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Title: Evaluation of benefits and risks associated with the agricultural use of organic wastes of pharmaceutical origin

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Keywords: Sludge; Anaerobic Digestate; Compost; Fertilizer; Phytotoxicity; Recycle

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Abstract: Industrial fermentations for the production of pharmaceuticals generate large volumes of wastewater that can be biologically treated to recover plant nutrients through the application of pharmaceutical-derived wastes to the soil. Nevertheless, benefits and risks associated with their recovery are still unexplored. Thus, the aim of the present work was to characterize three potential organic residues (sludge, anaerobic digestate and compost) derived from the wastewater generated by the daptomycin production process. The main parameters evaluated were the physico-chemical properties, potential contaminants (heavy metals, pathogens and daptomycin residues), organic matter stabilization and the potential toxicity towards soil microorganisms and plants.
The results showed that all the studied materials were characterized by high concentrations of plant macronutrients (N, P and K), making them suitable for agricultural reuse. Heavy metal contents and pathogens were under the limits established by European and Italian legislations, avoiding the risk of soil contamination. The compost showed the highest organic matter stabilization within the studied materials, whereas the sludge and the anaerobic digestate were characterized by large amounts of labile organic compounds. Although the pharmaceutical-derived fertilizers did not negatively affect the soil microorganisms, as demonstrated by the enzymatic activities, the sludge and the anaerobic digestate caused a moderate and strong phytotoxicity, respectively. The compost showed no toxic effect towards plant development and, moreover, it positively affected the germination and growth in lettuce and barley. The results obtained in the present study demonstrate that the valorization of pharmaceutical-derived materials through composting permits their agricultural reuse and also represents a suitable strategy to move towards a zero-waste production process for daptomycin.

Response to Reviewers: Response to reviewers' comments:

Reviewer #1:

The paper is based on a interesting experimental activity performed to evaluate the risk associated to organic residues of pharmaceutical origin when they are spread on the soil as fertilizers.
In the current paper the methodology is well discussed and commented. In my opinion the work can be useful for the reading of the Journal but significant revisions are needed before publication.

Comment No. 1
Explain how the results of the work can be extended to a more general case considering that the samples of the organic residues are related to a specific plant. Are the samples representative of organic residues from a generic plant that treats the type of wastewater discussed in the paper. Otherwise the work can be considered only a case study and the results are quite limited for scientific purposes.

Response No.1
The authors thanks the reviewer for the interesting and significant comment. Although the experiment was carried out with organic residues derived from a specific pharmaceutical plant, the results could be extended to generic wastewaters derived from antibiotic fermentation processes. For these reasons, the authors amended both the "Introduction" and "Conclusion" sections as reported below. Moreover the authors added a sentence to highlight the total antibiotic consumption in EU in the "Introduction".

Comment No. 2
The organic residues derive from different processes that don't permit the comparison in term of risk analysis; in particular, the compost derives from the aerobic treatment of digestate, so it is quite obvious that it is more stabilized (and probably less risky) if compared with digestate. Please clarify these aspects

Response No. 2
The authors agree with the reviewer that composted materials are obviously more stabilized than those derived from anaerobic digestion processes. In the present experiment, the composting represented also a strategy to complete the daptomycin degradation. Thus, the comparison of the sludge, the digestate and the compost was necessary to evaluate the different effectiveness of biological treatments for daptomycin degradation. In order to clarify that, the authors modified the text to better explain this aspect.

Comment No. 3
The dilution impact of agriculture by-products is not adequately discussed in the paper.

Response No. 3
The authors agree that the dilution impact of agricultural by-products on the daptomycin and heavy metals concentrations was not enough discussed in the "Results and Discussion" section. Thus, the text was amended accordingly.

Comment No. 4
Abstract, line 23: don't use the term "organic fertilizers". The terminology could be used if the sludge, digestate and compost will meet always the legal requirements for soil use; that it is not true, so it should be better a term as "organic residues"

Response No. 4
The authors agree with the reviewer and they accepted the suggestion.

