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Comparison of three sequential extraction procedures to describe metal fractionation in anaerobic granular sludges

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Abstract

In the last few decades, several sequential extraction procedures have been developed to quantify the chemical status of metals in the solid phase. In this study, three extraction techniques (modified [A. Tessier, P.G.C. Campbell, M. Bisson, Anal. Chem. 51 (1979) 844]; [R.C. Stover, L.E. Sommers, D.J. Silvera, J. Water Pollut. Con. F. 48 (1976) 2165]; and the Bureau Communautaire de Reference (BCR) [K.F. Mossop, C.M. Davidson, Anal. Chim. Acta 478 (2003) 111]) were applied to study the distribution of trace (Co, Ni, Zn and Cu) and major (Mn and Fe) elements in two different anaerobic granular sludges from full-scale methanogenic wastewater treatment plants. The Stover scheme displayed a higher number of fractions that induces a poor recovery compared to the other schemes. The sequential extraction scheme recommended by BCR and the modified Tessier scheme gave similar trends and are sufficiently repeatable and reproducible for application in fractionation studies. However, the BCR scheme seems to be of limited utilisation to study anaerobic matrixes because the extraction stage for its reducible fraction may release substantial amounts of trace elements bound to the organic/sulfides fraction, and consequently, the recovery of trace elements in the oxide fraction may be overestimated at the expense of the oxidisable fraction. As a final conclusion, the modified Tessier scheme seems to be the most suitable scheme to study the metal partitioning in anaerobic granular sludges.

Keywords: Anaerobic granular sludge; Sequential extraction; Standard reference materials; Major and trace elements; Reproducibility

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1. Introduction

In anaerobic reactors, trace elements are not only present as free ions, but as different chemical species because of the typical process conditions that prevail in these reactors, e.g., pH, redox potential, alkalinity, concentrations of phosphorus and sulfur. Precipitation of metals, e.g., as sulfides, phosphates or carbonates, may also affect their bioavailability [1–3]. The behaviour of metals in anaerobic sludge granules is further complicated by their biosorption to extracellular polymers [4]. Consequently, there is a need for fractionation or selective dissolution procedures to study the fate of trace elements in anaerobic bioreactors [5].

The total metal content is a poor indicator of metal bioavailability, mobility or toxicity, when these properties basically depend on the chemical association of the different components of the sample. Single and sequential extraction methods have been widely applied to characterise the chemical forms in which trace metals are present in soils, sediments and sludges. The application of these techniques has been recently reviewed by Filgueiras et al. [6]. In all sequential extraction schemes, extractants are applied in order of increasing reactivity so that the successive fractions obtained correspond to metal association forms with lesser mobility. The extractants commonly used in sequential extraction schemes fall generally within the following groups: unbuffered salts, weak acids, reducing agents, oxidising agents and strong acids [6]. The main problems of the sequential extraction are the non-selectivity of extractants and

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trace element redistribution among phases during extraction [7]. In addition to these inherent problems of non-selectivity of extractants and trace metal redistribution among sequential extraction steps, the sample pretreatment is also a crucial stage which introduces bias.

Despite these disadvantages, which are well reported in the literature, sequential extraction methods are still widely used for the evaluation of availability and mobility of trace metals in solid matrixes (see [6] for review). These include, for example those by Tessier et al. [8] and the Commission of the European Communities Bureau of Reference (BCR) based on the schemes of Salomons and Förstner [9] and Kersten and Förstner [10]. Some of these schemes may be variants of one another with minor variations in the chemical extractants and operating conditions. For instance, the Stover et al. [11] scheme differentiates the organic matter and sulfides fractions and thus allows the differentiation between the main fractions involved in metal sorption in anaerobic materials (e.g., carbonates, sulfides and organic matter) [1].

Few studies have reported on metal fractionation in anaerobic granular sludge from upflow anaerobic sludge-bed (UASB) reactors. The main objective of the present study is to compare the extraction efficiencies of three operationally defined extraction procedures, i.e., the Stover, Modified Tessier and revised BCR procedures, when applied to leaching of metals from anaerobic methanogenic granules from full-scale UASB reactors. This paper compares and discusses in detail the results of the fractionation of key metals important for anaerobic metabolism (Co, Ni, Cu, Zn, Mn and Fe) within two different anaerobic granular sludges.

2. Materials and methods

2.1. Source of anaerobic granular sludge

Methanogenic granular sludges were obtained from a full scale expanded granular sludge bed (EGSB) reactor treating alcohol distillery wastewater (Nedalco, Bergen op Zoom, the Netherlands) and from a full scale UASB reactor treating wastewater of four paper-mills (Industrie-water Eerbeek B.V., Eerbeek, The Netherlands). The sludges were harvested anaerobically and stored at 4 °C until analysis, which were performed within 1 month after sampling. In this study, wet sludge was used, in order to be able to preserve the anaerobic conditions in the sludge as recommended by Lake [12] and Lombardi and Garcia [13]. The effects of different preservation methods on metal fractionation in polluted sediments by using Tessier's sequential extraction procedure have been examined by Rapin et al. [7] and Bordas and Bourg [14]. Lake and McLaren [15] studied the effect of sludge drying on the metal distribution between fractions. Whatever the drying methods applied (air-drying, freeze-drying or oven drying), the partitioning of trace metals is strongly influenced both on sediment and in sludge.

2.2. Reagents and materials

Ultra pure water (Milli-RO, Millipore, Bedford, MA, USA) was used in preparing stock solutions of all reagents, which were of analytical-reagent grade or suprapur quality (Merck, Darmstadt, Germany) and were checked for possible trace metal contamination. All glassware and plastic material used was previously treated for a week in 10% (v/v) suprapur nitric acid and rinsed with ultra pure water.

