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Research article

The fertilising potential of manure-based biogas fermentation residues: pelleted vs. liquid digestate

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ABSTRACT

Spreading of manure on agricultural soils is a main source of ammonia emissions and/or nitrate leaching. It has been addressed by the European Union with the Directives 2001/81/EC and 91/676/EEC to protect the environment and the human health. The disposal of manure has therefore become an economic and environmental challenge for farmers. Thus, the conversion of manure via anaerobic digestion in a biogas plant could be a sustainable solution, having the byproducts (solid and liquid digestates) the potential to be used as fertilizers for crops.

This work aimed at characterizing and assessing the effect of digestates obtained from a local biogas plant (Biogas Wipptal, Gmbh), either in the form of liquid fraction or as a solid pellet on: (i) the fertility of the soils during an incubation experiment; (ii) the plant growth and nutritional status of different species (maize and cucumber). Moreover, an extensive characterization of the pellet was performed via X-ray microanalytical techniques.

The data obtained showed that both digestates exhibit a fertilizing potential for crops, depending on the plant species and the fertilizer dose: the liquid fraction increases the shoot fresh weight at low dose in cucumber, conversely, the solid pellet increases the shoot fresh weight at high dose in maize. The liquid digestate may have the advantage to release nutrients (i.e. nitrogen) more rapidly to plants, but its storage represents the main constraint (i.e. ammonia volatilization). Indeed, pelleting the digestates could improve the storability of the fertilizer besides enhancing plant nutrient availability (i.e. phosphate and potassium), plant biomass and soil biochemical quality (i.e. microbial biomass and activity). The physical structure and chemical composition of pellet digestates allow nutrients to be easily mobilized over time, representing a possible source of mineral nutrients also in long-term applications.

1. Introduction

Spreading of manure on agricultural soil is a main source of ammonia emissions and/or nitrate leaching [1]. Therefore, the European Union (EU) restricted the use of manures to a limited amount (170 kg N/ha/year) over a specified period [2, 3] with the Directives 2001/81/EC and 91/676/EEC ensuring, greater protection of the environment and human health. The disposal of manure has therefore become an economic challenge for farmers, as the amount of waste produced is often greater than the limit allowed [4]. Thus, the conversion of manure via anaerobic digestion (e.g. in a biogas plant) could be a sustainable solution since it produces two byproducts (solid and liquid digestate), which have the potential to be used as fertilizers and/or soil amendments in the crop management [5, 6]. In fact, digestates consist of substantial amounts of mineral elements such as nitrogen (N), phosphorus (P), potassium (K), essential for plant growth [7]. Moreover, recent evaluations indicate that biogas plants are very energy-efficient and represent an environmentally friendly technology [8] which can reduce greenhouse gases (GHGs) emissions, especially if locally available sources (i.e. manure, crop residues, etc.) are used.

The physical state of the digestates is primarily dependent on the conversion processes of the biomass [9]. One of the main issues is the

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high water content of the digestates produced (90–95%) [10], which makes the transport of these sub-products (e.g. from a biogas plant to an agricultural field) difficult and not economical [11]. The separation of the two phases of digestates (i.e. solid and liquid) to produce pellets [6] can represent a solution to more efficiently store and transfer this precious nutrient source to longer distances and to increase the nutrient recycling opportunities [12]. However, it has been demonstrated that pelletizing of digestates limited the nitrogen (N) release in pot experiments [13], while incubation experiments showed a net N immobilization after the application of pellets [6, 14]. Moreover, the application of the solid fraction of digestates resulted in significant lower yields compared to the liquid digestates and mineral fertilizers [15, 16]. This solid phase is therefore characterized as an organic fertilizer similar to solid manure but with high N and P contents, appropriate for the application to arable lands to enhance the soil humus formation [6] and to increase the soil organic matter (SOM) content in soils poor in organic matter [17]. On the other hand, liquid phases are characterized by low dry matter and more available P and high N (mainly as NH4-N) and high K contents [6, 18]. The application of these digestates to the field showed similar yields and N uptake to those of commercially available N fertilizers [15, 16].

Yet, the characteristics of both the liquid and solid by-products depend on the feedstock and the technological processes applied during the anaerobic digestion in the biogas plants. Thus, the present study aimed at characterizing and assessing the effect of digestates obtained from a local biogas plant (Biogas Wipptal, Gmbh), either in the form of liquid fraction or as a solid pellet on: (i) the fertility of the soils during an incubation experiment; (ii) the plant growth and nutritional status of different species (monocots- Zea mays L. and dicots- Cucumis sativus L.). To date, there is little information on the effect of pelletized cow manure-based digestate and its effect on the availability of nutrients. In particular, the analysis by X-ray microanalytical techniques, allowed the determination of a detailed distribution of chemical species within the pellets responsible for the nutrient availability and thus the fertilizing capacity of the digestate.

2. Materials and methods

2.1. Digestates

The digestates, one solid digestate in pellets (DP) and one liquid (DL) used in this work were obtained from the Biogas Wipptal plant in Vipiteno, Italy (http://www.biogas-wipptal.it/en/life-optimal-2012/programm-life-2007-2013.html). Commercially available samples of cow manure (CM) and urea (UR) were purchased and used as references of organic and inorganic N fertilizers.

All digestate samples were obtained using the composite sample technique: more than 5 subsamples (approx. 1.0 kg) were collected and mixed in order to obtain a composite sample. A subsample was analysed according to the European methods for fertilizers [19]. Dry weight and ashes were determined as weight residue at 105 °C and 550 °C, respectively. The pH was measured in the water extract (3:50 w:v) after 30 min of shaking at room temperature (RT). The electrical conductivity was determined in the filtered water extract (1:10 w:v) after 30 min of shaking at RT. Total organic C was determined by wet oxidation with potassium dichromate. Total N was measured, after wet acid mineralization, using a Kjeldda distillation instrument (K355 Büchi, Switzerland). The ammonium (NH4) and nitrate (NO3) N was determined after extraction with 1 M KCl (1:10 w:v) and steam distillation with magnesium oxide for NH4 and reduction with Dewarda alloy for NO3. Total organic N was calculated subtracting the inorganic N to total N [20]. Total P, S, and metals were determined by microwave wet acid digestion (Start-E, Milestone, USA) and by inductively coupled plasma optical emission spectroscopy (ICP-OES, Spectro Arcos, Germany). Available Cu and Zn were extracted with DTPA and determined by ICP-OES [21]. Enumeration of fecal coliforms (Escherichia coli) and Salmonella was obtained in agreement with the procedure ISO 7251 [22] and USEPA 1682 [23].

