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A Preliminary Study of the Effect of Bioavailable Fe and Co on the Anaerobic Digestion of Rice Straw

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Abstract: Rice straw is an abundant and sustainable substrate for anaerobic digestion (AD), but it is often deficient in essential trace elements (TEs) for proper microbial growth and metabolism. A lack of TEs leads to AD imbalances and suboptimal biogas yields. However, the total TE concentration is not a sufficient indicator of the amount of TEs available to the microorganisms. Therefore, this study investigated the degree of bioavailability of iron (Fe) and cobalt (Co) during the AD of rice straw, and correlated it to the biomethane yields and volatile fatty acids (VFAs) produced. When the two TEs were dosed at 205 μg Fe/g TS and 18 μg Co/g TS of rice straw, the biomethane production was approximately 260 mL CH_4/g VS, i.e., similar to that obtained when Fe and Co were not added. Despite an increased bioavailable fraction of 23 and 48% for Fe and Co, respectively, after TEs addition, the AD performance was not enhanced. Moreover, VFAs did not exceed 250 mg HAc/L both in the presence and absence of added TEs, confirming no enhancement of the methanogenesis step. Therefore, the bioavailability of Fe and Co was not a limiting factor for the biomethane production at low total VFAs concentration.

Keywords: anaerobic digestion; bioavailability; biogas; rice straw; sequential extraction; trace elements

1. Introduction

In the last two decades, pressing issues—such as world population increase, global warming, growing energy demand, and the need for national energy security and rural economic development—have been the driving forces urging for a switch from traditional fossil fuels towards sustainable energy sources [1]. Biomethane, produced through the anaerobic digestion (AD) process, has the potential to yield more energy than any other available biofuel, such as bioethanol or biodiesel [2], and is normally used in combined heat and power (CHP) units for the production of heat and electricity or directly injected in the gas grids.

Biomethane can be produced from a wide range of lignocellulosic materials (LMs), with the term LM referring to any plant dry matter, which is mainly composed of carbohydrate (i.e., cellulose and hemicellulose) and aromatic (i.e., lignin) polymers [3]. Waste LMs represent the most promising renewable organic feedstock for biogas generation, since their production does not compete for arable land [4]. More than 3 trillion kilos of LMs are available from agricultural sources every year and methane yields from the AD of crop residues are in the range of 3200 to 4500 cubic meters per hectare per year [5]. Rice straw is one of the most common agricultural wastes and its biogas production potential is appealing to both developed and developing countries [6]. After rice harvesting, rice straw can be used as a source of feed for ruminant livestock [7], being highly rich in polysaccharides. However, in many cases the straw is left unused or burnt in the fields, causing serious environmental problems [8]. Methane (CH_4) and carbon dioxide (CO_2) are considered as the major gases responsible for global warming [9], thus it is essential to reduce the self-decomposition or field burning of rice straw and employ the generated biogas for useful purposes without releasing it into the atmosphere.

In recent years, several studies have focused on the role of trace elements (TEs) within anaerobic processes [10]. The impact of different TEs on the AD of food waste, wastewater sludge and energy crops has largely been investigated, and positive effects of TE addition on the biogas production have been observed [11–13]. In contrast, the TE requirements of biogas reactors operating with agricultural residues have rarely been reported in the scientific literature, despite these types of feedstock usually show a low content of essential TEs [14,15]. The microbial growth and the whole anaerobic fermentation process depend on the optimal supply of TEs and their availability to the microorganisms [16]. The requirement of methanogenic archaea for TEs such as iron (Fe), nickel (Ni), cobalt (Co), molybdenum (Mo), selenium (Se), and tungsten (W) has been documented [17,18]. However, the recommended values are spread over a wide range of concentrations, suggesting that the presence of a certain TE does not necessarily imply that the microorganisms are able to take up the TE and incorporate it into the catalytic center of the enzymes [19].

