whereas in B they ranged from 11% to 65%. For single elements, deviation of B from A was 18–101% for iron, 61–200% for copper, 27–103% for nickel, 27–155% for zinc and 97–122% for cobalt. Molybdenum was in some cases below the limit of detection, therefore no reliable comparison could be performed.

Elements in carbonate fraction (f2) were not present in several cases. Where they could be found, their values ranged between 1% and 7% and did not differ between both extraction schemes.

Differences in yields of elements belonging to the organic/sulphide fraction (f3) showed no general trend to shift. This fact indicates, that the biomass and sulphide bound elements present in this fraction were insensitive to pretreatment, similar to the carbonates in f2.
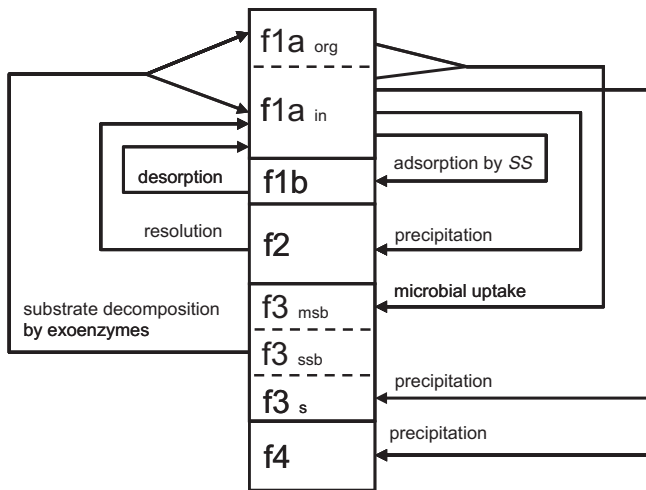
Fig. 3. dynamic flux of elements between extracted fractions in anaerobic digestion. Fractions 1–4: f1a org = dissolved organic, f1a in = dissolved inorganic, f1b = exchangeable, f2 = carbonates, f3 msb = microbial suspended biomass, f3 ssb = substrate suspended biomass, f3 s = sulphides, f4 = residual; SS = suspended solids.

It was observed that f3 was correlated to TS because this extraction step involves release of trace elements bound to organic matter as well as dissolution of metal sulphide precipitates.

In case of iron low TS values led to a minimal difference between the values gained from A and B. For the sample from plant I (slaughterhouse waste) with low TS concentration of 2.7% the difference in f3 was 10% related to A. However, the sample with the highest TS concentration (plant III, grass/maize silage, 12.6% TS) showed a difference of about 72%. The pig manure/maize silage substrate sample (plant II) with TS of 5.7% led to a difference of about 42%.

Another significant factor influencing the analytical results of f3 was the particle size.

Applying Method A (median particle size after grinding: 27.3 μm) relatively low CVs were measured. E.g. in case of iron the values were 3.0%, 0.9% and 0.7% for samples from plant I, II and III, respectively. In contrast, method B utilizes the original samples without grinding. According to a sieve fraction analysis, in all samples about 50% (w/w) of the particles had a particle size larger than 0.7 mm. The maximum particle length was between 35 and 45 mm. This larger particle size caused a significantly higher variation of the obtained analytical results. For iron the CVs were, 9.1%, 8.5% and 3.9% (plant I, II, III).

Similar results were observed with other elements as well, especially when focusing on samples with higher TS content. The determined concentration of copper in f3 was 28% and 12% of total element content applying A and B, respectively. The large particle size of solids, when applying B, led to a high CV of about 17.3% compared to 2.9% for A. In this experiment we did not investigate the influence of different temperatures and reaction times on the extraction yield in f3.

Certain other limitations of the modified Tessier scheme are already known. Beside the formation of metal oxides, which reduce the amount of bioavailable elements, pretreatment in terms of drying led to a pH shift as well. This is expected to influence the distribution in the fractionation process. During the pretreatment a pH shift from 7.8 up to 9.0 within the first 60 min was observed. This was probably caused by destruction of the carbonate buffer system due to purging of CO_2. The solubility of elements is strongly related to the pH as it can be seen in Fig. 4. The figure shows the soluble fractions of elements and their speciation for the case of equimolar concentrations of Ni-, Fe-, Co-, Cu- and Zn- ions in the presence of sulphides, phosphates and carbonates at different pH