2.2. DP characterization

The internal structure and volumes distribution of pellets were investigated by high-resolution micro X-ray computed tomography (μXCT). The analyses were carried out at the Micro X-ray Lab of the University of Bari (Italy) using a SkyScan 1272 (Bruker GmbH, Germany) μXCT scanner. For image acquisitions, a W micro-focus source (<5 μm spot size) working at 70 kV and 142 uA was employed, using a 0.5 mm Al filter to improve signal to noise ratio. Three intact DPs of about 1.0 cm (h) x 0.5 cm (w) were fully scanned with a pixel size of 2.0 μm, a rotation step of 0.1 deg (within the range 0–184 deg) and an exposure of 2033 ms per frame. Flat field correction, frame averaging (3) and random movement (10) were also applied for acquisition optimization. After analysis and shadow projections reconstruction (obtained using the NRecon software, version 1.6.10.4, InstaRecon®), the 3D rendering, volumes segmentation and their quantification were elaborated by the software CTvox (version 3.1.1 r1191) and CTAnalysyser (version 1.15.4.0 + 4), both from Bruker microCT®. The reported results can be considered representative of all the samples analysed.

For micro X-ray fluorescence (μXRF) analyses, three DPs were prepared as thin sections by embedding intact DPs in epoxy resin (L.R. White Resin, Polyscience Europe GmbH, Germany). After hardening, the DPs were cut both transversally and longitudinally and glued onto a glass slide. Finally, the thickness was reduced to 100 μm using a diamond abrasive disk. One transversal and one longitudinal sections were prepared from each DP for a total of 6 thin sections mapped by μXRF. The reported results can be considered representative of all the samples analysed.

μXRF analyses were carried out on the Micro X-ray Lab of the University of Bari (Italy) using an M4 Tornado spectrometer (Bruker Nano Gmbh, Germany, Berlin), equipped with a Rh target (50 kV, 600 μA) and poly-capillary optics, which provide a spot size of about 25 μm. Two XFlash® silicon drift detectors (area of 30 mm2, FWHM <140 eV at the Mn-Ka), each positioned at 45° to the incident X-ray beam, were used to collect the X-ray fluorescence signal. Analyses were performed under vacuum (20 mbar), using a sampling step of 20 μm and a cumulative 50 ms dwell time. X-ray fluorescence hyperspectral data were processed using PyMca 5.1.3 [24] and Datamuncher [25], as proposed by [26]. Brighter pixels in μXRF maps correspond to relative higher concentrations of the element. The maps of the different elements have different scales and cannot be compared. Scatterplots were obtained by plotting the intensity of the K-line fluorescent signal collected from each pixel of the μXRF maps for one element vs another element.

2.3. Soil

A typical vineyard soil, hereafter called Hirsch, was collected from the surface (0–0.2 m) in Termeno, in the Province of Bolzano, Italy. The soil was sampled from several sites (~8) distributed over an area of 5000 m2, and more than 100 kg of fresh soil was obtained. A subsample was air dried, milled and sieved at 2 mm for soil analysis in agreement with SSSA methods [27]. The main soil properties are listed in Table 1.

2.4. Soil incubation experiment

2.4.1. Experimental design

The soil, milled and sieved, was preincubated at 20 ± 2 °C and 50 % of full water holding capacity for 14 days. The two digestates, DP (milled and sieved at 0.5 mm) and DL, and two N fertilizers, cow manure (CM) and urea (UR), were added to the soil at different amounts: zero (no digestates); 75 mg N kg⁻¹ (1x) and 300 mg N kg⁻¹ (4x) for DP, DL, CM
and UR, corresponding approximately to 180 and 720 kg ha\(^{-1}\) of N. The lowest dose (1x) corresponds to the amount commonly used in the field (2.14 and 9.0 Mg ha\(^{-1}\) on dry matter basis for DL and DP respectively). The highest dose (4x) was chosen assuming that the N potentially mineralizable of these products would have been around 25 % of total N [6] (Table 2). Each treatment was carried out in triplicate and the pots were incubated at 20 ± 2 °C in the dark for 7 weeks. Moisture was kept as constant as possible during the incubation by weighing the pot each week, and adding distilled water if necessary. After 0, 7, 14, 21, 28, 35, 42, 47, 54 and 63 days of incubation the pots were sampled and analyzed for extractable inorganic N (NO\(_3^-\)-N and NH\(_4^+\)-N). At the end of the incubation, the pots were sampled and the microbial biomass C and N, the mineralizable of these products would have been around 25 % of total N [6] (Table 2).

### 2.5. Pot experiments

#### 2.5.1. Plant growth

A pot experiment using Hirsch soil and cucumber (Cucumis sativus L. cv. Chinese long) and maize (Zea mays L. hybrid PR33T56, Pioneer Hi-Bred Italia S.r.l) was set up with a protein of five treatments: Control (no addition), solid digestate (DP) 75 mg N kg\(^{-1}\) soil DW (1x), solid digestate 300 mg N kg\(^{-1}\) soil DW (4x), liquid digestate (DL) 37.5 mg N kg\(^{-1}\) (0.5x) and liquid fertilizer 75 mg N kg\(^{-1}\) (1x). Plants were grown in a climate chamber under controlled conditions (14 h, 24 °C, 70% RH during the day; 10 h, 19 °C, 70% RH during the night), for 4 weeks. Soil was kept at 60% water holding capacity during the experiment by weighing the pots every other day and adding, if necessary, tap water.

#### 2.5.2. Measurement of plant growth

During the growing period, SPAD index of fully expanded leaves was determined using a portable chlorophyll meter SPAD-502 (Minolta, Osaka, Japan). Measurements were carried out twice a week on both basal and apical leaves (at least two per plant), and five SPAD measurements were taken per leaf and averaged. At the end of the experiment, cucumber and maize plants were collected and fresh weight (FW) was assessed. Leaves tissues were then oven-dried at 65 °C until constant weight was reached and stored for subsequent analyses.