Comment No. 5
Abstract line 24. Heavy metals concentration can be considered a physic-chemical property; clarify why did you separate the terms;

Response No. 5
The authors separated the heavy metals from the other physico-chemical properties in order to highlight their pollution potential, as discussed in the “Results and discussion” paragraph.

Comment No. 6
Line 99 pag 5. Use "freezed"

Response No. 6
The text was corrected as suggested by the Elsevier Language Editing Service.

Comment No. 7
Line 164 pag 7. How many controls did you use?

Response No. 7
The authors carried out a control for each species tested and 5 replicates were used for each control.

Comment No. 8
Line 175 pag 8 "30, 22.5, 15 and 7.5 ton/ha" are not concentration but agriculture application doses; add the real concentrations used in lab tests

Response No. 8
The authors agree with the reviewer and they modified the text adding the concentrations used in the laboratory experiment.

Reviewer #2

Comment No. 9
This Ms reports original results of a laboratory experiment to assess the properties of three different treatments on a waste water from pharmaceutical industry. The experimental work is accurate and results properly reported and discussed. The manuscript is basically clear, but in my opinion, it would improve by an language revision by a native english speaking.

Response No. 9
The authors thank the reviewer for the comments. The suggestion of making a language revision by a native English speaker was accepted and the authors employed the Elsevier Language Editing Service to accomplish the objective.

Comment No. 10
The DH acronim has been used either for "Humificattion degree" or for "Dehydrogenase activity"

Response No. 10
The authors decided to use the DH acronym only for the Dehydrogenase activity abbreviation.

Comment No. 11
Table 1: Units are not clear(g g-1 or % ?)

Response No. 11
The authors decided to better explain the units as “%” in order to make them clearer.

Comment No. 12
Figure 4: Authors shold comment GI > 100% of the control.

Response No. 12
The GI > 100% of the control is mainly due to a hormone-like action that is quite common in well stabilized organic residues such as composted materials. The authors decided to clarify this aspect by modifying the text as described below.

*Graphical Abstract

***Highlights (for review)**

- The agricultural use of pharmaceutical derived organic wastes was assessed
- Physic-chemical parameters, contaminants and potential toxicity were evaluated
- All the organic materials are characterized by high macronutrient contents
- Sludge and anaerobic digestate reuse faces environmental issues
- The compost shows high organic matter stabilization and absence of toxicity

Evaluation of benefits and risks associated with the agricultural use of organic wastes of pharmaceutical origin

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Abstract

Industrial fermentations for the production of pharmaceuticals generate large volumes of wastewater that can be biologically treated to recover plant nutrients through the application of pharmaceutical-derived wastes to the soil. Nevertheless, benefits and risks associated with their recovery are still unexplored. Thus, the aim of the present work was to characterize three potential organic residues (sludge, anaerobic digestate and compost) derived from the wastewater generated by the daptomycin production process. The main parameters evaluated were the physico-chemical properties, potential contaminants (heavy metals, pathogens and daptomycin residues), organic matter stabilization and the potential toxicity towards soil microorganisms and plants.

The results showed that all the studied materials were characterized by high concentrations of plant macronutrients (N, P and K), making them suitable for agricultural reuse. Heavy metal contents and pathogens were under the limits established by European and Italian legislations, avoiding the risk of soil contamination. The compost showed the highest organic matter stabilization within the studied materials, whereas the sludge and the anaerobic digestate were characterized by large amounts of labile organic compounds. Although the pharmaceutical-derived fertilizers did not negatively affect the soil microorganisms, as demonstrated by the enzymatic activities, the sludge and the anaerobic digestate caused a moderate and strong phytotoxicity, respectively. The compost showed no toxic effect towards plant development and, moreover, it positively affected the germination and growth in lettuce and barley. The results obtained in the present study demonstrate that the valorization of pharmaceutical-derived materials through composting permits their agricultural reuse and also represents a suitable strategy to move towards a zero-waste production process for daptomycin.

