Abstract

This study aims to investigate the effect of anaerobic digestion (AD) on P species and how the different species are distributed in the digestate and digestate fractions, i.e. liquid and solid fractions. To do so, six full scale AD plants were used in this work and representative biomass samples were collected for investigation. P fractionation proceeded by adopting fractionation protocols consisting in step-by-step extraction with different solvents, (i.e. $NaHCO_3$, HCl and NaOH-EDTA). Subsequently P species in the different fractions were identified by using ^{31}P-NMR.

On average, AD did not affect P distribution into the different fractions, i.e. on a relative basis (mg kg^{-1} fresh matter - FM): i. ingestates P-$NaHCO_3$ of 410±152 ($n = 6$), P-HCl of 256±91 ($n = 6$) and P-NaOH-EDTA of 217±97 ($n = 6$), ii. digestate P-$NaHCO_3$ of 401±144 ($n = 6$), P-HCl of 277±85 ($n = 6$) and P-NaOH-EDTA of 229±98 ($n = 6$). On the other hand solid/liquid separation differently distributed P, i.e. liquid fraction P-$NaHCO_3$ of 269±142 (n = 6), P-HCl of 237±75 (n = 6) and P-NaOH-EDTA of 208±80 (n = 6); solid fraction P-$NaHCO_3$ of 984±234 (n = 6), P-HCl of 633±348 (n = 6) and P-NaOH-EDTA of 467±224 (n = 6). However, different trends were registered depending on the AD feed. ^{31}P NMR analyses revealed that inorganic P compounds dominated the spectra of all biomasses and fractions, with orthophosphate as the predominant species. On the other hand, when present, organic phosphorus compounds were typically represented by monophosphate esters, DNA and phospholipids, with a predominance of monophosphate esters.

Keywords: anaerobic digestion; phosphorus speciation; ^{31}P NMR; renewable fertilizers

1. Introduction

According to estimates from the Food and Agriculture Organization (FAO), only a fifth of the phosphorus (P) used for food production is actually consumed in the form of food, with 90% of the P which enters the system as organic and mineral fertilizers later becoming dispersed in the environment, mainly in water bodies and soils (FAO, 2017). As a consequence of that the total amount of phosphorus (P) brought to the soil today, on a world scale, is more than twice that considered a safe planetary boundary, i.e. a safe operating space for humanity while avoiding the risk of ecosystems' deterioration (Steffen et al., 2015). All this is causing environmental pollution in terms of eutrophication of freshwater and other water bodies (Correll, 1998) and there are numerous examples of water quality impairment associated with phosphorus pollution (Boesch et al., 2001). Long-term manure application to agricultural systems has been reported to be highly responsible for soil P accumulation and for the massive transfer of it to water bodies (Sims et al., 2000; Borda et al., 2011). Intensive animal production is mainly concentrated in specific areas (e.g. Po Valley, Flanders, Catalonia etc.) and so, manure/slurry management vs. soil-P overloading today represents one of the main issues requiring a push towards a more sustainable animal breeding and raising system.

In the last decade, the implementation of the Nitrates Directive (EU, 1991) at EU level has helped to improve the management of livestock effluent with regard to the loads of nitrogen, but until now no attention has been paid to the P in manure and its use in agriculture. In 2013, the European Commission pointed out, for the first time, by publishing a Consultative Communication on the Sustainable Use of Phosphorus in agriculture (EU, 2013), the unsustainability of phosphorus management. Fertilizer production is expected to reach 201.66 million Mg by the end of 2020 (FAO, 2017), with an average annual increase of 1.9% (Hein

and Leemans, 2012). Phosphate rock reserves are declining with time and, although diverse projections have been made, its reduction starting from 2050 onwards (Vaccari et al., 2018) can be foreseen. Moreover, the current quantities of phosphate rock are concentrated in a few countries, i.e. China and Morocco, this latter possessing about 74% of recognised P world deposits (Desmidt et al., 2015; Kataki et al., 2016; Sørensen et al., 2015, Azam et al., 2019), a situation which could potentially create international tension (Vaccari et al., 2018). Consequently, it is difficult to forecast precisely the extent of supplies of phosphate rock and the ability of these supplies to meet world demand for P in the long term, above all for EU-countries which import about 86% of P (IFA, 2014).