#### 2.5.3. Plant available elements in soil

DTPA-extractable fractions of nutrients were extracted from approximately 10 g of soil with 20 mL of extracting solution (0.005 M DTPA, 0.01 M CaCl\(_2\) and 0.1 M TEA adjusted to pH 7.3) according to Sparks (1996) [27]. Nutrient concentrations were subsequently determined by ICP-OES.

#### 2.5.4. Plant tissue analysis

Dried cucumber and maize leaves were homogenized and approximately 0.3 g of each sample were acid digested with concentrated ultrapure HNO\(_3\) (650 mL L\(^{-1}\); Carlo Erba, Milano, Italy) using a single reaction chamber microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA). Concentrations of macro- and micronutrients were then determined by ICP-OES using tomato leaves (SRM 1573a) and spinach leaves (SRM 1547) as external certified reference material. Total organic carbon (TOC) and total nitrogen (TN) of leaves tissues were determined using a Flash EA 1112 elemental analyzer (Thermo Scientific, Germany).

#### 2.6. Statistical analyses and data handling

#### 2.6.1. Soil analysis

The statistical analysis followed a completely randomized design and one way analysis of variance (ANOVA) was carried out. The ANOVA assumptions were verified through Bartlett’s test for homogeneity of

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**Table 1. Main physico-chemical characteristics of the soil used in the experiments.**

| Characteristics                      | Soil          |
|--------------------------------------|---------------|
| Texture (USDA)                       | silt-loam     |
| Clay (%)                             | 16.9          |
| Silt (%)                             | 50.8          |
| Sand (%)                             | 32.2          |
| pH (water)                           | 7.8           |
| pH (KCI 1 M)                         | 7.4           |
| Total carbonates (% CaCO\(_3\))      | 15.6          |
| Cation exchange capacity (cmol(+)/kg)| 16.9          |
| Total organic C (%)                  | 1.35          |
| Total N (%)                          | 0.143         |
| C/N ratio                            | 9.4           |
| Available P (mg/kg)                  | 27            |
| Exchangeable K (mg/kg)               | 329           |
| Exchangeable Ca (mg/kg)              | 2270          |
| Exchangeable Mg (mg/kg)              | 263           |
| DTPA extractable Cu (mg/kg)          | 70            |
| DTPA extractable Fe (mg/kg)          | 7             |
| DTPA extractable Mn (mg/kg)          | 12            |
| DTPA extractable Ni (mg/kg)          | 0.3           |
| DTPA extractable Zn (mg/kg)          | 4.4           |

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**Table 2. Summary of product doses used in the experiments (ds = dry soil, DW = dry weight, FW = fresh weight).**

| Dose of N          | Dose of liquid digestate | Dose of pellet digestate |
|--------------------|--------------------------|--------------------------|
| mg kg\(^{-1}\) ds | kg ha\(^{-1}\)           | Mg ha\(^{-1}\) DW        | Mg ha\(^{-1}\) FW        |
| 75                 | 180                      | 2.14                     | 24.4                     |
| 150                | 720                      | 8.57                     | 97.4                     |

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Soil pH and electrical conductivity (EC) were determined in agreement with SSSA methods [27]. Available P was extracted with 0.5 M sodium bicarbonate at pH 8.5 and determined with ascorbic acid-ammonium molybdate reaction [31]. Exchangeable cations were extracted with 1 M ammonium acetate at pH 7 and determined by ICP-OES [27]; these data were used to determine the Mg/K ratio as an indicator of soil fertility.

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variances and Shapiro-Wilk's test for normality of distributions. The significance of all test was assessed at α = 0.05. Post hoc HSD Tukey's test was performed to investigate differences between treatments when ANOVA returned a significant global test. Data are expressed on oven dried basis, and statistical analysis were performed using R version 3.4.4 (R Core Team, 2018).

### 2.6.2. Plant analysis

The results are presented as means of five replicates ± standard errors (SE). Statistical analysis was performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, San Diego California, USA). ANOVA was carried out, and means were compared using Tukey's test at P < 0.05.

### 3. Results

#### 3.1. Digestates

Table 3 shows the main characteristics of DL and DP digestates. As expected, the total solids (or dry weight) content was lower in DL than DP. Conversely, the ashes (on DW basis) were higher in DL than DP, therefore the DP had a higher content of volatile solids (or organic matter) than DL. These results are in agreement with the productive process of digestates: the DL process concentrates the soluble salts (increases the ashes and decreases the volatile solids), while the DP process concentrates the organic matter (increases the volatile solids and decreases the ashes). The pH was alkaline in all digestates and resulted highest for the DP (9.75, Table 3); similar results were observed for anaerobic digestates from animal wastes [32, 33]. The electrical conductivity (EC) ranged from 3.5 to 4.6 dS m⁻¹ and was higher in DL than DP; this is due to liquid separation from the solid phase. In each case, the EC value fell in the typical range for anaerobic digestates [33, 34], and was lower than 5 dS m⁻¹, a criterion suggested as a limit for the use of an amendment without dilution before the application to the soil [35]. Total organic C (on dry weight basis) was similar in both digestates ranging from 36 to 42% in the DL and DP, respectively; the total N was higher in DL (8.4% DW) than DP (2.0% DW), the C/N ratio resulting <5 for DL and >20 for DP. In DL half of total N was present as NH₄⁺ (4.4% DW), while in DP the inorganic forms of N were negligible, and organic N was higher than 85% of total N. For all the other total macronutrients such as P, K, magnesium (Mg) and sulphur (S), the DL showed a higher content than the DP. In the case of total micronutrients and heavy metals, instead, the higher concentrations were observed for DP. The most abundant micronutrients were iron (Fe), manganese (Mn) and zinc (Zn). In any case, the concentrations of total heavy metals (such as Cd, Cr, Cu, Hg, Pb, etc.), available copper (Cu) and Zn and microbiological indicators (Escherichia coli and Salmonella spp.) were lower than the limits fixed by the current European legislation for the use of sewage sludge in agriculture [36] and by the Italian regulation for fertilizers [37].