A deficiency in the required supply of TEs can lead to AD imbalances, mainly resulting in the accumulation of volatile fatty acids (VFAs). An acidification of the reactor leads to suboptimal biogas yields or, in the worst cases, a complete failure of the AD process [20]. On the other hand, supplying an excessive amount of TEs could provoke inhibitory or toxic effects to the microorganisms, and eventually to the environment through the application of high TE content digestates in agricultural fields [21]. When it is impossible to recover methanogenesis, the fermentation of lignocelluloses can be stopped at the production of VFAs, which are known to be the building blocks for many biorefinery applications [22].

In addition to a detailed physicochemical analysis of the feedstock and inoculum used in the anaerobic reactors, the bioavailability of TEs should be determined as well. Indeed, the total and the bioavailable amounts of each TE can be substantially different from each other [23], making the determination of the total TE concentration insufficient to fully evaluate the effect of a lack or excess of TEs on AD [21]. Bioavailability is defined as the degree to which TEs are available for metabolic activity, and is usually rather difficult to determine given the complexity of an AD system in terms of ongoing processes, compounds, and microorganisms involved [24]. Therefore, the bioavailable TE fractions are approximated with sequential extraction techniques to assess the distribution pattern of each analyzed TE [25]. The rationale behind this procedure is that, during a chemical sequential extraction, the pool of bioavailable TEs decreases after each extraction step [23]. The first TE fractions to be extracted are the water soluble and the exchangeable, which are commonly considered highly bioavailable [23,26]. Thus, an appropriate knowledge of the presence of these two fractions for each considered TE is essential for an adequate supplementation strategy in AD systems [19].

Fe and Co are among the most used TEs in anaerobic bioprocesses, but the influence of their bioavailable fractions on the AD of a lignocellulosic material was investigated for the first time in this study. Therefore, the main objective of this study was to determine whether the bioavailability of Fe and Co could be a limiting factor for biomethane production using rice straw as a substrate. Fe was added both alone and in combination with Co to batch bottles fed with rice straw and inoculated with an agro-zootechnical digestate. A sequential extraction technique was applied to assess whether the supplemented TEs could enhance the bioavailable fractions of Fe and Co and, thus, affect the biomethane production yield of rice straw. Biochemical methane potential (BMP) tests were conducted under mesophilic conditions to determine the biomethane production yield and the VFA concentration under each operating condition.

2. Materials and Methods

2.1. Feedstock and Inoculum

Rice (*Oryza sativa*) straw, obtained from agricultural fields in Pavia (Italy), was used as the sole substrate in this work. After collection, the straw was manually cut down to a particle size smaller than 4 mm. The inoculum used in this work was a agro-zootechnical digestate from a full-scale AD plant treating buffalo manure and milk whey from a mozzarella factory located in Capaccio (Italy). The inoculum was degassed by incubating it under mesophilic conditions (37 ± 2 °C) for 4 days before starting the experiments. The physicochemical characterization of the rice straw and the inoculum is reported in Table 1.

Table 1. Physicochemical characterization of the rice straw and inoculum used in the BMP tests in terms of solids, nitrogen, trace elements, and lignocelluloses. Analyses were performed in triplicate.

Parameter	Rice Straw	Inoculum
TS (%)	94.2 ± 0.2	4.3 ± 0.2
VS (%)	80.0 ± 0.7	2.8 ± 0.1
TKN (g N/kg TS)	11.2 ± 0.2	27.1 ± 1.3
Iron (Fe) (μg/g TS)	477 ± 81	4634 ± 57
Copper (Cu) (μg/g TS)	17 ± 5	19 ± 0.2
Zinc (Zn) (μg/g TS)	62 ± 25	69 ± 2.0
Cobalt (Co) (μg/g TS)	<1.0	2 ± 0.1
Nickel (Ni) (μg/g TS)	2 ± 0.0	10 ± 0.1
Selenium (Se) (μg/g TS)	<1.0	<1.0
Sodium (Na) (mg/g TS)	0.4 ± 0.0	13.8 ± 1.4
Magnesium (Mg) (mg/g TS)	1.1 ± 0.0	12.7 ± 0.4
Potassium (K) (mg/g TS)	14.5 ± 0.4	83.2 ± 7.1
Calcium (Ca) (mg/g TS)	9.1 ± 0.6	33.5 ± 1.6
Cellulose (%)	28.6 ± 0.2	-
Hemicellulose (%)	19.5 ± 1.2	-
Lignin (%)	17.3 ± 0.3	-

TS: total solids; VS: volatile solids; TKN: total Kjeldahl nitrogen.

