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Agricultural Reuse of the Digestate from Anaerobic Co-Digestion of Organic Waste: Microbiological Contamination, Metal Hazards and Fertilizing Performance

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Abstract The aim of this study was to evaluate the agricultural reuse of the digestate products (DPs) obtained from mesophilic anaerobic co-digestion of different organic wastes (sludge, cattle slurries and organic fraction of municipal solid wastes). At this scope, the content of faecal indicators and pathogens as well as the heavy metal concentration of DPs was monitored. The fertilizing performance of the DPs was also investigated. Co-digestion trials were performed using laboratory-scale (LRs) and pilot-scale reactors (PRs). The microbiological analysis of DPs showed the common presence of *Salmonella* and an inadequate reduction of indicator organisms during the digestion process, both in the LRs and the PRs. Moreover, the presence of pathogens (e.g. *Listeria monocytogenes*) in some DP samples highlighted the importance of the microbiological quality evaluation of the DPs to study the possible health risks for consumer. In several samples of DPs, the Cu, Ni and Zn contents exceeded the maximum admissible concentration for fertilizer, as specified by Italian law, suggesting possible environmental contamination if the DPs are used for agricultural purposes. Considering the fertilizing performance, significant differences of growth parameters were observed only for the DPs that were produced by LRs. In conclusion, this work can be considered as a preliminary study to evaluate the possible agricultural reuse of the digestate obtained from different organic wastes.

Keywords Anaerobic co-digestion · Agricultural reuse · Fertilizer · Faecal indicator bacteria · Pathogenic bacteria · Heavy metals

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1 Introduction

Millions of tons of solid waste are produced annually from municipal, industrial and agricultural sources. The decomposition of these organic wastes results in large-scale contamination of land, water and air (Nasir et al. 2012). However, the European Commission has set the ambitious goal of increasing energy from renewable

sources to 20 % in 2020 compared to 8.5 % in 2005 (EREC 2008). To reach this goal, the use of all existing renewable energy sources must increase. Anaerobic digestion is a suitable option for the production of renewable energy in the form of biogas, which can be used to treat organic wastes such as manures, slurries, food processing wastes, sewage sludge and organic fraction of municipal solid waste (Rajeshwari et al. 2000; Ward et al. 2008).

In anaerobic digestion, co-digestion is used to describe the combined treatment of several wastes with complementary characteristics. The co-digestion of combined wastes results in a high methane yield compared to single waste digestion, which is one of the main advantages of this anaerobic technology (Nasir et al. 2012). There are several studies in the literature that address the utilization of co-digestion, such as co-digestion of the organic fraction of municipal solid wastes (OFMSW), cattle manure and agricultural residues (Amon et al. 2007; Macias-Corral et al. 2008), organic solid wastes and sewage sludge (Murto et al. 2004) or more specific wastes (Demirel et al. 2013; Parawira et al. 2004; Traversi et al. 2013).

In addition to biogas, anaerobic digestion generates a digestate product (DP) that can be used as an agricultural fertilizer because the nutrients present in the raw input material after the digestion process remain as accessible compounds in the mineralized sludge (Alkanok et al. 2014; Diaz et al. 2011; Lehtomaki and Bjornsson 2006). The diverse origins of the input material used for biogas production indicate that biogas plants produce fertilizers that vary in nutrient content. At present, considering the Italian laws, the DPs derived from the co-digestion of OFMSW must be considered waste (D.Lgs. n. 205/2010). There is a lack of specific regulation on the use of OFMSW or its derivates in agriculture: only the agricultural use of wastewater digestion sludge (D.Lgs. 99/1992) and animal manure (D.M. n. 109/2006; D.M. n. 29819/2009) is regulated in Italy, and DPs derived from animal by-products (including animal faeces) fall under the European regulation on animal by-products (Commission Regulation n. 142/2011).