In order to understand the dynamics of nutrient availability from DP, this material was further characterized by µXCT and µXRF. As shown in Figure 1 and in the video (Video 1), the pellets are characterized by a heterogeneous structure, showing volumes with different density (evidenced by the different greyscale values) and empty spaces (cracks and pores). In particular, a very complex fracture network was observed, with cracks within a wide range of widths (from 10 to 400 microns) and lengths (up to 6 mm in transversal sections) that appear highly connected. Such fractures cross the whole section of the pellet, so that the inner regions are practically connected to the external surfaces. About 92–96% is constituted by organic matter with different densities (i.e. more or less compact, from dark grey to light grey in the images), 3–7% are voids and pores (black), and about 0.5–1.0% are more dense particles (appearing light grey or white in the images). The size of these particles (equivalent diameter) generally varies from about 10 microns to 300 microns (with some particles even up to 2 mm in certain pellets) and are characterized by a chemical composition different from that of the organic matrix, reflecting the presence of elements with Z values above those of H, C, N and O, likely mineral macro and micronutrients. Similarly [38], by using µXCT, observed a very heterogeneous distribution of inorganic material inside biochar.

**Table 3. Main properties of the organic fertilizers used in the experiments.**

| Properties          | Digestates | Cow Manure |
|---------------------|------------|------------|
|                     | Liquid (DL) | Pellet (DP) |
| Dry weight (% FW)   | 8.8        | 89         | 92.1 |
| Ash (% DW)          | 39         | 18         | 28.8 |
| pH (water)          | 8.77       | 9.75       | 7.1  |
| Electrical conductivity (dS/m) | 4.6        | 3.5        | 9.4  |
| Total Organic C (% DW) | 36        | 42         | 33.9 |
| Total N (% DW)      | 8.4        | 1.97       | 3.21 |
| NH₄ N (% DW)        | 4.4        | 0.04       | 0.44 |
| NO₃ N (% DW)        | 0.02       | 0.06       | 0.02 |
| Organic N (% DW)    | 4.0        | 1.87       | 2.75 |
| C/N ratio           | 4.2        | 22         | 11   |
| Total P₂O₅ (% DW)   | 4.3        | 2.0        | 2.8  |
| Total K₂O (% DW)    | 10.7       | 1.8        | 2.3  |
| Total MgO (% DW)    | 3.6        | 1.4        | 1.1  |
| Total SO₄ ( % DW)   | 3.4        | 1.2        | 1.8  |
| Total Fe ( % DW)    | 0.25       | 0.29       | 0.46 |
| Total Cd (mg/kg DW) | 0.1        | 0.4        | <0.1 |
| Total Cr (mg/kg DW) | 10         | 16         | 34   |
| Total CaO (mg/kg DW)| <0.2       | <0.2       | <0.2 |
| Total Cu (mg/kg DW) | 10         | 59         | 91   |
| Total Hg (mg/kg DW) | 0.2        | 0.2        | 0.2  |
| Total Ni (mg/kg DW) | 11         | 11         | 13   |
| Total Pb (mg/kg DW) | 11         | 6          | 5    |
| Total Mn (mg/kg DW) | 360        | 218        | 402  |
| Total Zn (mg/kg DW) | 135        | 242        | 326  |
| DTPA Cu (mg/kg DW)  | 18         | 3.8        | NA   |
| DTPA Zn (mg/kg DW)  | 64         | 1.1        | NA   |
| *Escherichia coli* (cfu/g) | <10    | <3        | <10  |
| *Salmonella* spp. (MPN/25 g) | absent | absent | absent |

FW = fresh weight; DW = dry weight; cfu = colony forming unit; MPN = most probable number; NA = not analyzed.
sections – Figure 4), and therefore could be in the form of Fe (hydr)oxy-
ides. Despite the distribution of Zn and Mn is quite homogeneous, these
elements in some points correlate with Fe and Ni hotspots (data not
shown). Copper is not detectable in thin sections because of its low
concentration (Table 3) and does not appear concentrated in hotspots.
Therefore, we can assume that it is uniformly distributed within the
whole organic matrix, as visible from a distribution map obtained from a
bulk (not thin-sectioned) sample (Figure 5). Similar results for Cu and Zn
were observed by [40] in biosolids produced from the treatment of
wastewaters, where Cu speciation was consistently dominated by sorp-
tion to organic matter whereas Zn partitioned mainly to iron oxides.

Other elements like lead (Pb), mercury (Hg) and chromium (Cr)
which have been detected in the pellet (Table 3) were below the detec-
tion limits of μXRF for elemental distribution mapping.

3.2. Soil incubation

3.2.1. Inorganic N release and net N mineralization

The results of cumulative NH$_4^+$ and NO$_3^-$ released in soil treated with
the digestates, cow manure and urea are reported in Figure 6. The
inorganic N was found in the soils mostly as NO$_3^-$, and the higher con-
centrations were observed in soils treated with the highest application rate (4x). A significant release of NH$_4^+$ in soil was found only during the
first 2 weeks of incubation and with the 4x application rate (Figure 6); the
highest release was found in the treatments with DL and urea. In the soil,
the net inorganic N release (N$_{\text{net}}$) was positive for all fertilizers tested,
excluding the DP (Figure 7). In the case of urea and DL, the N$_{\text{net}}$
increased faster and reached a maximum after 2–3 weeks. The DP treated
soil showed a negative trend reaching a negative value of N$_{\text{net}}$ at the end
of the incubation (-50 mg N kg$^{-1}$ DW); this indicates that DP induced a N
immobilization in soil. The soil treated with cow manure fluctuated around zero for the first four weeks (Figure 7), then increased positively
and reached a maximum, around 150 mg N kg$^{-1}$ DW in the case of the
highest application rate (4x).