Increases in world P demand will be partially slowed down by decreases in the use of P in those areas characterized by intensive animal production and soils with high available P as the result of the excess of manure spread in recent decades. Such a decrease is due to both economic factors, since extra phosphorus on P-saturated land brings no benefit for crops, and environmental regulations aimed at tackling water pollution (EU, 2014).

It is therefore evident that there are still high margins to improve the efficiency of use of phosphorus, and in particular for the aspects which concern the recovery of this element: once the system boundary has been drawn, key potential recovery points can be identified. While phosphorus recovery from wastewater has received perhaps the most attention (Peng et al., 2018; Chrispim et al., 2019), less has been made of the recovery from manure/slurries (Cordell et al., 2011).

Anaerobic digestion (AD) has been proposed as a biotechnology able to produce renewable energy but also able to transform manure/slurries into valuable fertilizers because of organic matter degradation and stabilization, and nutrient (N and P) mineralization (Tambone et al., 2009, 2010; Möller and Müller, 2012). Because of these features, anaerobic digestion could

represent a biological pre-treatment facilitating subsequent operations for nutrient separation and recovery (Sigurnjak et al., 2019; Zangarini et al., 2020). Although many data are available with reference to N speciation during anaerobic digestion and subsequent solid/liquid (S/L) separation (Tambone et al., 2017), there is a lack of information about the transformation of phosphorus during AD. This aspect is important in view of the use of digestate as a renewable fertilizer to replace common mineral fertilizers (Riva et al., 2016). In this regard, literature until now focused its attention mainly on the nitrogen species (i.e. organic-N, ammonia and nitrate), with digestate being presented as a substitute for mineral N fertilizers (Möller and Müller, 2012; Tambone et al., 2010). Moreover, digestates have a ratio between N, P and K which is not favourable for plant requirements, but since nitrogen is the most limiting element, usually the digestate additions are adjusted to give the optimal nitrogen input (Sogn et al., 2018). As a consequence of that, the amount of phosphorus applied can be too heavy, with consequences of soil P overload and water eutrophication phenomena. For this reason, a better knowledge of P speciation in digestate with particular reference to P availability is important.

The aim of this paper is to fill this gap in knowledge by studying P speciation during anaerobic digestion and subsequent solid/liquid separation in six full-scale AD plants characterized for different in-feed mixtures and process parameters. To do so, P chemical fractions were studied by chemical fractionation and the obtained fractions investigated by ^{31}P-NMR. Complete mass balance was also performed also to track P fluxes to the different fractions.

2. Materials and methods

2.1 Organic biomass sampling and chemical characterization

Ingestates, digestates, liquid fractions of digestates and solid fractions of digestates were sampled from six mesophilic full-scale anaerobic digestion plants located in the Lombardy Region (North Italy). Plants were characterized by different hydraulic retention times (HRT) and feed-stock mixtures (Table 1). The samples taken were stored in 2 litre PTFE bottles without headspace before chemical characterization.

On fresh samples, the Total N-Kjeldahl (TKN) and ammonia (TAN) were analysed according to the analytical method established for wastewater sludge (APHA 1998) as well as pH (US Department of Agriculture and US Composting Council, 2002). Total solids (TS) and volatile solids (VS) were determined following standard procedures (APHA, 1998). On dry samples, total P and K contents were quantified, after acid digestion (EPA, 1998), by inductively coupled plasma mass spectrometry (ICP-MS by Varian, Fort Collins, USA); standard samples (National Institute of Standards and Technology, Gaithersburg, MD, USA) and blanks were run with all samples to ensure precision in the analyses. All the sampling and the analyses were carried out in triplicate.

2.2 Sequential extraction of phosphorus

The sequential extraction of the different P species was performed according to the procedure suggested in Turner and Leytem (2004) with minor changes. This procedure involved the sequential extraction of different P fractions by using $NaHCO_3$, NaOH-EDTA, and HCl.