Keywords

Sludge, Anaerobic Digestate, Compost, Fertilizer, Phytotoxicity, Recycle

1. Introduction

In the past few decades, the enormous demand for life-saving drugs, such as antibiotics, has led to the development of the pharmaceutical manufacturing industry. Indeed, in 2015, the European Union population-weighted mean consumption of antibiotics for systemic use in the community (i.e., hospitals) was 22.4 defined daily doses per 1000 inhabitants per day (ECDC, 2016). Antibiotic manufacturing can involve a complex series of mainly batch processes in which numerous raw materials are often used and large volumes of wastewater are generated (Tang et al., 2011; Oktem et al., 2008). These processes are characterized by high values of biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) and are usually stabilized through physical and/or chemical or biological processes. In particular, physical and chemical treatments are not always suitable for wastewater treatment due to their low efficiency for dissolved COD removal and high consumption of chemicals (Oktem et al., 2008). Conversely, biological treatments are efficacious systems because they reduce the high COD concentrations. In particular, the aerobic stabilization of pharmaceutical-derived wastewaters is considered one of the most common strategies for the disposal of these wastewaters. At the end of the biological wastewater treatment, the residual sludge is usually disposed by landfill and incineration, although the Council Directive 86/278/EEC (CEC, 1986) encourages their agricultural reuse, preventing “harmful effects on soil, vegetation, animals and man” (Martín et al., 2015). The residual sludge can be reclaimed for agricultural land, producing several benefits to the soil, e.g., improving nutrient and organic matter content. Although some researchers have noted the potential risks of soil contamination by pathogens, heavy metals and emerging contaminants present in the sludge (Alvarenga et al., 2015; Verlicchi and Zambello, 2015), there is still a lack of evidence concerning the suitability of pharmaceutical sludge for agricultural reuse in terms of potential toxicity and soil benefits.

Recently, integrated biological systems combining anaerobic digestion and the composting process have been applied to industrial and high-strength wastewaters. Previous studies have shown that the integrated treatment can be considered operationally and economically advantageous, due to the energy recovery through biogas production and nutrient supply from the digestate (organic residues from biogas plant) and/or through compost agricultural reuse (Cucina et al., 2017; Bustillo-Lecompte and Mehrvar, 2016; Aquilanti et al., 2014). Although digestates and composts are widely considered to have high agricultural qualities (Solé-Bundó et al., 2017; De Bertoldi, 2013), these organic materials derived from pharmaceutical-wastewaters treatment have not been characterized yet. Furthermore, the agronomical and environmental implications derived from their application to the soil should be evaluated using different parameters and indicators. Particular attention should be paid to the macronutrient content, potential toxicity and stabilization of the organic matter (Solé-Bundó et al., 2017). The evaluation of several soil enzymatic activities after amendment and in vivo bioassays are useful tools to assess the potential toxicity towards soil microorganisms and plants, respectively (Solé-Bundó et al., 2017; Alburquerque et al., 2012; Bastida et al., 2012). Organic matter stabilization can be evaluated through the quantification of CO_2 emissions and the water extractable organic matter (WEOM) content in amended soils (Pezzolla et al., 2013). Moreover, bio-accumulative organic contaminants and pathogens need to be assessed, as recommended by the European Directive draft (CEC, 2003).

Hence, the aim of the present study was to evaluate the benefits and risks associated with the agricultural use of three different potential organic fertilizers (sludge, anaerobic digestate and compost), derived from a pharmaceutical manufacturing wastewater. This wastewater could be considered representative of wastewaters derived from antibiotic fermentation processes (Cucina et al., 2017; Coskun et al., 2012; Chen et al., 2011). The effect of these materials on soil organic matter stabilization and their potential toxicity towards soil microorganisms and plants were investigated through a soil microcosm experiment and in vivo bioassays.