2.2. Trace Elements Dosing Strategy

The representative concentrations of Fe and Co, together with some other TEs present in the rice straw and the agro-zootechnical digestate, are detailed in Table 1. Based on the results of previous studies [27,28], the bottles containing rice straw were inoculated with an agro-zootechnical digestate and Fe was added in a concentration of 205 μg Fe/g TS of rice straw. Analogously, another set of batch bottles containing rice straw was supplemented with a cocktail of Fe and Co. The amount of Co supplemented in the BMP tests was 18 μg Co/g TS of rice straw, determined on the basis of the recommended values from the literature [28], adjusted according to the results obtained by Mancini et al. [15]. Stock solutions were prepared using the $FeCl_3 \cdot 6H_2O$ and $CoCl_2 \cdot 6H_2O$ salts (analytical grade, Sigma-Aldrich, Germany), and the required amount of each TE was injected in the batch bottles at the beginning of the experiment.

2.3. BMP Tests

The biochemical methane production (BMP) tests were conducted at 37 (±2) °C in 250 mL glass bottles, loaded with 2.5 g of rice straw and 142.0 g of the agro-zootechnical digestate, in order to maintain an inoculum to substrate ratio of 2.0 g VS/g VS. Deionized water was added to reach a working volume of 150 mL into each bottle. Blank samples containing only inoculum and deionized water were also prepared in order to determine the biomethane production of the inoculum, which was then subtracted from the production of the BMP tests containing rice straw. Five bottles were prepared for each configuration, sacrificing two of them on day 5 for sequential extraction analysis and leaving a triplicate until day 40. The biomethane production was measured using a liquid displacement method, as also described by Mancini et al. [29]. The biogas from each BMP preliminary passed through an alkaline solution at 12% NaOH to entrap the CO_2, while releasing the only CH_4. Then, the CH_4 entered a glass container filled with deionized water. The volume of water collected in a graduated cylinder was considered equal to the amount of CH_4 produced. The VFA analysis was performed by daily sampling 1.0 mL of the liquid phase from each bottle during the first 10 days of the BMP tests and on day 15.

2.4. Sequential Extraction Protocol

To investigate the chemical speciation of the TEs in the BMP tests containing (i) only inoculum, (ii) rice straw, (iii) rice straw supplemented with Fe, and (iv) rice straw supplemented with Fe and Co, a sequential extraction technique was used following the procedure used by Ortner et al. [23]. The applied technique is founded on the principle of the Tessier extraction method, which uses different extraction solvents in order to solubilize specific fractions of metals [30]. In particular, the total TE amount was divided into five different fractions, which were determined following the scheme reported in Table 2.

Table 2. Extracting agents and conditions applied in the sequential extraction protocol to determine the highly bioavailable (F1 and F2) fractions of Fe and Co supplemented to the rice straw used in this study for AD.

Fraction	**Extracting Agent**	**Extracting Conditions** [a]		
		pH	**Temperature**	**Shaking Time**
F1—water soluble fraction	-	-	-	-
F2—exchangeable fraction	10 mL 1M NH_4CH_3COO	7.0	25 °C	60 min
F3—carbonate fraction	10 mL 1M CH_3COOH	5.5	25 °C	60 min
F4—organic matter and sulfide fraction	10 mL H_2O_2 (30% w/w)	2.0	35 °C	180 min
F5—residual fraction [b]	10 mL HNO_3	-	-	-

[a] Samples were centrifuged at 3000 rpm for 15 min. [b] The residual fraction was determined using the same procedure adopted for the total concentration.

The bioavailability of TEs decreases in each subsequent fraction from F1 to F5, with the water soluble (F1) and exchangeable (F2) fractions being considered highly bioavailable [26]. The fractions F3-F5 are either bound to particulate matter or precipitates and the TE mobilization depends on the aqueous solubility, which is generally poor in the case of metal carbonates (F3) and sulfides (F4). The residual fraction (F5) is non-extractable and non-dissolvable, and is commonly considered not bioavailable [23].