values in a MinteqA2 simulation of thermodynamic equilibrium. All elements showed higher solubility at low pH. The change in solubility was largest within the physiological pH range of 7–8. Ni exhibited the highest solubility at low pH, only a small amount was precipitated as NiS. Under alkaline conditions solubility decreased rapidly due to the formation of $Ni(OH)_2$, which is transformed into NiO at thermodynamic equilibrium. Co, Zn and Fe formed at low pH partially the corresponding insoluble phosphates. Increasing pH led to additional formation of carbonates and hydroxides, which dehydrated to the corresponding oxides at equilibrium point. It has to be considered that in environmental matrices equilibrium will be never reached at any time due to permanent dynamic behavior. Although the simulation is not able to reflect the real situation completely, it is a useful tool to estimate the formation and transformation fluxes of inorganic electrolyte species under defined and restricted conditions, as it was done by Aquino and Stuckey [16] for the investigation of the influence of complexing agents on anaerobic digestion.

Beside the pH dependence Tack et al. [36] demonstrated that the redox potential of the sample plays a major role in element speciation for a couple of trace elements. In their experiments they observed a higher solubility of copper and zinc at higher redox potential. Copper showed a significant higher solubility (10–90 mg/kg TS) at a redox potential of around +60 to +370 mV and pH range between 1 and 2. In comparison solubility of copper was almost zero at −220 to −150 mV.

In contrast to that, iron showed opposite effects. A significant higher solubility was given at low pH (between 1 and 6) and high redox potential. No significant influence of redox potential on solubility could be obtained for nickel and cobalt.

Shakeri Yekta et al. [39] reported about the influence of reduced sulphur compounds on speciation. They found out that approximately 20% of sulphur bound in FeS was oxidized to elemental sulphur during the first extraction step of the modified Tessier scheme. However, iron was supposed to be adsorbed by organic matter, but there, up to 27% of FeS remained unextracted. They proposed the partial forming of jarosite out of dissolved FeS, which can occlude cobalt. This fact might lead to a higher concentration of cobalt in the residual fraction.

To get an insight into occurring chemical reactions during fractionation from a theoretical point of view, simulation of ionic equilibria in the pH-range 5.5–8.0 was performed in MinteqA2. By this means the results showed a complete solubility of Ni, Co, Zn and Cu at pH 5.5 (corresponded with f2) which was according to the analytical data generated. However, at pH 7 Zn and Cu partially precipitated as smithsonite and azurite and thus did not reflect the fractionation data obtained. Furthermore iron showed a complete
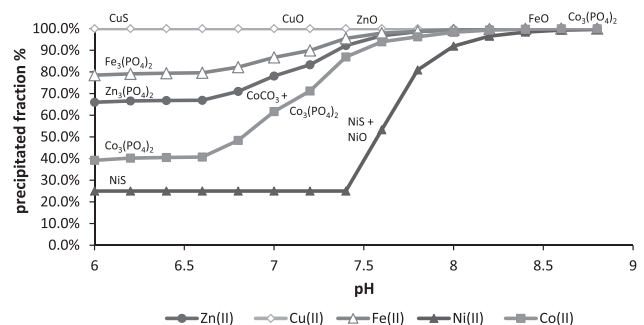
Fig. 4. MinteqA2 simulation: speciation of 5 trace elements (each 1 mM) in the presence of sulphide (1.25 mM), phosphate (1.25 mM) and carbonate (1.25 mM) at different pH. Charge balance was given by chloride and sodium. Predominant precipitated species are shown in the appropriate pH region.

contrary behavior. At pH 7 Fe was almost completely bound to insoluble siderite and at pH 5.5 only 13.7% was dissolved.

The underestimation of these fractions indicates that Fe was partially bound to complexing agents such as siderophores, e.g. excreted by microbes to enhance solubility and uptake of Fe ions. For that reason, calculation of ionic equilibria without considering complexing agents does not fully reflect the situation in biogas slurries. However, it can give a first understanding of transformation and precipitation of ionic species.

The evaluation of the applicability of both methods (A and B) was completed by investigation of the recovery rate (Table 3).

All elements exhibited a good recovery rate between 90% and 110%. Poor recovery was only observed at very low concentration. Comparing both methods, a slightly better recovery rate for method A could be noticed, presumably due to better homogeneity, as discussed above in connection with the coefficient of variation. This is in accordance to Baeyens et al. [40] who did similar observations in their sequential extraction experiments comparing a pretreated (lyophilized) and a wet sediment sample analyzing Fe, Mn and Pb.