2. Material and methods

2.1 Organic materials and sampling

The pharmaceutical sludge (PS) was provided by the ACS Dobfar SpA plant in Anagni (Rome) after the aerobic stabilization of a pharmaceutical wastewater, which was derived from the daptomycin fermentation. The wastewater was experimentally treated through anaerobic co-digestion with some agricultural by-products, and the obtained digestate (AD) was later used as a substrate for the composting in order to produce a high-quality organic amendment (compost, CM). All the processes were described in detail by Cucina et al. (2017). Representative samples of PS, AD and CM were cooled and stored at 4 °C for transport to the laboratory, and the sampling points of each type of material are outlined in Figure 1. Once in the laboratory, the samples were divided into three aliquots: one aliquot was stored at 4 °C for the analytical determination, one was frozen at -18 °C, and the third was freeze dried for the determination of daptomycin residues.

2.2 Characterization of organic materials

Total solids (TS), volatile solids (VS) and total organic carbon (TOC) were analyzed according to Standard Methods (APHA, 2005). The pH and the electrical conductivity (EC) were measured in a solid/water suspension (1:10 w/v) by using a glass electrode and a conductivity probe, respectively. Total volatile fatty acids (TVFA) were determined according to the HACH Lange methodology and expressed as g of acetic acid kg^{-1}. Fresh samples were used for the determination of total Kjeldahl-N and NH_4^+–N by means of macro and micro-Kjeldahl distillation methods, respectively (APHA, 2005). Total organic N was calculated by the difference between total Kjeldahl-N and $NH4^+$–N. Total P was measured spectrophotometrically after digestion of the samples with concentrated $H_2SO_4/HClO_4$ and humification degree was determined, both as described by Massaccesi et al. (2013).

For the metals determination, samples were digested in HNO_3 at 200 °C in a microwave oven (maximum power 800 W, Milestone Inc. ETHOS One, Sorisole, Italy) and then analyzed by flame atomic absorption spectroscopy using a Shimadzu AA-6800 apparatus (Shimadzu Corp., Tokyo, Japan). Total K and total Na were determined through the flame photometric method. Total Hg was determined by a cold-vapor generator coupled with an atomic absorption spectroscopy apparatus. Pathogens (*Salmonella* spp. and *Escherichia coli*) were determined for the fresh samples according to Standard Methods (APHA, 2005). Analysis of daptomycin residues in the organic materials was conducted as described by Cucina et al. (2017). Briefly, 10 mg of freeze-dried samples was dissolved in 50 mL of $CH_3CN/NH_4H_2PO_4$ 0.45 M solution (80/20% v/v). The obtained solutions at different dilution rates (1:2, 1:5 and 1:20) were analyzed in a Perkin-Elmer PE 200 HPLC system, and the results were confirmed using the standard addition method. For the analysis, a daptomycin reference standard (Sigma Aldrich, St. Louis, MO, USA), a column IB-Sil C8-HC (5 mm x 250 mm x 4.6 mm Phenomenex) and a pre-column IB-Sil C8 (5 mm x 30 mm x 4.6 mm Phenomenex) were used.

2.3 Soil incubation experiment

A soil microcosm experiment was conducted to evaluate how the pharmaceutical-derived organic materials affect the soil organic matter processes. Freeze-dried PS was applied to an agricultural soil (sandy-clay texture), according to the maximum dose allowed by the Italian legislation concerning agricultural reuse of sludge (30 tons ha^{-1}, Decree 99/92). The application doses used for AD and CM were then calculated to apply an equivalent quantity of organic C to the soil, and the freeze-dried samples were then used for the amendment. For each treatment, 30 cylindrical glass jars (250 mL) were filled with 200 g (dry weight) of soil to allow for ten destructive samplings after 0, 2, 4, 7, 10, 14, 17, 30, 45 and 60 days of incubation. After amendment, soil samples were incubated aerobically and in non-leached conditions for 60 days at 20 °C and at 80% of the water-holding capacity to ensure good biomass activation, as suggested by Pezzolla et al. (2013). CO_2 emissions were evaluated by using an alkaline trap and subsequent titration. Fresh soil samples were then divided in two portions: (1) air dried for the WEOM determination and (2) frozen at -20 °C for the enzymatic determinations. Water extractable organic C (WEOC) was extracted from the organic fertilizers and from the amended soils as described by Solé-Bundó et al. (2017) and the C concentration in the extracts was determined through a C-analyzer (Analytic Jena-Analyzer multi N/C 2100S). WEOM was calculated by the equation suggested by Pribyl (2010):