2.3. Analysis

The total suspended solids (TSS) and the volatile suspended solids (VSS) concentration were determined according to standard methods [16]. The carbonates content was estimated by loss on ignition at 950 °C [17]. The sludge's pH determination was performed following the dilution of 20 g of sludge sample with 20 mL of ultra pure water and stirred thoroughly for 5 min [15].

A Varian Vista-MPX CCD simultaneous inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Varian, Australia) was used for metal determinations. The following wavelengths were employed: 228.802, 213.598, 259.940, 259.372, 216.555, 202.548, and 181.972 nm for Co, Cu, Fe, Mn, Ni, Zn, and S, respectively. A WTW® pH meter was employed for pH adjustments of the reagents and the pH determination of the sludge.

2.3.1. Pseudo-total metal analysis

The pseudo-total metal content of sludge was determined by digestion with the aqua regia procedure [18]. For metal determination, 0.5 g TSS of anaerobic granular sludge was treated with 10 mL of aqua regia in TEFLON® PFA (PerFluoroAlkoxy resin) digestion vessels, in a temperature controlled microwave oven Milestone ETHOS E (Milestone Inc.; Monroe, CT). The application of closed pressurized vessels in combination with microwave heating allows a considerable reduction of the decomposition time for various matrices and an improvement in precision [18]. The control temperature was programmed to 170 °C and maximum microwave power was applied from the beginning (1000 W). If the temperature of one of the 12 vessels exceeded the programmed value, the power was regulated automatically and the temperature was held constant for the remaining digestion time. The heating period was 26 min. After 30 min of cooling, the digests were filtered through a Schleicher & Schuell 589 filter paper into volumetric flasks. The digestion vessels were then rinsed with ultra pure water and the washes were also filtered in the volumetric flasks. The sample volume was completed to 100 mL with ultra pure water.

2.3.2. Sequential extraction procedures

This study compared the sequential extraction procedures developed by Tessier et al. [8] with some modifications [19], Stover et al. [11] and the Commission of the European Communities Bureau of Reference (revised BCR protocol) [20].

Table 1
Operating conditions required in the revised BCR sequential extraction method

Fraction	Extracting agent	Extraction conditions	
		Shaking time[a]	Temperature
F1. Exchangeable + water and acid soluble	40 mL CH_3COOH (0.11 M, pH = 7)	16 h	20 °C
F2. Iron and manganese oxides	40 mL NH_2OH–HCl (0.5 M, pH = 1.5)	16 h	20 °C
F3. Organic matter and sulfides	20 mL H_2O_2 (30%, pH = 2) and then 50 mL CH_3COONH_4 (1 M, pH = 2)	1, 2, 16 h	20, 85, 20 °C
F4. Residual	10 mL demineralised water and 10 mL aqua regia (HCl/HNO_3, 3:1)	26 min	Microwave-oven[b]

[a] Shaking was applied at 30 rpm.

[b] Extraction of the residual fraction in the microwave was equal to the pseudo-total extraction method.

Table 2
Operating conditions required in the modified Tessier sequential extraction method

Fraction	Extracting agent	Extraction conditions	
		Shaking time[a]	Temperature
F1. Exchangeable	10 mL NH_4CH_3COO (1 M, pH = 7)	1 h	20 °C
F2. Carbonates	10 mL CH_3COOH (1 M, pH = 5.5)	1 h	20 °C
F3. Organic matter and sulfides	5 mL H_2O_2 (30%, pH = 2)	3 h	35 °C
F4. Residual	10 mL demineralised water and 10 mL aqua regia (HCl/HNO_3, 3:1)	26 min	Microwave-oven[b]

[a] Shaking was applied at 150 rpm.

[b] Extraction of the residual fraction in the microwave was equal to the pseudo-total extraction method.

The extractants used, the extraction conditions, and the nominal forms of metal considered to be extracted are outlined in Tables 1–3 and were performed as described below. When wet sludges were analysed, the amount of material used was determined from the moisture content to provide the required solid material. The volume and concentration of the first extractant was then adjusted so that the extraction conditions were identical to those used for dry material. The water content of the sludges is reported in the Section 3.1. Between each extraction, the sludge was washed by treating it with 25 mL of ultra pure water, centrifuging and discarding the supernatant. These procedures were followed in order to remove any remaining reagent and metals present in the sludge residue from the previous extraction.

2.3.2.1. Revised BCR scheme. The revised BCR scheme applied (Table 1) is a three-step sequential extraction procedure that was proposed to harmonize and validate the methods used in fractionation studies in soils and sediments [20]. The original BCR procedure was also applied to several solid matrixes such as soil [21], sludge [22], and sediment [23]. Compared to the original BCR procedure, the revised protocol involves the use of an increased concentration of $NH_2OH{\cdot}HCl$ and lower pH as described by Mossop and Davidson [20]. It was designed based on an acetic acid extraction of approximately 1 g TSS of granular sludge (step 1), hydroxylamine hydrochloride extraction (step 2) and hydrogen peroxide oxidation and ammonium acetate extraction (step 3). These extractions are associated to the exchangeable, water and acid soluble phase (bound to carbonate, step 1), the reducible phase (bound to iron and manganese oxides, step 2) and the oxidizable phase (bound to organic matter, step 3). In a natural sediment, soil or sludge, the metals bound to sulfide are mainly leached in step 3 [24]. To extract the residual phase (step 4), a mixture of 2.5 mL HNO_3 (65%) and 7.5 mL HCl (37%) (aqua regia digestion) was added to the residue and the filter from fraction 3. After microwave destruction, the samples of step 4 were paper filtered and diluted to 100 mL with ultra pure water.