Fresh material with a TS content of about 1.0 g was collected on day 5 from two bottles of each different BMP configuration. This sample was placed in centrifugation tubes, which were filled up with deionized water to a total weight of 50 g. After centrifugation at 3000 rpm in a Rotina 420 centrifuge (Hettich, Germany) for 15 min, the supernatant, representing the water soluble fraction (F1), was collected and stored at 4 °C until further analysis. As reported in Table 2, 1.0 g of dried material was extracted with ammonium acetate to determine the exchangeable fraction (F2), which was analyzed in the supernatant. The pellet obtained from this extraction step was further extracted with acetic acid, in order to quantify the TEs bound as carbonate (F3). Then, the pellet was extracted with hydrogen peroxide, and the TEs in the supernatant represented the fraction bound to the organic matter and the sulfide precipitates (F4). Finally, the pellet remaining at the end of the extraction process was digested with nitric acid in the microwave in order to determine the residual fraction (F5). The entire procedure was performed ensuring no contact with oxygen.

2.5. Analytical Methods

Total (TS) and volatile solids (VS) were determined by thermally drying (i.e., at 105 °C) and incinerating (i.e., at 575 °C) the samples, according to the method described by Sluiter et al. [31]. Total Kjeldahl nitrogen (TKN) was measured according to the Kjeldahl method [32]. The rice straw was analyzed for its structural carbohydrates and lignin content according to the H_2SO_4 hydrolysis-based procedure described by Sluiter et al. [33]. The TE content and VFA production were determined by inductively coupled mass spectroscopy (ICP-MS) (X-Series, Thermo Fisher Scientific, Waltham, MA, USA) and gas chromatography (GC) (Varian 430-GC, Varian Inc., Palo Alto, CA, USA), respectively, as described by Mancini et al. [15]. The samples for total TE determination were prepared by digesting 0.5 g of dried material with 10 mL of 65% concentrated HNO_3 in a microwave (MARSXpress, CEM, Matthews, NC, USA) at 175 °C for 20 min.

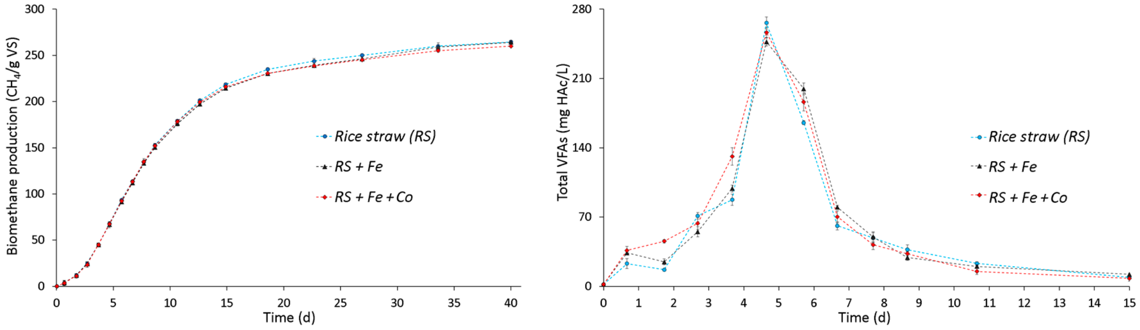
2.6. Statistical Analysis

The statistically significant difference between the biomethane yields obtained in the different BMP tests, in the presence and in the absence of Fe and Fe + Co, was determined by a paired *t*-test. The statistical package of Microsoft Excel 2016 (Microsoft Corporation, Washington, DC, USA) was used considering a 95% confidence interval.

3. Results and Discussion

3.1. Effect of Fe and Co Addition on Biomethane Production and VFA Accumulation from Rice Straw

Figure 1a shows that the cumulative biomethane production of the rice straw in the presence of the agro-zootechnical digestate was 264 mL CH_4/g VS. However, the addition of Fe did not result in an increased biomethane production yield, which remained at 263 mL CH_4/g VS (Figure 1a), i.e., similar to that obtained in the absence of Fe dosing. Despite the Fe addition, the VFA production also remained unchanged compared to the batch bottles where no TE was supplemented (Figure 1b).