According to the literature, the physico-chemical properties of DPs have been widely investigated, whereas fertilization studies are still scarce (Abubaker et al. 2012; Garfi et al. 2011; Ning et al. 2011; Nishikawa et al. 2012; Tambone et al. 2010). However, the DPs are not harmless products because they contain heavy metals and may also contain organic pollutants, such as pesticides and pathogenic bacteria, that are introduced to the soil ecosystem by their application. Heavy metals can be present in the input material used for biogas production and are not altered in the anaerobic digestion process (Sager 2007); therefore, they may be concentrated due to mass reduction during the process (Dabrowska and Rosinska 2012; Govasmark et al. 2011).

The application of digestate on fields can potentially spread pathogens from one farm to another, causing crop contamination. The potential health risk of digested residues from biogas plants is partly dictated by the substrates that are treated in the plants; for instance, organic wastes may contain pathogenic bacteria, depending on the source and type of waste. In particular, wastes of animal and human origin can contain various pathogenic bacteria (e.g. *Salmonellae*, *Enterobacter*, *Clostridiae* and *Listeria*), parasites (e.g., *Ascaris*, *Giardia* and *Cryptosporidium*), viruses (e.g. norovirus, enterovirus, rotavirus, Hepatitis A virus) and fungi (*Candida*, *Aspergillus* and *Trichophyton*) (Sahlstrom 2003; Sidhu and Toze 2009; Venglovsky et al. 2006). The possible presence of pathogens can be expected also in the OFMSW (Hassen et al. 2001) even if there is no specific information about pathogen contamination of OFMSW in the literature. Some studies have posited that pathogens can survive after anaerobic digestion (Sidhu and Toze 2009), and the growth of the remaining viable bacteria after the application of DP to land has been demonstrated for some bacterial species (Bonetta et al. 2011a; Johansson et al. 2005).

The aim of this study was to investigate the content of faecal indicators and pathogens (*E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, *Giardia* spp. and *Cryptosporidium* spp.) as well as the heavy metal concentration of DPs obtained from mesophilic anaerobic co-digestion of sludge (anaerobic and thickened), cattle slurries and OFMSW. Moreover, this study includes an experiment conducted in a greenhouse that investigated the fertilizing performance of the DPs on *Sorghum bicolor*.

This work is a portion of a larger multidisciplinary project DigestedEnergy concerning the improvement of biomass anaerobic digestion process in order to produce biogas, the integration of the process in waste and working refuse management and treatment cycle and the implementation of anaerobic digestion systems targeted to medium-small sized urban, industrial and rural entities.

2 Materials and Methods

2.1 Anaerobic Digestion Reactors and Sampling

The study of the optimization of anaerobic co-digestion was carried out using two different experimental devices: two laboratory-scale reactors (LRs) and two pilot-scale reactors (PRs). These reactors are located in the Società Metropolitana Acque Torino SpA (SMAT) area in Castiglione Torinese, Turin, Italy. More specific characteristics of reactors have been previously reported by Novarino and Zanetti (2012) and Traversi et al. (2012). Briefly, the LRs have working volumes of 10 L, the digestion process took place at mesophilic temperature (38±2 °C) and the reactors were fed daily with 500 mL of organic waste substrates. The DP collection was performed 40 days after the start of each new digestion trial, a period chosen based on two successive hydraulic retention times (HRTs): one (20 days) to ensure total replacement of the material inside the reactors and one (20 days) to allow the process to stabilize. The PRs have working volumes of 800 L, the digestion process took place at mesophilic temperature (38±2 °C) and the reactors were fed daily with 40 L of organic waste substrates. The DP collection was performed 30 days after the start of each new digestion trial: 20 days of HRT plus 10 days for the digestion process to stabilize. A total of eight trials for each reactor was carried out. The reactors were inoculated with a mixture of anaerobic sludge (75 %) and cattle slurry (25 %) and were fed with a mixture of pre-treated (pressure extruded or turbo-mixed) OFMSW and thickened sludge. The pressure extruder separates the organic fraction from the total amount of waste because of the exerted pressure whereas the turbomixing system uses a turbomixing chamber that rotates at high velocity and crushes the substrates.