P extracted in $NaHCO_3$ is considered readily soluble, in contrast with P extracted in NaOH-EDTA and HCl which represents the poorly soluble P fraction, i.e. P associated with amorphous iron/aluminium hydroxides and organic matter, and Ca^{2+}, Mg^{2+} and NH_4^+ -phosphates, respectively.

In brief, the first extraction was performed by using a 0.5 mole L^{-1} NaHCO$_3$ solution (1:60 dry biomass/solution ratio) for 4h. After centrifugation at 10,000 rpm for 30 min and then filtration through a 0.45 μm cellulose-nitrate membrane, the residual fraction was extracted for 16 h with 1 mole L^{-1} HCl solution (1:60 dry biomass/solution ratio) or with a 0.5 mole L^{-1} NaOH plus 50 mmole L^{-1} EDTA (1:20 dry biomass/solution ratio).

An aliquot of each supernatant was frozen at -80°C and lyophilized for NMR spectroscopy characterization. Another aliquot was mineralized and subsequently the P content was detected by ICP-MS analysis as previously described.

2.3 ^{31}P Nuclear Magnetic Resonance Spectroscopy

^{31}P Nuclear Magnetic Resonance Spectroscopy was performed by using 300 mg of lyophilized sample dissolved in 3 mL of a solution made by 1 mole L^{-1} NaOH and 0.1 mole L^{-1} EDTA (H$_2$O/D$_2$O 9:1) (pH > 13). The samples were vortexed and centrifuged at 5,000 rpm for 4 minutes at 20 °C. The supernatant was placed in a 10 mm NMR tube and the NMR spectra recorded on a Bruker AV600 spectrometer operating at a frequency of 600.13 MHz and 242.94 MHz for ^{1}H and ^{31}P nuclei, respectively. The ^{31}P spectra were acquired with a 10 mm BB probe at 20 °C and were referenced to external 85% H$_3$PO$_4$. P$_{90°}$=15 μs, D1=2 s, SW=7763, 975 Hz, NS ranging from 4,000 to 27,000 depending on the samples; chemical shifts (δ) were measured in ppm. The NMR spectra were transformed with a line broadening (LB=-2 Hz and GB= 0.02) by TOPSPIN software and the baseline was corrected using a polynomial function. All NMR signals were integrated and the sum of the integrals was assigned the value of the total phosphorus concentration (mg kg^{-1}) obtained by the ICP-MS analysis. The complete assignment of phosphorus species was performed following the

literature relative to the composition of the phosphorus species in soil, manure and slurry (Turner, 2004).

3. Results and discussion

3.1 Chemical characteristics of ingestates (I), digestates (D), liquid (LF) and solid fractions (SF)

Table 2 shows the main chemical characteristics of the samples studied. Low pH, characterizing ingestates (pH average 5.87±1.34) increased after anaerobic digestion (pH as average of 7.81±0.23) because of organic matter degradation (proteins) and ammonia production (Schievano et al., 2011; Möller and Müller, 2012). This fact was confirmed by volatile solids (VS) content that was, on average, lower for the digestates (VS of 75.66 ± 7.13 % Total Solids - TS) than ingestates (VS of 87.19 ± 6.91 % TS) as part of the organic carbon was transformed into CH_4 and CO_2 during anaerobic digestion. Nitrogen was preserved during the process, assuming similar values for digestate (TKN of 4.98 ± 0.96 g kg^{-1} Fresh Matter - FM) and ingestate (TKN of 4.70±0.71 g kg^{-1} FM). At the same time, ammonia content increased for digestate, but on an average basis, no statistical differences occurred, i.e. 1.61±0.74 g kg^{-1} FM for ingestates and 2.21±1.02 g kg^{-1} FM for digestates, respectively (Table 2). On the other hand, considering TAN/TKN ratio the ammonia increase was well documented for digestate *vs* ingestate (Tambone et al., 2017).