(**a**) (**b**)

Figure 1. Biomethane production (**a**) and VFA accumulation (**b**) during the AD of rice straw (i) without trace element supplementation, (ii) with added Fe alone, and (iii) with a cocktail of Fe + Co.

One of the first studies on Fe dosing in AD observed that the presence of Fe prevented VFA accumulation, especially at increasing organic loading rates (OLRs) [27]. A Fe concentration of approximately 250 mg/L was used to stabilize daily-fed batch digesters loaded with cow dung and poultry litter waste, observing an increased methanogenesis by 40%, linked to an enhanced utilization of VFAs and an increased number of methanogens in the reactor [27]. Fe addition was, thus, beneficial to both enhance the acetic acid consumption and increase the biogas production yield. In contrast, the VFAs did not accumulate up to inhibitory levels in this study, with the total VFA production exceeding 250 mg HAc/L only on day 5 (Figure 1b). This suggests that the amount of Fe required by the methanogens for the conversion of acetate to methane during the AD of rice straw was sufficiently high in the inoculum (Table 1, Figure 2) and an additional TE supplementation was likely not required. In contrast, in case the inoculum used had been collected from a TE-deficient digester, the external dosing of Fe would have been necessary to increase the biomethane production and make the process and biomass more robust, or would have allowed to observe an acute inhibitory effect on the microorganisms.

The stimulatory effect of Fe on biogas production has also been reported elsewhere. Moestedt et al. [34] reported a reduced VFA concentration linked to Fe addition during the anaerobic co-digestion of the organic fraction of municipal solid waste (OFMSW) and slaughterhouse residues, also supported by an increase of methanogenic communities, in semi-continuous laboratory scale reactors. Biogas production yields and rates increased by 9 and 35%, respectively. Besides stimulating the activity of microbial enzymes, the addition of Fe has also been observed to positively counteract the sulfide inhibition in reactors loaded with manure or OFMSW and beech sawdust [35,36]. Sulfide precipitated as ferrous sulfide, with a subsequent benefit for the biogas production yields. Therefore, these studies suggest that the AD of more rapidly biodegradable materials benefits more from Fe dosing than that of complex lignocellulosic substrates such as rice straw. In addition, the effect of supplemented TEs seems to prevail over that of elements already present in the inoculum/substrate mixture when using a semi-continuous operational strategy [34,36]. Indeed, the use of higher OLRs and more stressing conditions for methanogenic archaea better justifies the dosing of extra TEs.

Supplementing Co together with Fe in the BMP tests also led to negligible effects in terms of enhancement of the biomethane yield, despite the recognized importance of Co as co-factor for several enzymes involved in both the acetoclastic and hydrogenotrophic methanogenesis pathways (i.e., acetyl-CoA decarbonylase, Co dehydrogenase, methyl-CoM reductase and methyl-H4SPT:HS-CoM methyltransferase) [20]. The final biomethane production was 260 mL CH_4/g VS, i.e., not statistically different ($p > 0.05$) compared to those observed in the other BMP tests (Figure 1a). This result was again associated with the marginal effect of the TE addition in increasing the consumption of VFAs, with the VFA concentration remaining in the same range of that observed in the BMP test where TEs were not provided (Figure 1b).