A certain advantage of method A was that pretreatment allows larger sample amounts that are dried, grinded and homogenized prior to analyses. This leads to a reduction of the variation of the obtained data with significant gain of reproducibility. However, according to the gained experience, its main disadvantage is element shifting towards more insoluble fractions. Therefore method B, the adapted modified Tessier scheme (B), provides a much better representation of the bioavailable fractions.

4.2. Bioavailability of elements

In the following paragraphs only the outcome of method B is further discussed in detail for six selected elements. Focus was placed on the highly bioavailable fractions f1a and f1b and on the residual (nonbiovailable) fraction f4.

In terms of relative amount, iron generally exhibited the highest accumulation in the nonbioavailable fraction among the elements investigated. Most if it was found in the residual fraction (45–65%) and to a smaller share in the organic/sulphide fraction.

The highest bioavailability of iron was found in plant I. As a general tendency bioavailability of most elements was indirectly correlated to the dry mass which is the lowest in plant I. Nevertheless due to the high total amount of iron the absolute concentration in the easily bioavailable phase (f1a, f1b) is still quite high, 1790 (plant I), 885 (plant II) and 519 (plant III) mg/kg TS. Therefore sufficient supply with iron can be presumed. In comparison to iron, the total amounts of copper and zinc were much lower but generally the relative bioavailability was higher. Beside an increased share in fraction f1a/f1b also the amount in a transient state, i.e. the fractions which cannot be clearly designated bio- or nonbioavailable (f2, f3), was higher. Absolute amounts of nickel and cobalt were even around one magnitude smaller. Again the observed bioavailability was quite good. In these two cases highest relative bioavailability of these elements was found in plant III.

With respect to different behavior of elements within the investigated plants, the highest variance was observed for molybdenum. First of all it was not possible to find Mo in all types of fractions; in particular no molybdenum was measured in the residual fraction. Moreover, in plant II all molybdenum was presented in the biomass bound/sulphide fraction indicating a very low bioavailability of this element. In contrast in plant I und III the bioavailable fraction comprised 71.6% and 62.2% of the total amount.

Once more the difference between the two methods should be emphasized. By means of the method A the bioavailable fraction would have been largely underestimated. On the other hand determination of the total amount – the typical analytical approach in practice – leads to a false assessment of the level of nutrient supply as well.

5. Conclusion

Sequential fractionation is a useful method to assess bioavailability of nutrients and to locate reserve pools for nutrients, which can be mobilized. In this work we tested the modified Tessier scheme (A) and an adapted version (B) on applicability for element speciation in three different biogas slurries of slaughterhouse waste, grass/maize silage and pig manure/maize silage origin. Both methods gave reproducible results and fractionation patterns for six trace elements, which are essential for methanogenic communities in biogas processes. Method A showed better reproducibility and lower coefficients of variation than method B due to the homogenization in pretreatment. On the other hand pretreatment by drying and milling in method A not only required additional time-consuming working steps, it also led to artificial shifting of elements towards insoluble fractions and thus did not reflect the native condition of the sample. Especially the bioavailable fractions were much better represented by the method B therefore considered the more appropriate approach. This is of high interest in monitoring anaerobic digestion processes concerning trace element requirements. It could also be demonstrated, that in most cases remarkable amounts of elements exist in species not available for microbial uptake, which put the informative value of total element analysis into perspective.

Depending on the specific trace element a significant fraction, from 30% to more than 70%, is not directly bioavailable. This important aspect should be considered to guarantee sufficient supply of the microbial consortium with trace elements and at the same time to avoid overdosage.

Aware of the limitations of any extraction methods, e.g. discussed by Bacon and Davidson [41], Filgueiras et al. [18] and Gleyzes et al. [42] we assessed the sequential extraction by method B as highly applicable and valuable in practical control of biogas processes in terms of effort and information content.

Table 3
Comparison of recovery rates of elements (in %) applying method A (modified Tessier method) and B (adapted protocol). The recovery rate is defined as the sum of the amount of a single element in all fractions related to the total element content in the original sample.

Element	(A) SH	(B) SH	(A) GM	(B) GM	(A) PM	(B) PM
Fe	101	100	111	113	102	106
Cu	104	102	111	116	101	94
Zn	103	106	89	105	100	113
Ni	103	106	89	105	100	113
Mo	96	106	85	37	75	84
Co	101	102	106	112	97	64

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