Anaerobic and thickened sludge were obtained, respectively, by anaerobic digestion of sewage sludge and by effluent in a wastewater treatment plant located at Castiglione Torinese (Piedmont, north-west Italy). The OFMSW was collected in the Voghera town, and the pre-treatment phase was performed at the Aral waste treatment plant sited in Castelceriolo near the town of Alessandria (Piedmont). Cattle slurry was obtained by a farm located in Candiolo (Piedmont).

At the end of each digestion trial, the representative samples of DPs (~1.5 kg) were collected from the LRs and PRs in clean vessels and stored in two clean pots. One kilogram of sample was used for the evaluation of heavy metal content and fertilizing performance, and 0.5 kg was used for microbiological analyses.

The input substrates were collected every two trials (n=4) before the starting of the digestion process in clean vessels and stored in clean pot. They were kept cold during transportation and arrived at the laboratory within 24 h of sampling.

2.2 Microbiological Analyses

Microbiological analyses (indicators and pathogens) were performed on all types of input substrates (anaerobic and thickened sludge, cattle slurry, OFMSW) to characterize the microbial contamination of each material and on DPs collected at the end of the trials in LRs and PRs to evaluate the microbiological hazards related to their reuse as fertilizers.

2.2.1 Indicator Parameters

Each sample (50 g) was homogenized in sterile 0.9 % NaCl solution using a Stomacher Laboratory-Blender 400 (PBI International). Serial dilutions were prepared and inoculated in triplicate on specific agar media to enumerate bacterial indicators: mesophilic counts on Tryptic Soy Agar (TSA, Applichem) at 37 °C for 24 h, *Escherichia coli* on Tryptone bile X-glucuronide medium (TBX, Biolife) at 44 °C for 24 h, *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBG, Oxoid) at 37 °C for 24 h and faecal enterococci on Kanamycin Aesculin Azide Agar Base (KAA, Biolife) at 37 °C for 24–48 h. Bacterial counts were expressed as log CFU g^{-1} of wet matter. The presence of *Clostridium perfringens* in 1 g of sample was determined on Tryptose Sulphite Cycloserine Agar (TSC, Biolife) after anaerobic incubation at 42 °C for 24 h. Three to five suspected colonies (Gram-negative, catalase-negative) were confirmed with a reverse CAMP test. Briefly, cultures were inoculated at right angles within 1 to 2 mm of a β-haemolytic group B *Streptococcus* streak on Sheep Blood Agar plates (Biolife). After anaerobic incubation (37 °C for 18–24 h), a positive reverse CAMP test showed a "bow-tie" or "reverse arrow" pattern of hemolysis at the junction of the two cultures. A qualitative analysis was performed to detect helminth eggs. This test was based on the separating of helminth eggs from faecal material and concentrating them by means of a flotation fluid with an appropriate specific gravity. Briefly, 50 g of the sample

was suspended in 500 mL of a wash buffer (0.1 % Tween 80, Sigma). After mixing, the faecal suspension was poured through a tea strainer into a container. This procedure was repeated for two or three times. The suspension was left to stand for 20 min at room temperature, and the solid obtained was poured in different tubes. The flotation fluid ($ZnSO_4$ 33 %, density 1:200; Sigma) was added in each tube, leaving a convex meniscus at the top of the tube. Carefully, a coverslip was placed on top of the test tubes. The tubes were left to stand for 10–15 min at room temperature. After, the coverslip was raised carefully from the tube, together with the drop of fluid adhering to it and immediately placed on a microscope slide. The samples were observed with a microscope (×10 or× 40, Zeiss).