2.3.2.2. Modified Tessier scheme. The modified Tessier procedure has been used to study metal distribution in dechlorinating granular sludges [25] and methanogenic granular sludges [19]. The modified Tessier scheme is shown in Table 2. Step 1: *Exchangeable*: approximately, 1 g TSS of

Table 3
Operating conditions required in the Stover sequential extraction method

Fraction	Extracting agent	Extraction conditions	
		Shaking time[a]	Temperature
F1. Exchangeable	30 mL KNO_3, (1 M, pH 7)	16 h	20 °C
F2. Sorbed	48 mL KF (0.5 M, pH = 6.5)	16 h	20 °C
F3. Organically bound	48 mL $Na_4P_2O_4$ (0.1 M)	16 h	20 °C
F4. Carbonates	48 mL EDTA (0.1M, pH = 6.5)	2 × 8 h	20 °C
F5. Sulfides	30 mL HNO_3 (1 M)	16 h	20 °C
F6. Residual	10 mL demineralised water and 10 mL aqua regia (HCl/HNO_3, 3:1)	26 min	Microwave-oven[b]

[a] Shaking was applied at 200 rpm.

[b] Extraction of the residual fraction in the microwave was equal to the pseudo-total extraction method.

granular sludge was extracted with 10 mL of 1 M ammonium acetate (pH 7). After 1 h of shaking at 150 rpm at room temperature, the sample was centrifuged for 10 min at 4000 rpm. The liquid phase was decanted and diluted to 50 mL with ultrapure water. Step 2: *Carbonates*: to the residue of fraction 1, 10 mL of 1 M acetic acid was added. After 1 h of shaking at 150 rpm, it was centrifuged for 10 min at 4000 rpm at room temperature. The liquid phase was decanted and diluted to 50 mL with ultrapure water. Step 3: *Bound to organic and sulfide*: the residue from fraction 2 was treated with 5 mL of a 30% solution of H_2O_2 (pH 2 with nitric acid) and shaken at 35 °C and 150 rpm for 3 h. After centrifugation at 10,000 rpm, the samples were paper-filtered (Schleicher & Schuell 589, Germany) and diluted to 100 mL with ultrapure water. Step 4: *Residual*: a mixture of 2.5 mL HNO_3 (65%) and 7.5 mL HCl (37%) (aqua regia digestion) was added to the residue and the filter from fraction 3. After microwave destruction, the samples were paper filtered and diluted to 100 mL with ultra pure water.

2.3.2.3. Stover scheme. The sequential extraction procedure developed by Stover et al. [11] has been used for this type of biocatalyst by Ilangovan and Noyola [26], Espinosa et al. [27] and Leighton and Forster [28]. The extraction procedure used in the present investigation is the scheme originally described by Stover et al. [11], in which the reagents potassium nitrate (KNO_3), potassium fluoride (KF), sodium pyrophosphate ($Na_4P_2O_7$), sodium ethylene-diamine-tetra-acetic acid (EDTA) and nitric acid (HNO_3) were used for the selective solubilisation of the metals. Details of the extraction procedure are shown in Table 3. Shortly, Step 1: *Exchangeable*: approximately, 0.6 g TSS of granular sludge was extracted with 30 mL of 1 M KNO_3 (pH 7). After 16 h of shaking at 200 rpm at room temperature, the sample was centrifuged for 10 minutes at 4000 rpm. The liquid phase was decanted and diluted to 50 mL with ultra pure water. The solid residue was then washed with 30 mL of ultra pure water, which was discarded. This procedure was repeated with the other reagents, following the order presented in Table 3. Step 2: *Sorbed*: to the residue of fraction 1, 48 mL of 0.5 M KF (pH 6.5) was added. Step 3: *Organically bound*: to the residue of fraction 2, 48 mL of $Na_2P_2O_4$ (0.1 M) was added. Step 4: *Sorbed to carbonates*: to the residue of fraction 3, 48 mL of 0.1 M EDTA (pH 6.5) was added. Step 5: *Bound to sulfides*: the residue from fraction 4 was treated with 30 mL HNO_3 (1 M). Step 6: *Residual*: a mixture of 2.5 mL HNO_3 (65%) and 7.5 mL HCl (37%) (aqua regia digestion) was added to the residue and the filter from fraction 3. After microwave destruction the samples were paper filtered and diluted to 100 mL with ultra pure water.

2.4. Evaluation of analytical performance

The analytical performance of the laboratory procedures was evaluated by analysis of Certified Reference Material BCR-701 and CRM 146R. A two-sided *t*-test was used to check for significant differences from the reference content. Table 4 shows the data of three replicate analyses obtained for aqua regia extraction and Table 5 shows the revised BCR sequential extraction procedure, expressed as mg kg^{-1} of dry mass. Uncertainty is expressed as standard deviations; the values obtained are not significantly ($P > 0.05$) different from the certified values.

Table 4
Results obtained (mean ± standard deviation, $n = 3$) for aqua regia extractable (pseudo-total) metal content of CRM 146R sewage sludge from industrial origin

	Found value (mg kg^{-1})	Certified value (mg kg^{-1})
Co	6.35 ± 0.09	6.50 ± 0.31
Cu	744 ± 7	831 ± 16
Mn	278 ± 4	298 ± 9
Ni	53.3 ± 0.9	65.0 ± 3.0
Zn	2887 ± 29	3043 ± 58

The repeatability of the sequential extraction procedures was checked by subjecting triplicates to each procedure. Moreover, an internal check of the results of sequential extractions was made by comparing the sum of the amounts of the metals removed in each step of the procedures (M_S) with the results of the pseudo-total digestion (M_T). Recovery in the sequential extraction procedures was calculated as follows:

$$\text{Recovery}\ (\%) = \frac{\sum M_S \text{ in the individual fractions}}{M_T} \times 100 \qquad (1)$$

3. Results and discussion

3.1. Sludge characteristics

Table 6 presents the characteristics of the two anaerobic granular sludges investigated. The pH of the sludge is similar in the two sludges, which is similar to the pH value found in methanogenic bioreactors [29]. Eerbeek shows a lower organic matter (biomass + extracellular polymeric substances) content (73.9%) compared to the Nedaldo sludge (91.8%). The carbonate content is quite similar in the two sludges.