3.2.2. Soil biochemical indicators

Table 4 depicts the soil biochemical indicators determined at the end
of the incubation. The concentration of soil C$_{\text{ext}}$ ranged from 212 to 273
mg C kg$^{-1}$ DW and resulted significantly higher in soils treated with DP
and cow manure at the highest (4x) application rate than all other
treatments. Otherwise, the concentration of soil N$_{\text{ext}}$ was higher in UR 4x
(422 mg N kg$^{-1}$ DW) and DL 4x (303 mg N kg$^{-1}$ DW) compared to the
other treatments. Consequently, the C$_{\text{ext}}$/N$_{\text{ext}}$ ratio was higher in soils
-treated with DP and CM than DL and UR. The C$_{\text{mic}}$/N$_{\text{mic}}$ in agreement with

$$\text{C}_{\text{ext}} = \text{highest in the DP 4x (500 mg C kg}^{-1} \text{DW) and CM 4x (455 mg C kg}^{-1} \text{DW). Also in the case of N$_{\text{mic}}$, the highest value was observed for the}
$$
soils treated with DP 4x (66 mg N kg$^{-1}$ DW), followed by UR 4x (48 mg N
kg$^{-1}$ DW) and DP 1x (45 mg N kg$^{-1}$ DW). The C$_{\text{mic}}$/N$_{\text{mic}}$ ratio ranged
from 7.4 to 13.5, and was higher in the soils treated with CM 1x, CM 4x,
UR 1x and DL 1x compared to the untreated control and others treat-
ments. It is interesting to note that C$_{\text{mic}}$/N$_{\text{mic}}$ ratio was correlated with
N$_{\text{mic}}$ ($r = -0.77, P < 0.001, n = 27$) but not with C$_{\text{ext}}$/N$_{\text{ext}}$ ratio ($r = -0.16,
P = 0.41, n = 27$). Soil dehydrogenase activity (DHY) was significantly
affected by the treatments and application rate (Table 4). In particular,
DP and CM applied at the highest (4x) application rate exhibited a higher
DHY than DL and urea at the same application rate. By contrast, the DHY/
C$_{\text{mic}}$ ratio was not significantly affected by treatments (Table 4); this
indicates that DHY is highly correlated with C$_{\text{mic}}$ ($r = 0.87, P < 0.01, n = 27$).
The effect of the treatments on soil fluorescein diacetate hydrolytic
activity (FDA) was limited (Table 4) and ranged from 106 to 147 mg
fluorescein h$^{-1}$ kg$^{-1}$ soil DW; only DP at the highest application rate (4x)
resulted higher than UR 1x; the other treatments resulted not statistically
different. FDA and C$_{\text{mic}}$ were not correlated ($r = 0.18, P = 0.35, n = 27$),
therefore the FDA/C$_{\text{mic}}$ ratio was significantly affected by treatments
(Table 4); and was higher in DL (4x) than CM and DP for all application
rates.

3.2.3. Soil chemical indicators

Soil pH was not influenced by the treatments at the end of incubation
time (Table 5), moreover the soil electrical conductivity (EC) was
significantly affected by the treatments (Table 5), and increased in the
soil samples treated with DL, CM and UR at the highest (4x) application
rate. The soil available (Olsen) P was affected only by DP at the highest
(4x) application rate (Figure 8) showing the highest values (up to 60 mg
kg$^{-1}$). In the case of exchangeable cations (Ca, Mg, K and Na), a signif-

cant release of NH$_4^+$ and NO$_3^-$ in soil was found only during the
first 2 weeks of incubation and with the 4x application rate (Figure 6); the
highest release was found in the treatments with DL and urea. In the soil,
the net inorganic N release (N$_{\text{net}}$) was positive for all fertilizers tested,
excluding the DP (Figure 7). In the case of urea and DL, the N$_{\text{net}}$
increased faster and reached a maximum after 2–3 weeks. The DP treated
soil showed a negative trend reaching a negative value of N$_{\text{net}}$ at the end
of the incubation (-50 mg N kg$^{-1}$ DW); this indicates that DP induced a N
immobilization in soil. The soil treated with cow manure fluctuated around zero for the first four weeks (Figure 7), then increased positively
and reached a maximum, around 150 mg N kg$^{-1}$ DW in the case of the
highest application rate (4x).

3.2.4. Plant growth

Table 6 shows the effect of the fertilization with DL and DP on the
growth parameters of cucumber and maize plants in terms of shoot fresh
weight (FW), chlorophyll content measured as SPAD index, N and C
content. Shoot FW was significantly affected by the fertilizers application
in both plants cultivated, even though the effect differed among the two
plant species. In fact, while cucumber leaves biomass increased only

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**Figure 1.** 3D rendering, coronal (COR), transversal (TRA) and sagittal (SAG) sections of a digestate pellet as imaged by Micro X-ray Computed Tomography (μXCT). Brighter colours in the images correspond to higher density particles. Black features corresponds to voids and fractures.
when DL was applied (independently of the dose), maize shoot biomass increased only upon the application of the highest concentration of DP and the lowest concentration of DL (Table 6). SPAD index was not affected by the application of either DP or DL in cucumber, while it increased in maize plants fertilized with DP (Table 6). Shoot total N did not significantly change in tissues collected from cucumber plants grown on soils fertilized with DP, while it decreased in those treated with DL as compared to controls. In contrast, the concentration of N detected in maize plants was significantly higher in plants cultivated with DL, while it decreased in the presence of DP. Regarding total organic C, its concentration was not affected by the applied fertilizations in cucumber plants, while it slightly increased in maize plants grown with the highest DP concentration (Table 6).

3.2.5. Plant nutrient concentration

In order to assess the effect of the manure-based biogas fermentation residues on the shoot ionome of both cucumber and maize plants, the concentrations of macro- and micro-nutrients were determined at harvest (Table 7). Overall, the concentration of macronutrients decreased in maize plants fertilized with both DP and DL, with the only exception of S, which increased in the shoots of plants fertilized with DL at the lowest dose. Regarding cucumber plants, the effect of fertilizers on macronutrients concentration was different: in fact, while Ca and Mg decreased only in the presence of DL, the application of DP in the soil induced an increase of P. Furthermore, S concentration was highest in the treatment with the highest DL dose (Table 7). The concentration of micronutrients in cucumber plants showed an increase of the bivalent cations Mn, Zn and Cu in the treatment DP 4x, while Fe content decreased slightly in all the treatments. Differently, maize plants showed a decrease of the concentration of all the micronutrients in the shoots, with respect to control, even if this decrease was less pronounced for Zn and Cu in the DL treatments (Table 7).

3.2.6. Soil fertility indicators of pot experiment

Figure 9 shows the concentrations of ammonium ($\text{NH}_4^+$), nitrate ($\text{NO}_3^-$) and P and the cation exchange capacity (CEC) of the soils collected at the end of the pot experiments, where either cucumber or maize plants were cultivated. The concentration of NH$_4^+$ resulted significantly higher in all cucumber-grown soils treated with both DP and DL, while a different pattern was observed in maize-grown soils, where the concentration increased only in the presence of DP at the highest concentration (Figure 9E). However, in maize-grown soils amended with DP at both doses, the concentration of NO$_3^-$ was significantly decreased compared to the control (Figure 9F), whereas in cucumber, it increased of about 15% in soils amended with DP 4x (Figure 9B). The available P increased in all soils fertilized with both DP and DL, independently from the plant species (Figure 9C-G). The CEC decreased in all cucumber-grown soils treated with digestates, while it increased in maize-grown soils amended with the highest DL dose.