2.2.2 Pathogens

Salmonella analysis (25-g sample): After pre-enrichment in Buffered Peptone Water (BPW, Oxoid) (24 h at 37 °C), an aliquot (100 μL) was inoculated into Rappaport-Vassiliadis broth (10 mL, RV, Biolife) (18–24 h at 42 °C), and another aliquot (1 mL) was inoculated into Selenite Broth base (9 mL, SB, Biolife) (24 h at 37 °C). Both the RV and SB broths were streaked on Bismuth Sulphite Agar (BSA, Biolife) and Xylose Lysine Desoxycholate Agar (XLD, Biolife) and incubated at 37 °C for 24 h. Colonies with typical *Salmonella* morphology were confirmed with the agglutination test (Biolife) and with biochemical tests using the Biolog Microbial Identification System (BIOLOG, Inc.). This method tests the ability of a microorganism to utilize or oxidize different carbon sources. Tetrazolium violet is used as a redox dye. Briefly, bacteria were grown on Biolog Universal Growth agar at 37 °C for 24 h. Colonies were suspended in a 0.4 % saline solution, and the inoculum density was adjusted to the specified turbidity range. The bacterial suspensions were inoculated in a Biolog GN Microplate and were incubated at 37 °C then manually read after 24 h. The pattern of purple wells was compared with a specific database by Biolog_ Microlog 2 software.

L. monocytogenes analysis (25-g sample): After pre-enrichment in Fraser Broth Half concentration (Oxoid) (30 °C for 24 h), an aliquot (100 μL) of the pre-enrichment broth was inoculated into 10 mL of enrichment Fraser Base Broth (Oxoid) (24 h at 30 °C). Aliquots of pre-enrichment and enrichment broths were streaked on Listeria Palcam Agar Base (Biolife) (37 °C for 24 h) and ALOA Agar (Biolife) (30 °C for 48 h). Colonies with typical *Listeria* morphology were confirmed as *L. monocytogenes* by real-time PCR (iQ-Check *Listeria monocytogenes* Kit, BioRad).

E. coli O157:H7 analysis (25-g sample): After enrichment in Tryptic Soy Broth (Biolife) supplemented with novobiocin (42 °C for 24 h), samples were subcultured onto MacConkey Sorbitol Agar (CT-SMAC, Biolife) plates by streaking (24 h at 37 °C). The suspected colonies were confirmed by multiplex PCR, as reported by Bonetta et al. (2011b).

The results of bacterial pathogen contamination were expressed as the presence/absence of pathogens.

Giardia and *Cryptosporidium* analysis (1-g sample): A 25 mL volume of 0.1 M phosphate-buffered saline (Sigma) was added to a centrifuge tube containing the sample and vortexed for 60 s. Another 25 mL volume of phosphate-buffered saline was then added to the sample, and the tube was inverted five times. The sample was left to stand for 60 min at room temperature. At the end of this period, 45 mL volume of liquid was transferred to a clean 50 mL centrifuge tube. The liquid was then brought to a final volume of 50 mL with filtered deionized water and centrifuged for 5 min at 1,050×*g*. After centrifugation, the top 45 mL of liquid was removed and discarded (Massanet-Nicolau 2003). The sample was suspended by vortexing and then purified with a commercial kit for immunomagnetic separation of *Giardia* and *Cryptosporidium* cysts and oocysts (Dynal) in accordance with the manufacturer's instructions. After purification, the presence and number of cysts and oocysts were determined by immunofluorescence with the *Cryptosporidium* Cell Test IF (Cellabs) and the *Giardia* Cell Test IF (Cellabs) (ISS 2007). The oocysts were counted with an epifluorescent microscope (Zeiss), taking into consideration the morphology, size and color of the particles. The results for cyst and oocyst contamination were expressed as the presence/absence and, when present, as the number of cysts and oocysts per gram of sample.