The total metal content of both sludges was quite different, which may be due to a variable industrial wastewater influent composition entering the two methanogenic bioreactors [30]. Total sulfur and total phosphorus content in the Eerbeek sludge is twice the content in the Nedalco sludge. The crystalline precipitate that could be detected using X-ray diffraction analysis was the $CaCO_3$ precipitate calcite in the Eerbeek sludge. In the Nedalco sludge, no crystalline Ca, P or Fe phase were detected. The other expected minerals such as sulfides (e.g., FeS or FeS_2) and phosphate (e.g., $Ca_5OH(PO_4)_3$) precipitates are very likely present in an amorphous form [31].

Table 5
Results obtained (mean ± standard deviation, n = 3) for sequential extraction analysis and aqua regia extractable (pseudo-total) metal content of BCR 701 sediment

	Step 1		Step 2		Step 3		Residual		Pseudo-total metal	
	Found value (mg kg^{-1})	Certified value (mg kg^{-1})	Found value (mg kg^{-1})	Certified value (mg kg^{-1})	Found value (mg kg^{-1})	Certified value (mg kg^{-1})	Found value (mg kg^{-1})	Indicative value (mg kg^{-1})	Found value (mg kg^{-1})	Indicative value (mg kg^{-1})
Cu	69.5 ± 2.0	49.3 ± 1.7	120.0 ± 1.5	124 ± 3	53.4 ± 1.3	55.2 ± 4.0	37.3 ± 2.0	38.5 ± 11.2	271.8 ± 5.1	275 ± 13
Ni	15.5 ± 2.0	15.4 ± 0.9	24.0 ± 0.3	26.6 ± 1.3	15.9 ± 0.2	15.3 ± 0.9	42.1 ± 3.0	41.4 ± 4.0	100.0 ± 1.0	103 ± 4
Zn	193.6 ± 2.6	205 ± 6	104.0 ± 2.0	114 ± 5	47.9 ± 1.0	45.7 ± 4.0	105.1 ± 2.7	95 ± 13	449 ± 2.0	454 ± 19

3.2. Comparison between the three extraction schemes

Results of the three extraction schemes for the major and trace elements are presented in Table 7 and Figs. 1 and 2. For each material and extraction, three samples were simultaneously analysed to determine the precision of the measurements. To compare the results obtained by methods that have a different name, the fractions were arranged into four "equivalents" defined in Table 8.

3.2.1. Repeatability and metal recoveries

Table 7a and b show that the BCR procedure provides a satisfactory recovery. In fact, the sum of the three steps and of the residue, expressed as percentages of the total concentration, varies from 90 to 100% for Nedalco sludge (Table 7a) and 97 to 103% for Eerbeek sludge (Table 7b). However, zinc and iron recoveries in Eerbeek show higher values, 111 and 119%, respectively. The modified Tessier scheme shows a satisfactory recovery as well, except for manganese (165%) and cobalt (142%) in Nedalco sludge and iron (118%) in Eerbeek sludge.

The relative standard deviation (RSD) values varied over a wide range and depended on the elements and the extraction stage. Very good precision, usually in the range 1–16%, was achieved for most of the elements analysed. In one case, the RSD value obtained was approximately 41% for cobalt in the Nedalco sludge. Such low precision was observed for measurements of the exchangeable fraction of the Modified Tessier scheme, in which the cobalt concentration was very low or close to their detection limits (Fig. 1a).

The Stover scheme shows a poor percentage recovery for iron in the Nedalco sludge and cobalt, manganese and iron in the Eerbeek sludge. The reproducibility of the Stover scheme is lower than the reproducibility of the modified Tessier and revised BCR schemes, which confirms previous observations made by Rudd et al. [32]. This is very likely due to the higher number of fractions of the Stover scheme, which involve numerous weight, volume, and analytical determinations, and losses of small amounts of material at each stage during centrifugation and decantation. Note that since no suitable certified reference sludge materials were available for validating sequential extraction data of sulphur, iron, cobalt and manganese, the source of variability could not be clearly identified. To ascertain the selectivity of the extraction schemes with respect to sulphur, sequential extraction and total microwave digestion were conducted for sulphur on all sludge samples investigated. Dollar et al. [33] showed that the majority of sulphur is extracted in the organic/sulfides fraction.

Table 6
Comparison of the basic characteristics of the two anaerobic granular sludges (mean ± standard deviation, n = 3)

Parameters	Eerbeek sludge	Nedalco sludge
pH of the sludge	6.8 (±0.1)	7.2 (±0.1)
Total suspended solids (TSS) (%)	22.6 (±0.2)	8.3 (±0.2)
Volatile suspended solids (VSS) (% of TSS)	73.9 (±0.2)	91.8 (±0.2)
Carbonates (% of TSS)	0.4 (±0.2)	0.8 (±0.2)
Total sulfur (mg g^{-1} TSS)	41.8 (±1.0)	22.7 (±0.1)
Total phosphorus (mg g^{-1} TSS)	6.6 (±0.1)	3.3 (±0.1)

3.2.2. Exchangeable and acid soluble fractions

The exchangeable and acid soluble fractions show the amount of each element that would be released by the anaerobic sludge if the conditions become slightly acidic (pH < 7.0). The comparison of leaching data between the exchangeable/carbonates fraction from the BCR scheme, the sum of exchangeable and carbonates fractions from the modified Tessier scheme and the sum exchangeable + sorbed + carbonates fractions of the Stover scheme shows some interesting trends. The amount of cobalt, nickel, zinc and copper that was extracted in these fractions is in the same order of magnitude between each sequential extraction scheme in the two sludges. The extraction results for the exchangeable/carbonates fraction indicate that three extraction methods are performing similarly. For instance, the concentration of extracted Cu is low, which is consistent with its chemistry being predominantly bound as sulfides, which do not dissolve from anaerobic solid phases at this stage [34].