$$WEOM = WEOC \cdot 2$$

2.4 Soil enzymatic activities

The potential toxicity of PS, AD and CM to soil microorganisms can be evaluated through the determination of several enzymatic activities in the amended soil, as described by Bastida et al. (2012). Fresh soil samples, amended with PS, AD and CM obtained as described in the previous paragraph (2.3), were used for the determination of the total dehydrogenase (expressed as mg of triphenyl formazane g_{soil}^{-1} h^{-1}), fluorescein diacetate (FDA) hydrolysis (expressed as mg of fluorescein g_{soil}^{-1} h^{-1}), urease (expressed as micromole of NH_4^+ g_{soil}^{-1} h^{-1}) and alkaline phosphomonoesterase (expressed as mg of paranitrophenol g_{soil}^{-1} h^{-1}) activities (Torres et al., 2016; Schumacher et al., 2015; Ameloot et al., 2014).

2.5 Plant bioassays

Three dicotyledonous (*Cucumis sativus* L., *Lepidium sativum* L., *Lactuca sativa* L.) and one monocotyledonous (*Hordeum vulgare* L.) plants were used for the seed germination tests. Water extracts were prepared from the freeze-dried samples of PS, AD and CM, as described by Cayuela et al. (2007). Pure extracts together with three dilutions (75%, 50% and 25% v/v in deionized water) were used as germination media, and the germination tests were carried out as described by Solé-Bundó et al. (2017). A control test for each species was carried out using deionized water as germination media (5 replicates for each control). After the incubation period (2 days for *L. sativum*, 5 days for the other species), the germination index (GI) was calculated as a percentage of the control.

The tests with the aquatic plant *Lemna minor* L. were conducted according to the standard ISO SO/WD 20079. The test was performed in triplicate in 100-mL beakers with a working volume of 50 mL. The same dilutions of the seed germination tests were used as growth media. Distilled water was used as control. Ten fronds of *L. minor* were used as inoculums. The test was conducted in a climatic chamber (25 ± 2 °C, light intensity 100 µE s^{-1} m^{-1}) for seven days. Plant dry weights were used to calculate the growth index (GrI) as a percentage of the control.

A modification of the method described by Pivato et al. (2016) was used to evaluate the influence of the pharmaceutical-derived organic fertilizers on the biomass accumulation of three dicotyledonous (*C.sativus*, *L.sativum*, *L.sativa*) and one monocotyledonous (*H. vulgare*) species. Decreasing doses of PS, AD and CM were mixed with an artificial soil (sand/sphagnum peat/expanded clay in the ratio 80/10/10 w/w) to obtain four different concentrations (10, 7.5, 5 and 2.5 g 100 g^{-1} of artificial soil, corresponding to agricultural doses of 30, 22.5, 15, 7.5 tons ha^{-1}, respectively). Non-amended soils were used as control. A total of 10, 7, 5 and 5 seeds of cress, lettuce, cucumber and barley were sown on the substrate, respectively. The test was conducted in a climatic chamber (25 ± 2 °C, light intensity 100 µE s^{-1} m^{-1}) for 15 days with a photoperiod of 16 hours of light and 8 hours of darkness. Plants were collected and dry weights were used to calculate the growth index (GrI) for each species as a percentage of the control.