Solid/Liquid (S/L) separation led to two different fractions (Table 2). The solid fraction was characterized by both higher TS (TS of 22.07 ± 2.74 % FM) and VS (VS of 85.30 ± 4.05 % TS) contents, but by both lower TKN and TAN contents than liquid fraction, i.e. TS of 4.99±1.35 % FM; VS of 66.89 ± 7.95 % TS, TKN of 4.23 ± 0.61 g kg^{-1} FM, TAN of 2.35 ±

0.88 g kg^{-1} FM. These data were in agreement with previous findings for similar samples (Tambone et al., 2017). As expected, the nitrogen in the liquid fraction contained a higher ammonia content (TAN/TKN of 54.32 ± 14.31) than the solid fraction (TAN/TKN of 25.68 ± 10.27) due to the higher water solubility of ammonia (Tambone et al., 2017).

3.2 Phosphorus fractionation by chemical extractions

The P fractionation of biomass samples was carried out to assess the effects on P fractions distribution of both the anaerobic digestion process and solid/liquid mechanical separation of digestates. Results obtained were referred to both fresh (Table S1) and dry matter (Table S2). However, data referred to FM allowed us to get feedback about the goodness of the P fractionation performed and total P recoveries (Table S1), avoiding results misinterpretation because of P "concentration effect" during the AD process due to organic matter degradation (Table S2).

The fractionation of P into the three fractions (NaHCO$_3$, NaOH-EDTA and HCl extraction) allowed, for all the biomasses studied, i.e. ingestate, digestate, liquid fraction of digestate and solid fraction of digestate, good P recoveries that ranged from 82.9% to 110% of the total P detected for all biomass categories (Table S1).

Average P fractions contents (mg kg^{-1} FM) were as in the following (Figure 1a): for ingestates P-NaHCO$_3$ of 410±152 (*n* = 6), P-HCl of 256±91 (*n* = 6) and P-NaOH-EDTA of 217±97 (*n* = 6) and for digestate P-NaHCO$_3$ of 401±144 (*n* = 6), P-HCl of 277±85 (*n* = 6) and P-NaOH-EDTA of 229±98 (*n* = 6) (Figure 1a). From the data it can be seen that AD, on average, did not lead to substantial differences between the three P-fractions. Subsequent S/L separation led to the following results: for liquid fractions (mg kg^{-1} FM), P-NaHCO$_3$ of 269±142 (n = 6), P-HCl of 237±75 (n = 6) and P-NaOH-EDTA of 208±80 (n = 6) and for solid fractions P-

NaHCO$_3$ of 984±234 (n = 6), P-HCl of 633±348 (n = 6) and P-NaOH-EDTA of 467±224 (n = 6) (Figure 1b). These data suggest that the P was preferably concentrated on a relative basis (mg kg^{-1} FM) into the solid fraction, which would appear to be characterized by a higher content of easily available P (P-NaHCO$_3$) (Figure 1b). However, the results so expressed do not consider the real mass balances after the S/L separation process, the effects of which will be discussed later. The same trend can be discussed for data referring to the dry matter are reported (Table S2).

3.3 Phosphorus distribution in the ingestate, digestate and relative fractions: a quantitative and qualitative approach.

3.3.1 P speciation studied *by ^{31}P NMR.*

Typical NMR spectra of ingestate, digestate, liquid and solid fractions are shown in Figure 2, and Figures S1-S10. The main peaks detected were attributed to inorganic P-forms, i.e. orthophosphate (6.1 ppm) and pyrophosphate (-4.4 ppm). In addition, various minor peaks, corresponding to P organic compounds were identified, i.e. a broad signal at -0.2 ppm assigned to DNA, and the small peaks in the monoester region (4.85-4.64 ppm and 5.23 ppm) attributed to α and β –glycerophosphate and phosphatidic acid, respectively. Other signals in the phosphate monoesters region (4.95-4.10 ppm) were due to inositol-phosphate and mononucleotides originating from the hydrolysis of RNA. The quantification of each P-species obtained by the single spectra is reported in Table 3. In some samples, it was possible to detect the phytic acid that showed signals at 5.95, 5.06, 4.70 and 4.56 ppm in a ratio of 1:2:2:1 (Figure 2, Figure S5 and S9).