The distribution of nickel shows a different behaviour in the Nedalco sludge compared to the Eerbeek sludge. Nickel shows a higher amount in the labile fraction like carbonates and exchangeable fractions compared to the organic matter/sulfides fraction in the BCR and modified Tessier scheme. In the BCR scheme, the labile fraction of nickel is shown in the exchangeable/carbonates fraction (36% of the total metal extracted). For the modified Tessier scheme, the carbonate and exchangeable fractions represent 53% of the total metal extracted. In contrast, the Stover scheme shows a

Table 7
Repeatability of the three extraction procedures (mean ± %RSD, n = 3)

Nedalco	Co	Ni	Cu	Zn	Mn	Fe[a]
(a) The Nedalco sludge						
Tessier						
Σ	26.6 (±41%)	147.8 (±6%)	686.7 (±6%)	1387.7 (±9%)	62.7 (±3%)	27.1 (±3%)
M_T	18.7 (±3%)	130.3 (±3%)	690.0 (±1%)	760.0 (±3%)	54.8 (±1%)	20.8 (±1%)
Recovery (%)	142	108	91	165	114	104
BCR						
Σ	18.6 (±10%)	130.5 (±6%)	708.6 (±6%)	817.3 (±6%)	49.2 (±5%)	24.4 (±5%)
M_T	18.7 (±3%)	130.3 (±3%)	690.0 (±1%)	760.0 (±3%)	54.8 (±1%)	20.8 (±1%)
Recovery (%)	100	94	93	90	95	97
Stover						
Σ	20.0 (±11%)	125.9 (±10%)	680.9 (±7%)	775.0 (±10%)	57.0 (±16%)	10.7 (±16%)
M_T	18.7 (±3%)	130.3 (±3%)	690.0 (±1%)	760.0 (±3%)	54.8 (±1%)	20.8 (±1%)
Recovery (%)	107	92	91	92	103	40
(b) The Eerbeek sludge						
Tessier						
Σ	60.5 (±5%)	41.7 (±13%)	130.5 (±3%)	208.9 (±6%)	238.4 (±1%)	46.9 (±1%)
M_T	59.4 (±3%)	38.3 (±2%)	124.6 (±4%)	195.7 (±4%)	275.0 (±3%)	39.8 (±2%)
Recovery (%)	102	109	105	107	87	118
BCR						
Σ	58.5 (±4%)	39.6 (±5%)	121.4 (±5%)	216.6 (±5%)	278.3 (±3%)	47.3 (±6%)
M_T	59.4 (±3%)	38.3 (±2%)	124.6 (±4%)	195.7 (±4%)	275.0 (±3%)	39.8 (±2%)
Recovery (%)	98	103	97	111	101	119
Stover						
Σ	50.5 (±13%)	39.7 (±5%)	137.7 (±8%)	210.9 (±19%)	185.8 (±5)	26.6 (±8%)
M_T	59.4 (±3%)	38.3 (±2%)	124.6 (±4%)	195.7 (±4%)	275.0 (±3%)	39.8 (±2%)
Recovery (%)	85	103	110	108	68	67

[a] Note that iron content is expressed as mg g^{-1} TSS, Σ represents the sum of the different extraction step and M_T represents the pseudo total metal concentration.

lower amount: the exchangeable, loosely sorbed and carbonate fractions represent only 26% of the total metal extracted. This is lower compared to the results from the BCR scheme (Fig. 1). Indeed, the EDTA extractant, incorporated in the Stover scheme specifically to extract carbonate metal species, has been shown of limiting efficiency to extract nickel carbonate from sewage sludge [32].

A significant amount of iron and manganese was released in this fraction by the BCR and modified Tessier scheme. For instance the modified Tessier scheme released in total 12.5 (±1%) mg g^{-1} TSS and 7.5 (±1%) mg g^{-1} TSS for respectively the Nedalco and Eerbeek sludges. Iron was mainly released in the carbonates fraction of the modified Tessier scheme (e.g., 42% of total iron extracted in the Nedalco sludge). Those results are not comparable with the results from the Stover scheme due a very low percentage recovery of iron in the Nedalco sludge (Fig. 1 and Table 6).

Manganese is mainly extracted in the exchangeable and carbonates fractions of the BCR and modified Tessier schemes, respectively, 39.5 and 72% of the total manganese extracted in the Nedalco sludge. As for the Nedalco sludge, the Eerbeek sludge shows that a great part of manganese is bound to the labile fraction. For instance, manganese is also released in the carbonates fraction in the three sequential schemes used: 53, 52, and 46% in the modified Tessier, BCR and Stover schemes, respectively. Lombardi and Garcia [13] described the same trends in anaerobic municipal sewage sludge applying a slightly modified version of the Stover scheme. Jong and Parry [34] showed that the carbonates fraction was an important binding phase for Fe in the solid-phase present in sulfate reducing bioreactors. This can be attributed to the solubilisation of $FeCO_3$ and $MnCO_3$ in acidic solutions of sodium acetate rather than to the attack of Fe and Mn sulfides [6,12]. Moreover, the low concentration of sulfur indicates that only a limited dissolution of sulfides occurred (Figs. 1 and 2).