2.3 Metal Analyses

Metal content (Cd, Cr, Cu, Hg, Ni, Pb, Zn and Fe) was evaluated in DPs to investigate the chemical hazard related to their reuse as fertilizers. Samples were pretreated with HCl/NO_3 for digestion in a microwave vessel at 200 °C for 30 min, and metal concentration was measured by a Varian Series ICP 820-MS (Palo

Alto, USA) equipped with a collision reaction interface (CRI) system.

2.4 Plant Growth Test

The plant growth test was performed with *S. bicolor* to study the DPs fertilizing performance: Two seeds were sown in vases filled with 120 mL of substrate (zeolite/vermiculite/agriperlite/peat; 1:1:1:2) and 20 mL of DP samples. Twelve replicate vases for each DP sample and control sample (substrate without DP sample) were prepared. Plants were grown in a greenhouse under controlled light, temperature and moisture conditions. After 1 month of growth, plants were harvested and analyzed for stem length and for the fresh and dry weights of the stem and root.

The results were expressed as the percentage of stem length and as the fresh/dry weight of stem and root compared to the control plants and statistically compared with ANOVA, Fisher test.

3 Results and Discussion

3.1 Microbiological Analyses

The results of the analysis of the microbial indicator parameters obtained from the substrates entering the process (input substrates) and from the DPs are reported in Table 1. Mesophilic bacterial counts and *E. coli* showed a slightly lower mean value in the sludge (anaerobic and thickened) than the other input substrates. On the other hand, no relevant differences were observed among the various input substrates for enterococci count, except for cattle slurry which had slightly lower mean value of this parameter compared to the other substrates. The concentrations of the bacterial indicator parameters examined in this study for sludge (anaerobic and thickened), cattle slurry and OFMSW were similar to, or sometimes lower than, those reported in other studies (Iwasaki et al. 2011; Sahlstrom et al. 2004; Sidhu and Toze 2009; Soupir et al. 2006).

The DPs produced by LRs generally presented analogous levels of bacterial contamination to those of input waste substrates, with the exception of mesophilic bacteria, which were present in the output samples at lower levels. On the other hand, the digestion process in PRs seemed to cause a reduction of indicator bacteria. This result is particularly important for enterococci, which are considered the best microbial indicators of vegetative bacterial pathogen reduction during the digestion process (Larsen et al. 1994; Viau and Peccia 2009).

By contrast, the anaerobic digestion process did not seem to reduce the number of positive samples for *C. perfringens*. The presence of *C. perfringens* contamination observed in this work in the DP samples was also reported in earlier studies (Bagge et al. 2005; Bonetta et al. 2011a). The persistence of *C. perfringens* in the digested residues could be an indicator of other pathogenic spore-forming bacteria.

The presence of helminth eggs was observed only in two samples of cattle slurry and in only one samples of DP in the LRs. Although helminth infections are a major concern in the developing world, in agreement with results obtained in this study, the occurrence of helminth eggs in digested biosolids has been also reported in other industrialized countries (Rubio-Loza and Noyola 2010; Sidhu and Toze 2009).

The number of pathogen-positive samples detected in the input wastes and in the DPs are presented in Table 2. *E. coli* O157:H7 was not found in any of the samples.

Salmonella was present in all of the sludge samples (anaerobic and thickened) and in one sample of OFMSW, but was never found in cattle slurry. The identification of species revealed in the sludge samples the presence of *Salmonella enterica* (67 %) and *Salmonella enterica* subsp. *arizonae* (33 %). *Salmonella* isolated from OFMSW sample was identified as *S. enterica*. In general, the presence of *Salmonella* spp. was reported in almost all DPs collected from the LRs and in some samples collected from the PRs; in all cases, it belonged to the species *enterica*. The presence of *Salmonella* in wastewater sludge such as in anaerobically digested sludge has also been reported in other studies (Dahab and Surampalli 2002; Sahlstrom et al. 2004; Sidhu and Toze 2009). The contamination of OFMSW samples could be ascribed to the use of thickened sludge as a diluent for OFMSW pre-treatment.