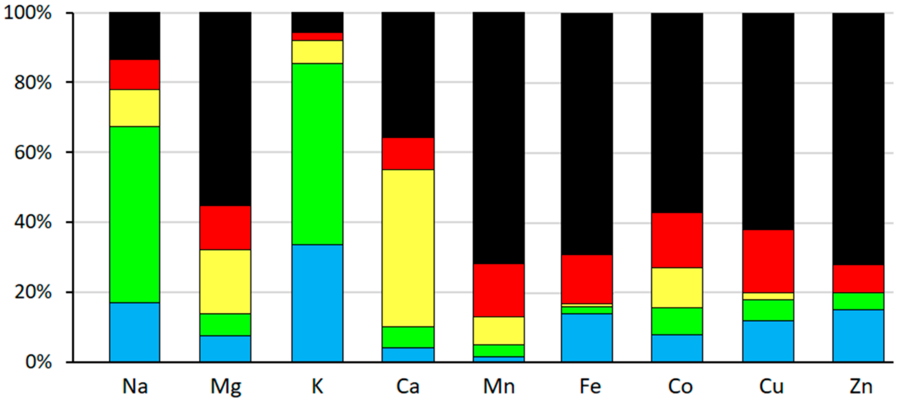
The results obtained in this study show that the supplementation of TEs has no significant effects when VFAs do not considerably buildup in the digestate. In the presence of recalcitrant LMs such as rice straw, hydrolysis should be first enhanced with a LM pretreatment phase in order to increase the concentration of VFAs [37]. Subsequently, TE supplementation may be considered to stabilize the AD process and promote a higher biomethane production. Khatri et al. [38], indeed, observed a significant improvement of the biomethane production and the digestion time of maize silage only when Fe addition was coupled to a NaOH pretreatment, which markedly enhanced the hydrolysis of the substrate. Also, the dosing of Se at 1 µg Se/g TS resulted in an increase of the biomethane potential of rice straw by approximately 8% [15]. Besides the fundamental role of Se in the formation of selenoproteins in archaea [39], Se has been reported to particularly prevent propionic acid accumulation and stimulate the enzymes responsible for the oxidation of formate, which is a breakdown product of propionate towards methane formation [40].

An analysis of the enzymatic activity would also offer an elegant way to better evaluate the effects of TE addition on the activity of the microbial enzymes, including cellulases that are responsible for the hydrolysis of the cellulose contained in the rice straw [41]. Zhang et al. [42] and Tian et al. [43] showed a positive effect of Fe and Ni in enhancing the biogas production yields from cow dung and *Phragmites* straw, respectively, ascribing this also to a stimulation of the cellulase activity. Ni has also been reported to be an essential co-factor for enzymes involved in both the acetoclastic and hydrogenotrophic pathways for methanogenesis [44].

3.2. Sequential Extraction and Bioavailable Fractions of Fe and Co

An optimum supply of TEs is essential for the growth and enzymatic activities of the microorganisms involved in the AD process in order to increase the biomethanation yields and rates. Quantifying the bioavailable amount, rather than the total amount, provides better information about the sufficient supply of TEs to the microbial consortium [19]. A sequential extraction technique was, thus, performed in order to rule out the possibility that the supplemented TEs were present under forms not directly bioavailable to the microbial population in the AD bioassays.

The TEs already present in the inoculum, fractionated into five categories, are shown in Figure 2.



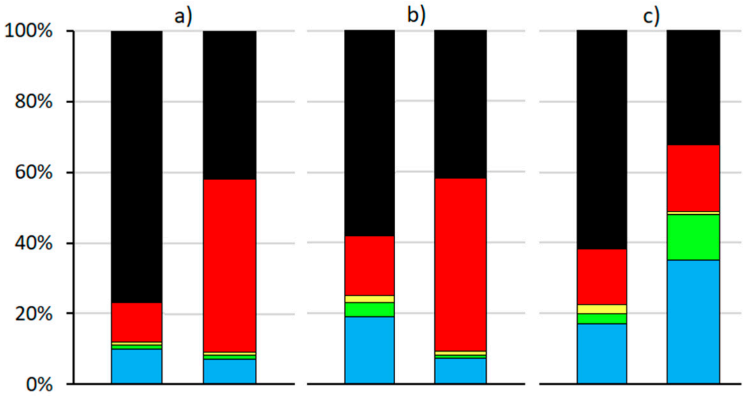
	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn
F1 🟦	2354	964	27970	1342	7	649	0.2	7	48
F2 🟩	6952	805	42972	2012	15	93	0.2	3	16
F3 🟨	1452	2329	5565	15093	35	46	0.2	1	0
F4 🟥	1188	1603	1932	3122	66	650	0.3	10	25
F5 ⬛	1865	7028	4718	11974	314	3196	1.1	35	229

Figure 2. The sequential extraction allowed to reveal the water soluble (F1), exchangeable (F2), carbonate-bound (F3), sulfide bound (F4), and residual (F5) fractions of trace elements (TEs) present in the original buffalo manure–cheese whey digestate used as inoculum in the biochemical methane potential (BMP) tests. All values are expressed as µg TE/g TS.