3.2.7. DTPA extractable metals in soils

The concentration of DTPA extractable metals (Cu, Fe, Mn and Zn) in cucumber and maize grown soils is shown in Figure 10. Available Cu did not change in soils where cucumber plants were grown, while its concentration was the highest in the DP 4x-treated soils and decreased in those soils fertilized with DL (Figure 10A-E). Regarding available Fe, its concentration was the highest in maize-grown soils supplied with the highest DP dose (4x); a similar trend was observed in cucumber-grown

Figure 2. Micro X-ray fluorescence (μXRF) distribution maps of Mg (red), Ca (blue), P (green), S (purple), Fe (yellow), Zn (light green), Mn (orange), and K (cyan) of a transversal section of digestate pellet. Brighter colours in the images correspond to relative higher concentrations. A screenshot picture of the section is also presented (central image, Video).
Figure 3. a) P vs. Mg scatterplot obtained using fluorescent K-line signals collected by micro X-ray fluorescence (μXRF) in each pixel of a section of digestate pellet. The green areas in the left image correspond to pixels where P and Mg are correlated according to the scatterplot (points inside the green box). b) K vs. S scatterplot obtained using fluorescent K-line signals collected by micro X-ray fluorescence (μXRF) in each pixel of a section of digestate pellet. The yellow and red areas in the left image correspond to pixels where K and S are correlated according to the scatterplot (points inside the yellow and red box, respectively).

Figure 4. Ni vs. Fe scatterplot obtained using fluorescent K-line signals collected by micro X-ray fluorescence (μXRF) in each pixel of three sections of digestate pellet.

Figure 5. Micro X-ray fluorescence (μXRF) distribution maps of S (purple) and Cu (yellow) of a longitudinal section (not thin-sectioned) of digestate pellet. Brighter colours in the images correspond to relative higher concentrations. A picture of the section is also presented (upper image).
Figure 6. Trend of ammonia (A, B) and nitrate (C, D) N in soil treated with different fertilizers (CK = control, untreated soil; CM = cow manure; DL = digestate liquid; DP = digestate pellet; UR = urea).

Figure 7. Net inorganic N release in soil treated with different fertilizers (CM = cow manure; DL = digestate liquid; DP = digestate pellet; UR = urea) and application rates: 75 mg N kg\(^{-1}\) (A) and 300 mg N kg\(^{-1}\) (B).
Soils, although in this case the differences were not significant. Similar trends, independently from the plant species, were observed for available Mn (Figure 10C-G), with the highest concentration of the metal detected in soils amended with the highest DP dose. The concentration of available Zn significantly increased in DP 4x-treated soils as compared to the other treatments in cucumber-grown soils, while it decreased significantly only in maize-grown soils supplied with DL (Figure 10H).

4. Discussion

Agricultural advantages and environmental risk of soil fertilization with anaerobic digestates from (animal) manure were recently reviewed [6, 32, 33, 34]; the use of digestates as organic fertilizers is considered beneficial since it supplies plant nutrients (such as N, P, K), improve soil structure and increases soil organic matter. However, potential to harm the environment and human health are matter of concern. In Teglia et al (2011) [32], a wide range of chemical, physical and biological indicators used to assess the agronomic quality of digestates were discussed; these authors concluded that the valorization of digestates in agriculture cannot exclude a full characterization of the products, which therefore remains the starting point for any evaluation of the agronomic quality of a digestate.

The two products obtained from the two-phase separation of anaerobic digestate from straw and animal manure are characterized, as expected, by a different composition of the organic and inorganic fractions (Table 3). The results reported are in agreement with other studies [13, 41]. A large amount of organic matter (volatile solids) is separated in pellet product (>80% on DW) than the liquid fraction (60% on DW). Also the N was fractionated in the two products: in particular, the NH₄-N/total N ratio was higher in DL (0.52) than in DP (0.02), indicating that the production process of DL recovered the more NH₄-N (and, probably, the pelletling process caused a large loss of NH₄-N from solid digestate). Finally, the pellet product showed a lower content of P₂O₅ (<2%), K₂O (<2%), MgO (1.4%) and SO₃ (1.2%) on dry weight basis. Therefore, the pellet product may be comparable to a solid animal manure, but with relative lower content in nutrients (such as, N, P, K). Conversely, the DL is characterized by high inorganic N and K, comparable to a mineral N-K fertilizer or animal urine.

Anaerobic digestates had significant effects on the inorganic N release in soil, with early presence of NH₄-N and a final accumulation of NH₄-N in soils treated with DP and urea (Figure 6). The difference between the two products reflect their composition (Table 3). The early presence on NH₄-N in DL 4x-treated soil is clearly due to the high content in ammonia-N immobilization). Soil N immobilization after anaerobic digestate application has been previously reported for biomass with C/N ratio higher than 25–30 [13]. The C/N ratio observed in DP (>20) is only partially in agreement with this finding, confirming that the C/N ratio is not an accurate indicators to predict the N mineralization in soil treated with anaerobic digestates. A significant increase in microbial biomass N was also observed in soil treated with DP respect to the control and soil treated with DL and urea (Table 4), confirming the N immobilization in this soil. These results suggest that liquid-solid separation and pelletization of solid fraction of anaerobic digestate produce an organic biomass characterized by organic matter with lower biodegradability in soil. The DL shows a lower C/N ratio (<5) and a large inorganic N release, as it has been observed in the past [6]. These findings are explicable considering that during the fractionation process the inorganic N (i.e. ammonia) was mainly recovered in DL (approximately 50% of total N is in inorganic form in this fraction).