L. monocytogenes was present in some samples of sludge and in one samples of OFMSW, but was never found in cattle slurry. This pathogen was found in a sole sample of DP obtained from the PRs. *L. monocytogenes* is a common contaminant of organic wastes and, in general, of biomasses. This bacterium is wide spread in the environment (Colleran 2000), but it is normally present in low numbers (Sidhu and Toze 2009). Different studies showed the *L. monocytogenes* contamination in

Table 1 Mean, minimum and maximum values (expressed as $\log_{10}$CFU g^{-1}) or percentage of positive samples of bacterial indicator parameters in input substrates and DPs

	N	Mesophilic count			*E. coli*			Enterococci			*C. perfringens* (%)	Helminth eggs (%)
		Mean	m	M	Mean	m	M	Mean	m	M		
Organic waste												
Anaerobic sludge	4	6.3	5.3	8.7	3.5	2.9	3.8	4.2	3.1	4.9	75	0
Thickened sludge	4	7.1	6.7	7.9	4.2	2.9	5.7	4.3	4.2	4.5	75	0
Cattle slurry	4	7.8	7.3	8.5	5.3	4.9	5.5	3.5	2.8	4.2	50	50
OFMSW	4	7.9	7.4	8.7	4.9	4.3	5.0	4.6	3.8	5.2	100	n.a.
DPs												
LRs	8	6.6	5.4	7.8	4.4	<2	5.6	4.8	4.6	5.0	71	13
PRs	8	6.1	5.1	7.0	3.6	<2	4.4	3.8	3.1	5.2	100	0

N number of samples, *n.a.* not analyzed, *m* min, *M* max

wastewater sludge (Sahlstrom et al. 2004; Sidhu and Toze 2009). As reported for *Salmonella*, the presence of *L. monocytogenes* in samples of OFMSW could be related to the mixing of OFMSW with thickened sludge in the pre-treatment. Many studies have reported that animal manure is generally contaminated by different pathogens (Sahlstrom 2003; Venglovsky et al. 2006) contrary to that observed in this study. The effect of the mesophilic digestion process on *Listeria* has not been investigated in depth; according to the results obtained in this study, a reduction, but not a complete elimination, was observed in the study conducted by Horan et al. (2004) using a laboratory-scale plant.

Giardia was revealed in all samples of sludge (mean 132 cysts g^{-1}), one sample of OFMSW (296 cysts g^{-1}) and all DPs (mean 11 and 67 cysts g^{-1} in LRs and PRs respectively); *Cryptosporidium* was observed only in one sample of LRs and PRs at a low concentration (6 oocysts g^{-1}). *Giardia* and *Cryptosporidium* (oo)cysts are frequently isolated from wastewater, although *Giardia* cysts are more frequently present in these samples compared with *Cryptosporidium* oocysts, which have a more seasonal distribution. Anaerobically digested biosolids can also be contaminated by these protozoa, but there are only limited information. Some previous studies reported that DPs may contain up to 10 g^{-1} *Cryptosporidium* oocysts and 10^2 g^{-1} of *Giardia* cysts (Chauret et al. 1999; Hu et al. 1996).

The results of the microbiological analyses of the DPs obtained from the LRs and PRs did not always coincide. A direct comparison between the data obtained in the two types of reactors cannot be made because,

Table 2 Number of pathogen-positive samples in input substrates used for anaerobic co-digestion and DPs

	E. coli O157:H7	*Salmonella* spp.	*L.monocytogenes*	*Cryptosporidium* spp.[a]	*Giardia* spp.[a]
Organic waste					
Anaerobic sludge	0/4	4/4	1/4	0/2	2/2
Thickened sludge	0/4	4/4	3/4	0/2	2/2
Cattle slurry	0/4	0/4	0/4	0/2	0/2
OFMSW	0/4	1/4	1/4	0/2	1/2
DPs					
LRs	0/8	7/8	0/8	1/2	2/2
PRs	0/8	3/8	1/8	1/2	2/2