3.2.3. Reducible fraction: Fe/Mn oxides fraction in BCR scheme

This fraction theoretically represents the contents of metals bound to iron and manganese oxides that are released if the matrix is subjected to reductive conditions. However, iron and manganese oxides are relatively scarce in this organic-rich, reduced granular sludge (confirmed by XANES analysis, data not shown). That is why the modified Tessier and the Stover methods were designed to work with anaerobic matrixes (e.g., no reducible fraction). The BCR method shows, nevertheless, a high liberation of iron (and manganese in a lesser extend) for the two sludges studied (Figs. 1 and 2). For instance, the Fe/Mn oxides fraction released amounted to 8.4 (±1.5%) mg g^{-1} TSS and 15.0 (±2%) mg g^{-1} TSS of iron respectively for Nedalco and Eerbeek sludges, which

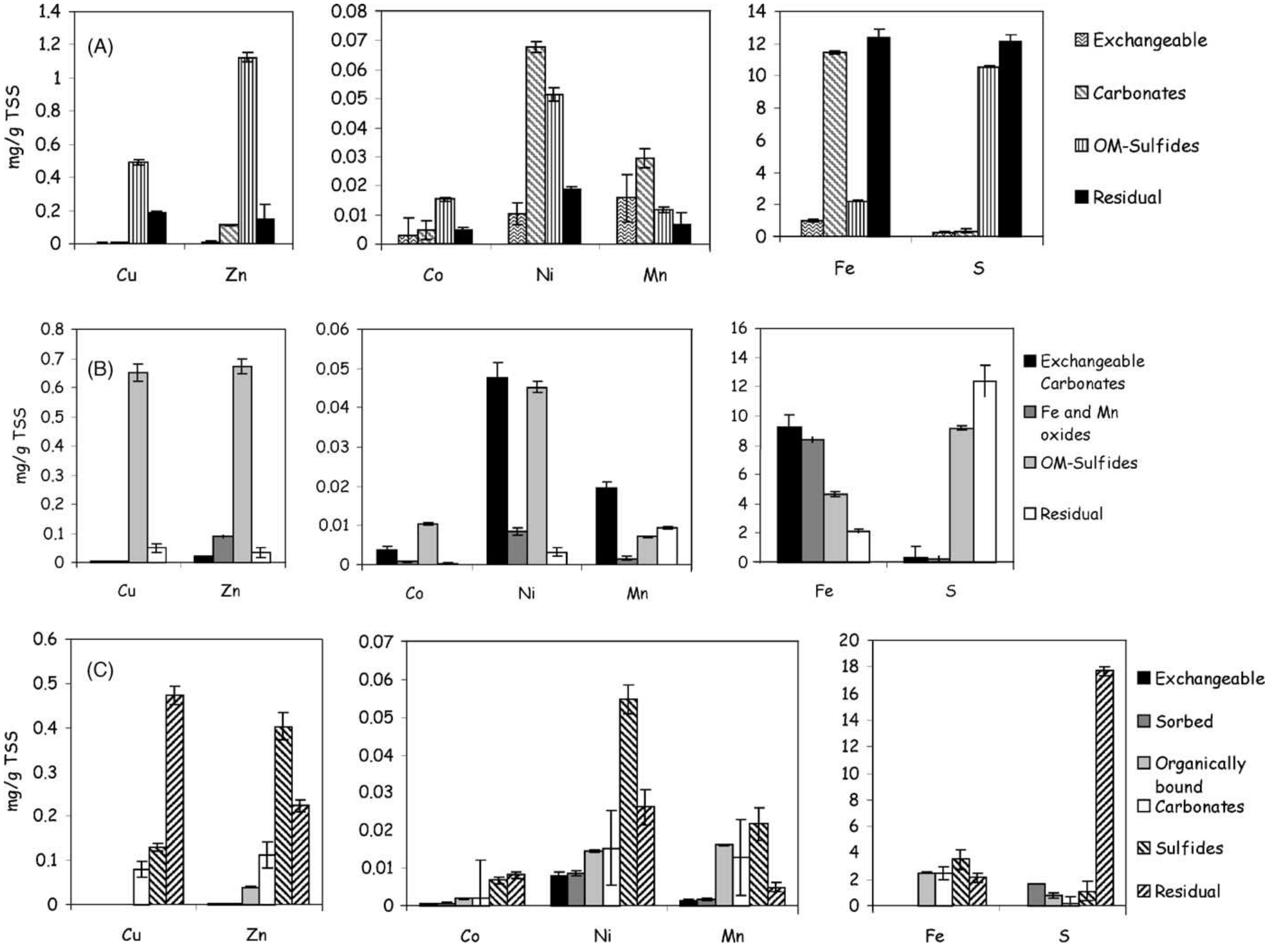

Fig. 1. Metal partitioning profiles in Nedalco sludge according to the modified Tessier (A), BCR (B) and Stover (C) scheme (mean ± standard deviation, $n = 3$).

is respectively 34 and 31% of total iron extracted in each sludge. Problems observed during sequential extraction of iron were also emphasized in the case of anoxic sediments [24,35]. Ngiam and Lim [35] showed that anoxic sediment samples were found to have been oxidized during the extraction stage for the reducible fraction under the BCR scheme, despite the maintenance of an oxygen-free environment. This artefact has resulted in an over-representation of the reducible fraction and an under-representation of the organic/sulfide fraction in the heavy metal speciation patterns of anoxic sediment samples [35].