2.6 Statistical analysis

All the reported data are the arithmetic means of three replicates. Two-way analysis of variance (ANOVA) was done to determine significant differences among the parameters analyzed at a level of significance of $P < 0.05$, whereas linear regression analysis was done to determine significant correlations between selected parameters at a level of significance of $P < 0.05$.

3. Results and discussion

3.1 Physico-chemical and fertilizing properties of pharmaceutical-derived organic materials

Physico-chemical characteristics of organic fertilizers depend strictly on the raw materials used for their production. Moreover, these properties are related to the biological process performed to obtain the fertilizer (aerobic digestion, anaerobic digestion, composting). Physico-chemical properties of the studied materials are reported in Table 1.

In the present study, all the organic wastes exhibited dry matter contents compatible with their origin (Table 1). In particular, PS and CM showed high TS content that made them solid products (14.3±0.8 and 51.9±1.0%, respectively), unlike AD, which can be considered a liquid material (3.8±0.3%). The management of liquid products such as AD entails technical issues due to the high cost of transportation and distribution; thus, the agricultural reuse of PS and CM may appear more appropriate (Alvarenga et al., 2015). pH values of all the samples were slightly alkaline (> 7.0). In particular, AD showed the lowest pH (7.4±0.0), probably due to the high content of TVFA in this material (48.0±1.30 g kg^{-1}). As expected, PS was characterized by the highest pH value (8.6±0.0) due to the addition of NaOH before the aerobic stabilization of wastewater. In fact, the wastewater is treated with NaOH to pH 12 in order to ensure microorganism inactivation and degradation of daptomycin residues. CM showed a pH value of 8.4±0.0, which was typical of mature compost and in the range established by the Italian law concerning fertilizers (Alvarenga et al., 2015; Gigliotti et al., 2012; Italian Decree 75/2010). However, pH values observed in all the materials (PS, AD and CM) allow their agricultural reuse without any negative effect on soil pH. Both salinity, estimated through the EC, and TVFA contents of the organic fertilizers may affect soil properties due to their phytotoxicity (Solé-Bundó et al., 2017; Di Maria et al., 2014; Alburquerque et al., 2012). EC was moderate in all the studied materials and ranged from 7.0 dS m^{-1} in CM to 12.1 dS m^{-1} in AD; these values cannot represent a potential risk for soil secondary salinization. Of the three materials studied, AD showed the highest TVFA content. High TVFA content may result in a phytotoxic effect, since it was demonstrated that these low weight organic acids are responsible for seed germination inhibition (Di Maria et al., 2014; Alburquerque et al., 2012). As expected, both PS and CM showed low contents of TVFA due to their mineralization during aerobic treatments (aerobic digestion and composting) (Said-Pullicino et al., 2007a). A moderate content of organic matter (as deduced from the volatile solids content) was found in the three organic fertilizers (Table 1). The VS/TS ratio ranged from 61.0% in the CM to 70.9% in the AD. The TOC content differed significantly among the studied materials (17.8, 34.0 and 28.1% for PS, AD and CM, respectively) due to the different biological treatments from which they originated. As expected, PS and CM, both derived from aerobic treatments, showed the highest degree of OM mineralization, leading to materials with an OM content similar to agro-industrial sludges and composts (Alvarenga et al., 2015; Tambone et al., 2010).

The OM content of the studied organic fertilizer was affected not only by the treatment conditions (aerobic, anaerobic or their combination) but also by the initial substrate composition. This could explain the TOC content observed in AD that was higher than TOC values commonly found for digested sludge (Solé-Bundó et al., 2017). The AD was obtained from the anaerobic co-digestion of the pharmaceutical wastewater and other agricultural by-products, characterized by high contents of organic matter such us corn silage, olive husk, bovine serum milk and pig slurry (Cucina et al., 2017).