[a] Only two samples were analyzed

Table 3 Metal content and Italian law limits measured in DP samples expressed as milligrams per kilogram (dry matter)

	LR		PR		Law limits	
	Mean±SD	Max	Mean	Max	LV fertilizer[a]	LV sludge[b]
Cd	1.4±0.3	1.6	0.6±0.7	1.6	1.5	20
Cr	250.6±187.0	560.3	96.9±58.1	240.0	–	–
Cu	447.6±111.2	561.0	159.9±102.3	420.0	230	1,000
Hg	0.7±0.1	0.8	0.5±0.5	1.1	1.5	10
Ni	239.0±89.2	355.9	107.8±61.4	220.0	100	300
Pb	104.1±17.2	126.0	52.6±23.6	91.0	140	750
Zn	2,818.4±560.1	3,753.7	655.0±234.5	1,200.0	500	2,500
Fe	21,129.3±6,171.0	29,837.7	13,700.0±2,022.7	16,000.0	–	–

[a] Limit value (LV) D.M. n. 29819/2009

[b] Limit value (LV) D.lgs. n. 99/1992

as observed in other studies, both volumes of input substrates involved in the digestion process and the microbial dynamics were different (Wagner et al. 2008).

Because no specific Italian law has been established to regulate the hygienic quality of DPs, the results of the microbiological analyses obtained in this study were compared with the maximum admissible concentration (*E. coli* <1,000 CFU g^{-1}) specified in the Italian law for fertilizers (D.M. n. 29819/2009). From this comparison, the microbiological analyses of the DPs reveal values for *E. coli* that are higher than the allowed limit in almost all LR digestates and in some PR samples. Moreover, considering the European regulations for animal by-products, all of the DP samples exceeded the standard for enterococci ($m=1\times10^3$ and $M=5\times10^3$ CFU g^{-1}) (Commission Regulation n. 142/2011).

The presence of *Salmonella* in almost all of the DP samples could represent a hygienic problem because the absence of *Salmonella* in 25 g of material serves as an indicator of bacterial pathogen absence and is considered the standard for the use of DP as a fertilizer. This standard is reported in the Italian laws for fertilizer (D.M. n. 29819/2009) and wastewater sludge (D.Lgs. n. 99/1992), as well as in European regulations of animal by-products (Commission Regulation n. 142/2011).

Considering that in this study the suitability of *Salmonella* presence as an indicator of bacterial pathogens has not been demonstrated, other indicators, such as viral contaminants, should be introduced and studied. In particular, an evaluation of the effectiveness of the anaerobic process and determination of the most effective post-treatment of DP, based on different input substrates and type of anaerobic process used, could be useful for indentifying specific indicators for each biogas plant.

3.2 Metal Analyses

The metal concentrations of the DPs are reported in Table 3. In several DP samples, the metal content exceeded the maximum admissible concentration under the Italian law for fertilizers (D.M. n. 29819/2009). In particular, the levels of Cu, Ni and Zn were found to be higher than the allowed levels in all of the DP samples from the LR and in a large number of the PR samples. The Cd concentration showed values higher than the

Table 4 Plant growth parameters measured after application of DP as fertilizer. Results were expressed as percentage respect to control (100 %)

DPs	Stem length	Fresh weight stem	Fresh weight root	Dry weight stem	Dry weight root
LRs	149.5±10.5	194.8±56.4	235.9±43.8	225.4±39.9	221.1±40.2
PRs	123.8±10.3	171.6±39.6	156.8±27.7	164.8±37.6	135.2±16.2

limits in two of the DP samples from the LR and in a sole PR sample. On the other hand, the concentrations of Hg and Pb were lower than the limits specified by law.