Distribution of different P fractions extracted from ingestate, digestate and digestate solid and liquid samples are shown in Tables 4 and 5. In almost all the samples, the most abundant P species detected by ^{31}P NMR was the inorganic P (Pi). When the ingestates and digestates were extracted using a solution of $NaHCO_3$, the Pi, which is considered soluble and readily available for plants, was dominated by orthophosphate species (Table 3). The pyrophosphate species, if present, amounted to only 1-2% of the total P (Figure 2 and Table 4). The low concentration of pyrophosphate species could be due to the P hydrolysis because of the high pH used during the extraction. The poorly soluble P species, such as calcium phosphates, and P associated with amorphous Fe/Al and organic matter, were recovered by the extractions with HCl and NaOH-EDTA (Table 4). In these extracts, orthophosphate was still the dominant P species and monophosphate esters were the most represented P species in terms of organic phosphorus. DNA and phospholipids, if present (Table 3) represented minor organic species. Monoester P compounds were probably produced by the hydrolysis of the phospholipid membrane of microorganisms during the AD process. In almost all cases, it was not possible to assign the signals to a specific monoester species because of the line broadening and the low signal to noise ratio. In this regard, only for plants C and E it was possible to identify the typical signals of phytic acids (Figures S5 and S9), the major phosphorus storage compound for seeds and cereal grains (Turner and Leytem, 2004; Weinhold and Miller, 2004). These data corresponded with the AD plant feedstock that, although with different percentages, generally contained seeds of energy crops (Table 1). Nevertheless, there was no correlation between the phytic acid content and the presence of energy crops (corn or sorghum silage) in the ingestate mixtures. When the LF and SF samples were extracted using a solution of $NaHCO_3$, the orthophosphate was the only P-species represented (Figure 3 and Table 5). Similarly, inorganic P (i.e. orthophosphate species) dominated the HCl and NaOH-EDTA extracts for

both liquid and solid fractions (Tables 3 and 5). As expected, the Po compounds were extracted mainly with NaOH-EDTA rather than by HCl. In this context, the characterization of the organic phosphorus compounds detected was more interesting. During the mechanical separation process, the concentration of the phytic acids was higher in the SF than in the LF. This fact was probably due to its low solubility which led to its being precipitated with Ca (Leytem et al., 2006).

3.3.2 P distribution in the extracted fractions.

In general, inorganic phosphorus (Pi) ranged from 62% to 98% (average 84%±12; $n = 6$) for ingestates and from 78% to 93% (average 88%±5; $n = 6$) for digestates (Table 4). Consequently, in ingestates and digestates the organic P (Po) amounted to 16%±12 ($n = 6$) and 12%±5 ($n = 6$) of total P, respectively. Generally, Po was found in the fractions extracted with HCl and NaOH-EDTA; more precisely HCl extracted from ingestate contained from 0% to 51% organic P compounds and NaOH-EDTA extracted contained from 8% to 56% of Po (Table 4). In effect, after anaerobic digestion, the values of Po extracted from digestates ranged between 2% - 25% for HCl extracts and 5% - 43% for NaOH-EDTA. Therefore, on average, these data did not suggest significant differences between Pi and Po from ingestates vs. digestates. Nevertheless, the analysis of each single plant showed different Pi and Po distributions (Table 4).

Plants A, D, E and F, which were characterized by the highest pig/cow slurry content in the feeds (from 40.5% to 84.3% w/w of the total in-feed) (Table 1), showed after anaerobic digestion a decrease in readily soluble P (extracted with $NaHCO_3$) and an increase of P associated with both organic matter and amorphous Fe/Al (Cheng and at., 2015; Eriksson et al., 2015) (extracted with P-NaOH-EDTA) (Table 4). The immobilization of soluble P already