Table 4. Soil extractable C (Cext), extractable N (Next), Cext/Next ratio, microbial biomass C (Cmic), microbial biomass N (Nmic), Cmic/Nmic ratio, soil dehydrogenase activity (DHY), specific dehydrogenase activity (DHY/Cmic), FDA hydrolytic activity (FDA) and specific FDA hydrolytic activity (FDA/Cmic), determined after 9 weeks of incubations of products into the soil (mean of three replicates). F ratio and standard error of the means (SEM) were reported at the end of the table. Different superscript letters indicates statistically different values within each column (P < 0.05).

|          | Cext mg kg⁻¹ DW | Next mg kg⁻¹ DW | Cext/Next ratio | Cmic mg kg⁻¹ DW | Nmic mg kg⁻¹ DW | Cmic/Nmic ratio | DHY mg h⁻¹ kg⁻¹ DW | DHY/Cmic mg h⁻¹ g⁻¹ | FDA mg h⁻¹ kg⁻¹ DW | FDA/Cmic mg h⁻¹ g⁻¹ |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-------------------|-------------------|-------------------|-------------------|
| CK       | 312             | 153             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| DL 1x    | 198             | 186             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| DL 4x    | 226             | 303             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| DP 1x    | 214             | 135             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| DP 4x    | 273             | 122             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| CM 1x    | 222             | 171             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| CM 4x    | 257             | 209             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| UR 1x    | 217             | 221             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| UR 4x    | 283             | 422             | 2.07            | 357             | 392             | 9.18e           | 83.1            | 233               | 134               | 374               |
| F₉,18    | 10.2***         | 47.1***         | 115***          | 7.53***         | 25.5***         | 9.68***         | 8.26***         | 0.50**            | 2.80***           | 5.75***           |

CK: control; DL: digestate liquid, DP: digestate pellet, CM: cow manure, UR: urea; 1x and 4x indicate the dose of N added: 75 and 300 mg kg⁻¹ on dry soil basis. DW = dry weight.

NS, *; **; *** not significant, or significant at P ≤ 0.05, 0.01, 0.001, respectively.

Table 5. Soil reaction (pH), electrical conductivity (EC), Ca/Mg ratio and Mg/K ratio determined after 9 weeks of incubations (mean of three replicates). F ratio and standard error of the means (SEM) were reported at the end of the table. Different superscript letters indicates statistically different values within each column (P < 0.05).

|          | pH   | EC dS m⁻¹ | Ca/Mg ratio | Mg/K ratio |
|----------|------|-----------|-------------|------------|
| CK       | 7.50 | 386       | 6.53        | 2.57       |
| DL 1x    | 7.57 | 462       | 6.30        | 2.29       |
| DL 4x    | 7.60 | 758       | 6.57        | 2.17       |
| DP 1x    | 7.51 | 382       | 6.26        | 2.30       |
| DP 4x    | 7.54 | 428       | 5.13        | 2.27       |
| CM 1x    | 7.54 | 437       | 6.10        | 2.44       |
| CM 4x    | 7.45 | 539       | 5.61        | 2.39       |
| UR 1x    | 7.48 | 486       | 6.31        | 2.61       |
| UR 4x    | 7.47 | 834       | 6.00        | 2.91       |
| F₉,18    | 1.06  | 53.5       | 19.6        | 29.3       |
| SEM      | 0.05 | 22.3      | 0.1         | 0.04       |

CK: control; DL: digestate liquid, DP: digestate pellet, CM: cow manure, UR: urea; 1x and 4x indicate the dose of N added: 75 and 300 mg kg⁻¹ on dry soil basis. DW = dry weight.

NS, *; **; *** not significant, or significant at P ≤ 0.05, 0.01, 0.001, respectively.
The kinetics of inorganic N release over time in soil treated with anaerobic digestates was slow in both cases; it increased over time for DL, and decreased for DP (Figure 7).

Generally, the organic fertilizers have a significant effect on soil biochemical indicators, such as microbial biomass and activities [42]. However, our results showed a different behaviour between pellet and liquid products. In the case of DP treated soil, an increase in extractable C and a decrease in extractable N was observed, resulting in an increase of extractable C/N ratio. As discussed above, an imbalance in available C respect to N induces a reduction of C use efficiency (CUE), and an increase of N use efficiency (NUE). This latter results in N immobilization by soil microorganisms [43], as observed in the soil treated with DP. In the case of DL treated soil, no differences were observed in Cext and Cmic respect to the untreated soil, but only in Next, that increases, and in Nmic, that decreases, showed differences. Therefore, the liquid digestate seems to act as an inorganic N fertilizer rather as an organic fertilizer. The indicators of the microbial activities seem to confirm these findings: the DHY increased significantly for DP and not for DL, while FDA was not affected by anaerobic digestates. The DHY is considered an indicator linked to the energy metabolism of microorganisms [44], and clearly correlates with available substrate for energy production such as Cext. Then, the observed increase of DHY may be explained by the Cext increase in DP treated soil. Moreover, the fact that the DHY/Cmic ratio, an indicator of microbial efficiency or stress, do not show any difference in each thesis, suggest that the anaerobic digestate does not induce stress about the energy metabolism of soil microorganisms. The FDA/Cmic ratio, an indicator of microbial efficiency, was lower in DP than in DL treated soils (especially at 4x dose), suggesting that in the DP case, the increase in Cmic

Figure 8. Soil available P (A), exchangeable Ca (B), exchangeable Mg (C), exchangeable K (D), exchangeable Na (E) determined after 9 weeks of incubations with different fertilizers and application rates (mean of three replicates; error bars indicate standard errors of the mean; different letters indicate a significant difference for P ≤ 0.05, HSD Tukey’s test).
was higher than the increase in FDA, resulting in an increase in microbial efficiency, conversely than the soil treated with DL.

The differences observed in the biochemical indicators explain the higher efficiency of DP compared to DL in enhancing P availability in the field. An excess of soluble (available) P will lead to an increased risk of run-off or leaching of P from soil to surface water bodies.

Concerning the effect of anaerobic digestates on soil exchangeable cations, an increase was observed for some cations (i.e., K⁺, Mg²⁺ and Ca²⁺) and a decrease for others (i.e., Ca²⁺). The effect of anaerobic digestates on soil exchangeable cations, the supply of K to soil was higher than Mg, reducing the exchangeable Mg/K ratio in treated soil. The Mg/K ratio in soil is an indicator of soil fertility in agreement with the "ideal" soil concept (the absolute amounts of available Ca, K, and Mg are not that important as their relative values [45]), and a Mg/K ratio of 2:1 is considered "ideal" [46]. According to this interpretative approach, the continuous applications of DL and DP can reduce the Mg/K ratio and induce a plant Mg deficiency. However, under the experimental conditions adopted, all the exchangeable cations concentrations observed fell inside the optimum range [46].