If the DPs are considered as sludge, then it is possible to compare the metal concentrations with the Italian law for wastewater treatment sludge (D.Lgs. n. 99/1992). In this case some DP samples produced by the LRs exceeded the allowed limits for Zn and Ni.

The high levels of some heavy metals observed in the DP samples are ascribed to contamination of the input substrates that were used for anaerobic digestion in this study. In fact, different authors have reported that wastewater sludge, cattle manure and OFMSW can contain hazardous substances such as heavy metals (Dong et al. 2010; Tulayakul et al. 2011; Uysal et al. 2010). Moreover, it is important to note that during anaerobic digestion, the composition of organic substances results in an increase of heavy metal concentration in the dry matter of sludge (Dabrowska and Rosinska 2012).

The heavy metal content lower than that showed in the DPs analyzed in this study was reported in other works focusing on DPs from food and garden waste (Govasmark et al. 2011), slurry (Jin and Chang 2011) or OFMSW (Dong et al. 2010; Garcia et al. 2012). A similar contamination was observed in the study of Dabrowska and Rosinska (2012) that analyzed the heavy metal content in DPs from wastewater sludge highlighting the role of this input substrate in the heavy metal contamination of the digestate.

The presence of significant amount of Cu, Ni and Zn in DPs suggests that there is a possibility of environmental contamination if the DPs are used for agricultural purposes. In addition to environmental concerns, the release of heavy metals (e.g. Cu, Zn, Pb and Cd) into soils, water and plants, through the use of DPs as fertilizers, could also pose public health risks throughout the food chain.

3.2.1 Plant Growth Test

The general effects of DPs used as fertilizers on relative biomass fractions (root, stem) and plant stem lengths are reported in Table 4. As shown for the DPs produced by PRs (compared to the control), neither the weight of the stem and root nor the stem length were affected by treatment with DPs, which produced parameter values that were similar to those in the control plant. Significant differences ($p<0.05$, ANOVA test, Fisher) of growth parameters with respect to the control were observed only for the DPs produced by LRs. The different fertilizing performance of DPs produced by the two types of reactors (LRs vs. PRs) could be ascribed to differences in the management practices and operating conditions of the digestion process, which lead to the production of fertilizers with varying contents of plant macro- and micro-nutrients, as well as chemical contaminants (Abubaker et al. 2012; Garfi et al. 2011).

Unfortunately, it is difficult to compare the results of this study to those obtained in previous studies because the fertilizing performance of DPs depends on the origin and composition of the feedstock and has been investigated under experimental conditions (pot vs. field trials) that vary in the plant tested (e.g. tomato, rice and potato) and the fertilization rate (Abubaker et al. 2012; Garfi et al. 2011; Ning et al. 2011; Nishikawa et al. 2012).

4 Conclusions

In this work, the possible agricultural reuse of DPs produced by the anaerobic co-digestion of sludge (anaerobic and thickened), cattle slurry and OFMSW was investigated from several different points of view.

The microbiological analysis of DPs performed in this study revealed the presence of *Salmonella* and an inadequate reduction of indicator organisms during the digestion process, both in the laboratory and pilot-scale reactors. Therefore, this contamination could make the DP unsuitable as an agriculture fertilizer. Moreover, the presence of pathogens (e.g. *L. monocytogenes*) in some DP samples highlights the importance of the microbiological quality evaluation of the DPs to study the possible health risks for consumer.

Considering the metal content of the DPs analyzed the high levels of Cu, Ni and Zn may cause environmental contamination. Thus, heavy metal pollution should be a concern when we apply DPs to soil, particularly in relation to the possible health risks for humans that are caused by some heavy metals (e.g. Cd, Cr and Pb).

The significant positive effect on plant growth observed with the DPs obtained from LRs demonstrates the need for further investigations. In particular, it could be interesting to study fertilizing performance on different plants using field trials.

In conclusion, this work can be considered as a preliminary study to evaluate the possible agricultural

reuse of the digestate obtained from different organic wastes.

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