The BCR technique produces negligible concentrations of Cu and low but significant concentrations of cobalt, nickel and zinc (Figs. 1 and 2). Lacal et al. [24] showed that metals bond to sulfide can be extracted in the reducible fraction of sludge from pyrite mining with the BCR scheme. This probably reflects the greater solubility of zinc sulfides, known to dissolve in acidified reagents at pH 5 [36], rather than destruction of Fe/Mn oxides in the granular sludges. Moreover, a modified Tessier method, supplemented with an AVS collection system instead of agitation during the pre-oxidation extraction steps, was carried out by Flyhammar [37]. The latter author's results confirm the earlier assumptions by Wallman et al. [36] that sulfide solid phases are dissolved in various extraction steps along the extraction sequence and not only in the "sulfide" fraction. Further evidence of this is the presence of low sulfur concentrations (Figs. 1 and 2) and a distinct hydrogen sulfide smell from the reaction vessel observed while performing the extractions.

The very low concentrations of Cu in this fraction (Figs. 1 and 2) could have resulted from the re-precipitation of Cu originally bound in non-sulfidic sludge phases (e.g., organic matter) as stable sulfides [36]. One of the problems which appears is that it releases substantial amounts of trace elements bound to the organic matter/sulfides fraction. Consequently, the recovery of trace elements in the oxides fraction may be overestimated at the expense of the oxidisable fraction [6,38] as shown in the present study with the BCR scheme (Figs. 1 and 2). To illustrate this last statement, the quantity of cobalt, nickel and zinc extracted in the

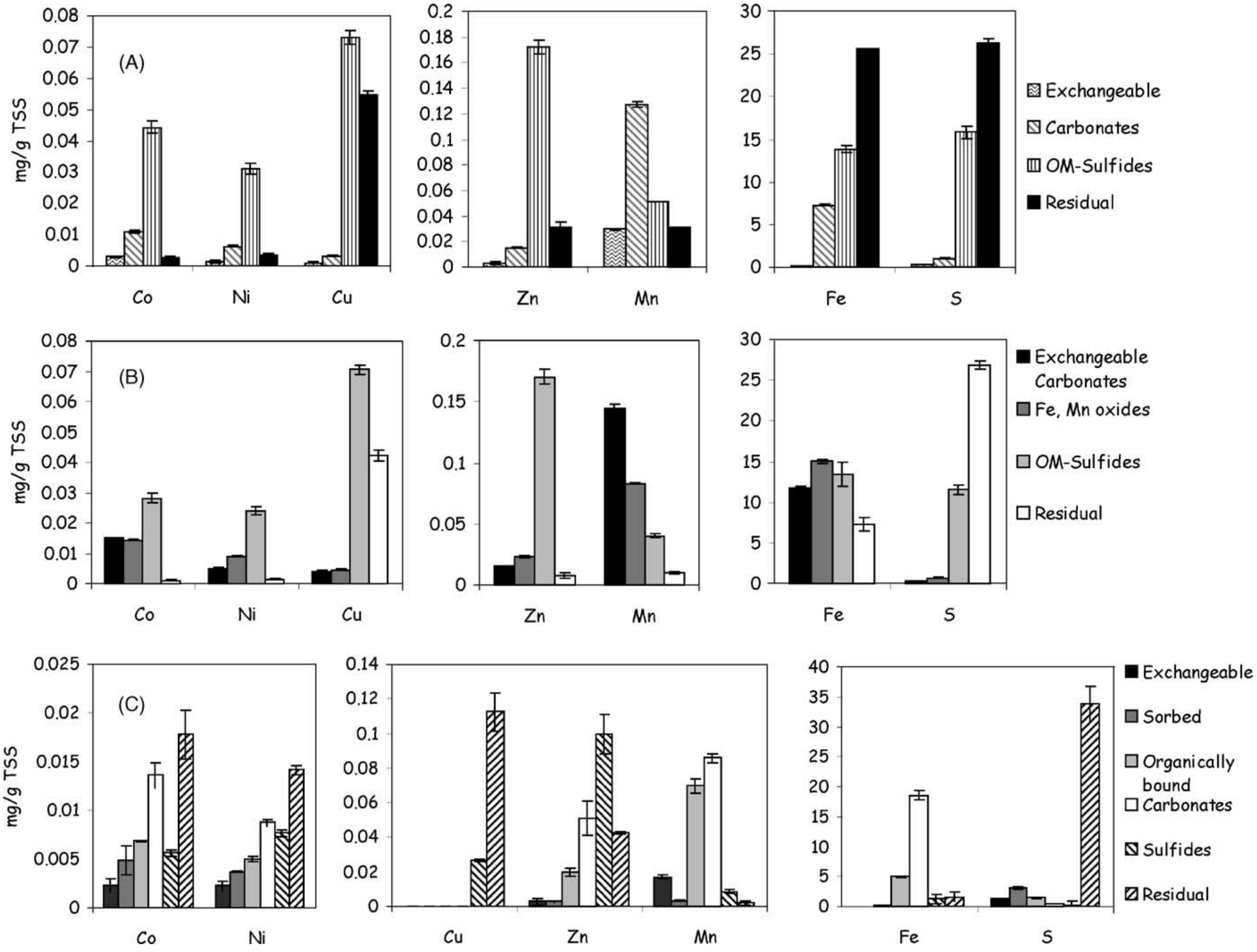

Fig. 2. Metal partitioning profiles in Eerbeek sludge according to the modified Tessier (A), BCR (B) and Stover (C) scheme (mean ± standard deviation, $n = 3$).

organic/sulfides fraction of the BCR scheme is always significantly lower than the quantity extracted in the modified Tessier scheme (Figs. 1 and 2).