Although the main plant nutrient present in the studied materials was nitrogen, TN content differed among the biomasses studied, following this order: AD > PS > CM (Table 1), as expected from the composition of the starting organic matrix and the biological processes that the matrices underwent (Cucina et. al, 2017; Solé-Bundó et al., 2017; Alvarenga et al., 2015; Tambone et al., 2010). As a consequence of the TOC and TN contents, PS and AD showed low C/N ratios relative to the CM (4.5, 3.6, and 10.4 for PS, AD and CM, respectively). These C/N values can be considered ideal for land application, avoiding the risk of soil N immobilization. It is well known that biomasses characterized by high C/N value may affect negatively the soil N-cycle through the immobilization of this important nutrient in the cell constituents of soil microorganisms (Nelson et al., 2011). Data obtained from the repartition of TN into ammonia and organic N gave interesting results. The high pH of the matrices and the aerobic stabilization caused ammonia losses during the aerobic digestion of the wastewater and the composting of the anaerobic digestate, resulting in low ammonia-N contents in PS and CM. Conversely, anaerobic digestion, causing the transformation of the organic N into ammonia-N, led to its increase in the AD (Tambone et al., 2010). Since organic N contributes to the medium and long-term N turnover in soil, PS and CM could act as more effective N sources for the crops in a long-term perspective. In contrast, the application of the AD may raise environmental issues due to its high ammonia-N content producing ammonia volatilization in the air or nitrate leaching into the soil.

The studied materials presented interesting contents of the other plant macronutrients (P and K). The highest P content was observed in the PS (2.0±0.1%), which was expected because of its tendency to combine with the solid fraction during the wastewater treatment process (Alvarenga et al., 2015). Conversely, the highest K content was observed in the CM (1.8±0.1%), due to the OM matter mineralization and the sequential concentration that occurred during composting. All the studied materials presented rather high sodium contents; as expected, the highest value of total Na was observed for the PS (1.30±0.20%) followed by AD and CM (0.91±0.04 and 0.69 ±0.01%, respectively). The Na content should be carefully considered when the pharmaceutical-derived organic fertilizers are applied to the soils to avoid salinization or other negative effects, e.g., colloid dispersion, loss of soil structure, or the inhibition of plant growth (Daliakopoulos et al., 2016).

From an agronomic point of view, humic-like substances are considered key indicators to evaluate the quality of fertilizers (Bernal et al., 2009). As expected, the humification degree increased from 41.4% in the PS to 62.3% in the CM, mainly due to the production of humic-like substances during the biological treatments. Thus, the application of the pharmaceutical organic wastes may represent an effective strategy to reclaim high quality organic matter to the soil. Nevertheless, CM application should be more recommended due to the lower salinity and ammonia-N content with respect to the other studied materials.

3.2 Environmental risks: heavy metals, pathogens and daptomycin residues

The potential risks of soil contamination related to the agricultural use of the pharmaceutical wastes were assessed through the determination of heavy metals, pathogens and the occurrence of daptomycin residues.

Heavy metal concentrations in the PS, AD and CM are reported in Table 2. Heavy metal contents were low if compared to typical values for biomasses usually applied in agriculture (Alvarenga et al., 2015; Chen et al., 2008) and even the total Cd content was lower than the detection limit of the method used for all the samples. These values were expected, since the raw materials used in the daptomycin fermentation process were always checked for their chemical quality, such as the presence of heavy metals that could interfere with the daptomycin production process. Thus, it can be assumed that the low heavy metal contents of the studied materials may be due to their low concentrations in the wastewater from which they originated. Moreover, the addition of the agricultural by-products in the biological treatments, as described in Cucina et al. (2017), may be responsible for a dilution effect of heavy metals in the organic residues. Thus, PS, AD and CM were within the legal limits established by European and Italian authorities for the agricultural reuse of sludge, digestate and compost (CEC, 2003; CEC, 1986; Italian Decree 75/2010; Italian Decree 99/92).

Since the absence/low content of pathogens (*E. coli* and *Salmonella* spp.) was observed in almost all the samples, it is possible to state that the pharmaceutical materials studied in the present work are well sanitized biomasses (Table 3). The raw materials used in the daptomycin fermentation process must be devoid of contaminant microorganisms.