In pot experiments with cucumber and maize, after 21 days of cultivation, we have observed a different effect of digestates on shoot FW than the liquid digestate and cattle manure. Considering that the products were applied on the basis of N content, a different amount of P was added with the different fertilizers (for the 4x dose, DP: 300 mg P₂O₅ kg⁻¹, DL 152 mg P₂O₅ kg⁻¹ and CM 263 mg P₂O₅ kg⁻¹) and this could explain the higher efficacy of DP compared to DL in enhancing P availability. Therefore, also the N/P ratio is an important indicator for the assessment of fertilizing properties of anaerobic digestates; in the case of low N/P ratios (i.e., <2), such as DP, it is necessary to consider the fate of the P in the field. An excess of soluble (available) P will lead to an increased risk of run-off or leaching of P from soil to surface water bodies.

Concerning the effect of anaerobic digestates on soil exchangeable cations, an increase was observed for some cations (i.e., K⁺, Mg²⁺ and Na⁺) and a decrease for others (i.e., Ca²⁺). The effect of anaerobic digestates on soil exchangeable cations, the supply of K to soil was higher than Mg, reducing the exchangeable Mg/K ratio in treated soil. The Mg/K ratio in soil is an indicator of soil fertility in agreement with the "ideal" soil concept (the absolute amounts of available Ca, K, and Mg are not that important as their relative values [45]), and a Mg/K ratio of 2:1 is considered "ideal" [46]. According to this interpretative approach, the continuous applications of DL and DP can reduce the Mg/K ratio and induce a plant Mg deficiency. However, under the experimental conditions adopted, all the exchangeable cations concentrations observed fell inside the optimum range [46].

In pot experiments with cucumber and maize, after 21 days of cultivation, we have observed a different effect of digestates on shoot FW
The DL treatment increased the shoot FW in the case of cucumber, but decreased it in the case of maize. Conversely, the DP treatment led to a reduction of the shoot FW in the case of cucumber, and increased it in the case of maize, as already observed in previous experiences with this crop and other plant species [15, 16].

The shoot N concentration was significantly lower in cucumber plants treated with DL and in maize treated with DP (Table 6); in other words, the shoot FW weight increased when shoot N decreased. Clearly, this is due to the different availability of N in soils treated with digestates. In the case of cucumber, we have observed in the soil a low concentration of NH$_4^+$ (2–3 mg N kg$^{-1}$ DW) and NO$_3^-$ (6–9 mg N kg$^{-1}$ DW) in each treatment (Figure 8). This suggests that in pot experiments with cucumber, the N in soil is a limiting factor for plant growth; further, the liquid digestate, as observed in the incubation experiment, is the product with the largest amount of available N (and positive net N mineralization balance) [6]. On the other hand, most of the N in the DP is not readily available for plant uptake at least in short time [13]. In the case of maize, we have observed a higher NO$_3^-$ (30–50 mg N kg$^{-1}$ DW) and a lower NH$_4^+$ (5–10 mg N kg$^{-1}$ DW) concentration. The soils treated with DP at both doses showed a lower NO$_3^-$ (and a higher NH$_4^+$) concentration. The DP-treated plants performed better in terms of shoot FW weight and SPAD index, despite the higher rate of N immobilization and an observed positive net mineralization balance of DP respect to the DL. Nonetheless, the high concentration of available NO$_3^-$ present also in soils amended with DP could not represent a limiting factor for maize growth, thus explaining the highest shoot FW of plants grown in this condition.

The soil treatment with digestates increased the P concentration in cucumber leaves (Table 7), in agreement with the increase of available P in the soil (Figure 3). In the case of maize, when the soil available P increased, the concentration of P in leaves decreased, when the soil was treated with digestates. This could probably be due to the high P demand of maize for growth compared to cucumber, particularly in the case of high N availability [47].

The anaerobic digestates generally decrease the concentrations of Ca, K and Mg in plant leaves, particularly in maize (Table 7). The higher Ca/N and the lower Mg/K ratio of DP than DL probably explain the lower
concentration of Ca and Mg in plants cultivated in the pots treated with DL [48].

In general, the soil treatment with digestates decreases the micronutrients concentration in leaves of cucumber and maize, except for Mn, Zn and Cu in cucumber treated with DP 4x (Table 7). The total Cu, Fe, Mn and Zn applied to soil with DP was higher than DL, in agreement with the observed relative high DTPA-extractable Cu, Fe, Mn and Zn in pots treated with DP (Figure 10); these partially explain the minor reduction of Cu, Fe, Mn and Zn concentration in leaves of plants treated with DP. Manganese, Zn and Cu are uniformly distributed within the DP matrix, as evidenced by μXRF maps (Figures 2 and 5) and thus probably bound to organic matter. Compared to maize, cucumber seems to be able to slightly better mobilize and take up these elements from DP (Table 7). Iron in DP appears mostly present as sparingly soluble (hydr)oxides particles (Figure 2) and therefore difficult to be mobilized for plant nutrition [49].

5. Conclusions

The major outcomes of this work are: (1) both liquid and pelleted manure-based digestate exhibit a fertilizing potential for crops; (2) this potential is highly dependent on the plant species and the fertilizer dose. Future studies will deepen the ability to deliver nutrients, the dosage and the formulation of the product. In fact, even though the liquid digestate may have the advantage to deliver nutrients more rapidly to plants, its storage represents the main constraint. Particular attention needs to be paid when deciding the dosage for digestates application, considering not only N but also P and K. Indeed, pelleting the digestates could improve the storability of the fertilizer besides enhancing plant nutrient availability (i.e. phosphate), plant biomass and soil biochemical quality. The physical structure and chemical composition of pellets allow nutrients to be easily mobilized over time thus making it a possible source of mineral.
nutrients also in long-term applications. These results will be confirmed in long-term field experiments.

Declarations

Author contribution statement
Fabio Valentuzzi, Luciano Cavanli: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Carlo Porfido: Performed the experiments; Analyzed and interpreted the data.

Roberto Terzano: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tanja Mimmo: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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