3.2.4. Oxidisable fractions: organic matter/sulfides fraction

The metals extracted from the organic matter/sulfides fraction determined for the Stover scheme was calculated by the addition of the amount of metals extracted from the organically bound and sulfides fractions shown in Figs. 1 and 2. The three schemes show the same fractionation features for cobalt, zinc and copper in the Nedalco sludges (Fig. 1). These metals are mainly bound to the organic matter/sulfides and residual fractions (75–99% of total metal extracted). The Stover fractionation results show that the metals extracted are quite different in the organically bound fraction and the sulfide fraction. In the Stover procedure, the sulfide fraction shows a higher amount of cobalt, copper and zinc extracted (34, 19, and 52%, of total metal extracted, respectively) compared to the organic fraction (9.5, 0, and 5% of total metal extracted, respectively). This result illustrates the great role played by the sulfide phase in the scavenging of metals in such anaerobic solid phases, as reported by Lake [12].

Concerning the metal extractability in oxidisable fraction, the calculated average percentage quantity of oxidisable metal (BCR or modified Tessier schemes) in this fraction for the Nedalco sludge increases in the order Mn < Fe < Ni < Co < Zn < Cu, which widely corresponds to the Irving–Williams order for the sequence of complex stability [39], but also to the stability of the respective sulfidic metal compounds. This observation is in agreement with the studies of Kersten and Forstner [40] on anoxic freshwater sediments and Jong and Parry [34] on solid-phase materials from a bacterial sulfate reducing bioreactor. For the Eerbeek sludge (Fig. 2), the average percentage quantity of oxidisable metal in this fraction (BCR or modified Tessier schemes) increase in the order Mn < Fe < Cu < Co $\cong$ Ni < Zn. This classification does not completely fit with that of the Nedalco sludge. This is very likely due to lower extractability of copper as described in the following section.

Table 8
Equivalent fractions used

Procedure	Equivalent fractions			
	Acid soluble	Reducible	Oxidisable	Residual
BCR	F1	F2	F3	F4
Modified Tessier	F1 + F2		F3	F4
Stover	F1 + F2 + F4		F3 + F5	F6

3.2.5. *Residual fraction*

A much higher percentage of copper is noticed in the residual fraction of the BCR and modified Tessier scheme of the Eerbeek sludge compared to the Nedalco sludge. The percentage of copper in the residual fraction (34–42 % of the total metal extracted) is higher than the residual percentage of cobalt, nickel and zinc (2–14 % of the total metal extracted). Copper is expected to be present as copper sulfide species due to the concomitant release of sulphur in the residual fraction. These results show that the copper sulfide species are more refractory to the H_2O_2 oxidation step compared to the nickel, cobalt and zinc sulfides especially in the case of Eerbeek sludge. Moreover, the Eerbeek sludge contains a much higher content of total sulphur (likely as sulfide), iron and organic matter which would explain the lower extraction efficiency of copper (Tables 6 and 7). In some cases, some modifications of the initial Tessier scheme to dissolve the target phase have been attempted. For instance, an increased contact time (from 5 to 12 h compared to the initial Tessier protocol) or amount of H_2O_2 added in the oxidisable fraction was needed when applying the Tessier scheme to organic-rich sediments [33]. Dollar et al. [33] showed that these modifications allowed to extract the majority of sulphur in the organic/sulfides fraction.

In both sludges studied, the amounts of cobalt, nickel, zinc and copper extracted in the residual fractions are higher for the Stover scheme than for the BCR and the modified Tessier schemes. For instance, the residual fraction of cobalt, nickel and zinc (1–14% of the total metal extracted) in the modified Tessier and BCR schemes is much lower than the residual fraction of these metals in the Stover scheme (20–35% of the total metal extracted) (Eerbeek sludge, Fig. 2). These latter differences can be explained by the different strength of the extractant used in the modified Tessier and BCR schemes (H_2O_2) and Stover scheme ($Na_4P_2O_7$ + nitric acid 1 M) in the steps before the residual fraction extraction. The lower metal extracted in the modified Tessier and BCR scheme can be linked to the lowest amount of sulfur extracted simultaneously. The Stover scheme releases in the Eerbeek sludge only 1% of total sulfur in the sulfides fraction compared to the 84% in the residual fraction (Fig. 2). Moreover, the amount of sulfur extracted in the sulfides fraction with the Stover scheme is almost five times less than in the other schemes. This outlined the weakness of the reagent used in this step. Basically, the Stover scheme was designed with the addition of 1 M HNO_3 to dissolve the sulphidic fraction [11]. However, Oake et al. [41] suggested the addition of 6 M HNO_3 for increased efficiency in the extraction of sulfide forms (Lake [12]). However, even with higher HNO_3 concentration, Rudd et al. [32] showed that copper and zinc sulfides precipitates are relatively difficult to extract in the sulfides fraction of the Stover scheme. In this respect, Rudd et al. [32] suggested that it may be more appropriated to classify the sulfides and residual fractions as a single phase.

4. Conclusions

The results presented in this article imply that sequential extraction methods can be used to identify qualitative differences in the partitioning of metals in anaerobic granular sludges collected from full-scale anaerobic bioreactors. The sequential extraction scheme recommended by BCR and the modified Tessier scheme are sufficiently repeatable and reproducible for application in fractionation studies. However, the BCR scheme seems to be of limited utilisation to study anaerobic biofilm matrixes. The reproducibility of the Stover scheme is significantly lower than the reproducibility of the modified Tessier and revised BCR schemes. Moreover, the Stover scheme was shown to poorly extract sulfur from the sulfide fraction compared to the BCR and modified Tessier schemes and requires a further modification of the extractant used to extract the sulfidic precipitates in this fraction.

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