This fractionation shows that the highly bioavailable amount of the two TEs, given by the sum of F1 and F2 [23], was only 16 and 20% for Fe and Co, respectively, in the inoculum (Figure 2). The percentage decreased to 11 and 8% for Fe and Co (Figure 3a), respectively, when the rice straw was added to the buffalo manure–cheese whey digestate, due to the considerably lower Fe and Co content in the straw. The highly bioavailable amount of Fe (F1 + F2) increased to 23% (Figure 3b) when Fe was supplemented as the sole TE, and 20% (Figure 3c) when Fe was dosed with Co. Similarly, Co supplementation resulted in an increase of the bioavailable Co (F1 + F2) up to 48% (Figure 3c).

Despite the amount of bioavailable Fe and Co was higher in the batch tests where the two TEs were externally provided (Figure 3), the biomethane production remained unchanged compared to the BMP tests without TE dosing (Figure 1a). Therefore, a direct correlation between the increase of Fe and Co bioavailability (Figure 3) and improved AD performance (Figure 1) was not observed in this study.

A future work should be similarly addressed, but using a pre-treated rice straw which would result in a considerably higher VFA concentration to better assess the effect of bioavailable Fe and Co on the methanogenesis step. Also, the use of continuously-fed or semi-continuously-fed operating conditions might allow achieving high OLRs and higher VFA levels over longer operational times. This would permit a more thorough evaluation of the role of externally-dosed TEs over those already present in the inoculum or substrate. Finally, the use of mathematical models and sensitivity analysis tools would help to predict the fate of TEs supplemented to AD systems and discern among the most sensitive parameters affecting the biomethane production yields. Recently, Maharaj et al. developed a mathematical model to simulate the precipitation/dissolution [45] and complexation [46] of TEs in AD processes. Coupling their achievements to the TE sequential extraction approach used in this study can give a further impulse to the study of TE addition in AD.



	Fe	Co	Fe	Co	Fe	Co
F1 🟦	216	0.1	531	0.1	480	3.9
F2 🟩	25	0.0	112	0.0	81	2.0
F3 🟨	22	0.0	56	0.0	69	0.0
F4 🟥	241	0.7	475	0.7	450	2.3
F5 ⬛	1689	0.6	1621	0.6	1752	4.2

Figure 3. Water soluble (F1), exchangeable (F2), carbonate-bound (F3), sulfide bound (F4), and residual (F5) fractions of Fe and Co in the biochemical methane potential (BMP) tests on day 5. Columns (**a**) show the speciation of Fe and Co in the rice straw without trace element (TE) addition. Fe and Co fractions in the rice straw with only Fe addition are displayed in columns (**b**). Fe and Co fractions in the rice straw when both TEs were externally dosed are shown in columns (**c**). All values are expressed as µg TE/g TS.

4. Conclusions

The sequential extraction performed in this study showed that Fe and Co supplementation during the AD of rice straw resulted in significantly higher Fe and Co bioavailable fractions for the microbial metabolic activities. However, the biomethane production was not enhanced by the TE addition likely due to the elevated TE presence in the inoculum. The VFA concentration was similar in all the BMP tests performed, regardless the presence of bioavailable TEs. Hence, in the case of recalcitrant LMs such as rice straw, it is suggested to firstly promote the VFA buildup, for instance enhancing the LM hydrolysis or increasing the OLR in semi-continuous systems, and then use a TE dosing strategy to better stimulate the acetate conversion to methane.

Author Contributions: G.M. was responsible for conceptualization, methodology, investigation, data curation, and writing—original draft preparation; S.P. was responsible for data curation, writing—review and editing, and supervision; P.N.L.L. was responsible for supervision and writing—review and editing; G.E. was responsible for supervision, project administration, and funding acquisition.

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Conflicts of Interest: The authors declare no conflict of interest.

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