present in the slurries into Po compounds, such as phosphate monoester and/or DNA, because of microorganism growth during the anaerobic digestion (Sharpley and Moyer, 2000), probably contributed to this finding. Plant C was the only AD plant where, after the AD, the P inorganic and readily soluble P significantly increased (Table 4), i.e. from 38.6 % to 47.1% Ptot, with a consequent decrease of both P soluble in NaOH-EDTA and P associated with calcium (Table 4). As shown in Table 1, AD plant C was the only one fed with a high percentage (65.9% w/w) of pre-digested material that probably both contributed to total inorganic-P of the mix and acted as microbial inoculum, leading to a faster degradation of P-organic compounds. In this regard, as reported in Table 4, P-NaHCO$_3$ for the digestate of this plant was inorganic. For AD-plant B, the anaerobic digestion process did not seem to influence P distribution from ingestate to digestate; probably in this case inorganic P coming from slurry was immobilized in microbial bodies while at the same time organic-P from energy crops was mineralized.

From the above discussion a question arises: did the anaerobic digestion process, by P speciation, affect P availability? Data before discussed seem to indicate that AD favoured P immobilization because of a general decrease of the available P (extractable with P-NaHCO$_3$) and, since organic phosphorus content decreased, P was being immobilized as inorganic forms.

The inorganic P was the most abundant P-species in LF and SF samples (Tables 3 and 5) in agreement with previous work done (Bachman et al., 2016). In particular, Pi ranged from 76% to 97% total P for LFs (average 89%±8; $n = 6$) and from 81% to 99% for SFs (average 91% ± 6; $n = 6$). Again, Table 5 showed that the P distribution for the LFs was similar for Plants A, D and E (more or less the same percentage of P extracted with NaHCO$_3$, HCl and NaOH-EDTA). On the contrary, two AD plants (Plant C and F) were characterized by higher

percentages of readily soluble P content than the other AD plants. The liquid fraction from Plant B was characterized by a higher percentage of P bound to calcium (soluble in HCl) than the other AD-plants.

In terms of P speciation in solid fractions, data reported in Table 5 show a more homogeneous distribution within the AD plants studied, with a higher content of readily available-P for most of the plants in agreement with the literature (Bachman et al., 2016).

Comparing LFs and SFs samples in terms of different P availability, the data reported in Table 5 seem to indicate that the P soluble in $NaHCO_3$, i.e. the more available P, flowed to the solid fraction more than the liquid fraction. In this context, however, it should be noted that during mechanical separation only 30% of the total P flows into the solid fraction (Tambone et al., 2017) and that the remaining 70% of P flows to the LF. Consequently, in absolute terms, most of the available-P is found, after mechanical separation, in the liquid fraction. Moreover, taking into account that LFs are also characterized for higher ammonia content, these fractions of digestate can well be valorized as a renewable fertilizer.

Conclusion

This study describes the fractionation and speciation of the organic and inorganic P compounds deriving from different biomasses subjected to the anaerobic digestion process and on average, the AD process did not modify the relative distribution of P fractions. Nevertheless, taking into account the "concentration effect" due to organic matter degradation during the AD process, the P availability increased when it was compared with the dry matter content. The subsequent S/L separation indicated that available plant-P (extracted with P-$NaHCO_3$) was preferably concentrated into the solid fraction. Feed composition affected P

speciation so that different trends were observed among the six AD plants studied. In particular the high presence of manure/slurry led to a reduction of available P (extracted in $NaHCO_3$) because of its immobilization into microbial bodies during the AD process. On the other hand, the presence of large amounts of energy crops did not affect P distribution between the different fractions.

Only in one case was available P increased because of the presence of a large amount of digestate in the feed mix. ^{31}P NMR data showed that inorganic P dominated the different fractions extracted. Orthophosphate led the fraction extracted in $NaHCO_3$, but it was also well represented in the HCl and NaOH-EDTA fractions as well as phosphate monoesters.

CRediT authorship contribution statement

Stefania Mazzini: Investigation, Formal analysis, Writing original-draft. Gigliola Borgonovo: Formal analysis. Leonardo Scaglioni: Formal analysis. Floriana Bedussi: Formal analysis. Giuliana D'Imporzano: Formal analysis. Fulvia Tambone: Investigation, Formal analysis, Writing original-draft, Founding acquisition. Fabrizio Adani Writing original-draft, Founding acquisition.

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