Regarding daptomycin residues, the concentrations obtained in the PS and in the CM were lower than the detection limit of the method used. However, antibiotic residues were still detectable in the AD (4.50±0.24 mg kg^{-1}), despite the concentration of daptomycin being diluted in the anaerobic bioreactor as a result of the addition of the agricultural by-products. This result suggests that the anaerobic process may not be effective for the complete degradation of daptomycin. Thus, to avoid any possible risk of soil contamination, the agricultural reuse of PS and CM should be preferred over AD, although the daptomycin concentration in AD was low. The absence of daptomycin residues in PS and in CM may be due to the aerobic microorganisms that are able to mineralize the daptomycin residues through protease-mediated hydrolysis mechanisms, as described by Cucina et al. (2017). Thus, the comparison of PS, AD and CM showed that composting of the digestate resulted in increased agronomic properties and an absence of organic contaminants in the mature compost.

3.3 Effect of organic matter stabilization on CO_2 emissions

Variations in CO_2 emissions with time after the application of PS, AD and CM to the soil are shown in Figure 2(A). Whereas control soils showed relatively constant emission rates throughout the incubation period, the addition of PS and AD generally resulted in greater CO_2 fluxes, particularly in the first days after amendment. Similar results were obtained by other authors after amending soils with anaerobic digestate and compost (Solé-Bundó et al., 2017; Pezzolla et al., 2013; Köster et al., 2011; Alluvione et al., 2010). The highest emission rates were observed for soil treated with the AD within 2 days after amendment, and the daily respiration rate was significantly higher for both PS and AD amended soils, with respect to the control, until the 14th day of incubation ($P < 0.01$). During the experiment, CO_2 emissions tended to decrease steadily, reaching relatively constant values similar to those obtained for the unamended controls within 18 days. Conversely, CM-treated soils did not show significant differences in emission rates with respect to the unamended controls throughout the incubation period.

Cumulative CO_2 emissions at the end of the incubation period increased in the order CM < PS < AD (Table 4). The application of the PS and AD to the soil induced a remarkable effect on the cumulative mineralized C ($P < 0.01$). The total amounts of CO_2 released after 60 days of incubation for the PS and AD amended soils were 71.6 and 129.8 mg-C, respectively. Considering that the application doses were designed to yield the same TOC addition to the soil for all the samples, CO_2 emission was probably related to the different quantity and quality of labile organic C added with the amendment (Pezzolla et al., 2013). This idea is consistent with the higher biodegradability of PS and AD compared to CM. This observation can be demonstrated by the values of C-mineralization, expressed as the percentage of the added TOC that was mineralized at the end of the incubation (Table 4). When the PS and the AD were applied to the soil, 18.8% and 34.1%, respectively, of the organic C added with the amendment was mineralized and lost as CO_2 at the end of the incubation period (60 d). Conversely, when CM was added to the soil, this value was 2.5%, which is significantly lower than the values obtained with the PS and the AD ($P < 0.01$). The high values of mineralized C in the PS and AD amended soils was probably due to the so-called *priming effect*, defined as a strong short-term change in the turnover of soil organic matter caused by the amendment (Kuzyakov et al., 2000). Confirming this hypothesis, a high linear correlation was found between the mineralized C in the 60 days of the incubation experiment and the C/N ratio, a key biomass stability parameter (Bernal et al., 2009). Indeed, a significant linear correlation was found between the C/N ratio of the pharmaceutical organic wastes and the mineralized C at the end of the incubation period (R^2=0.7769, n=9, $P < 0.05$). Specifically, the higher the organic matter stabilization of the fertilizer, the lower the percentage of added TOC that was mineralized.

Figure 2(B) shows the WEOM evolution in the soils amended during the microcosm experiment. The application of the PS and the AD significantly enhanced the concentrations of WEOM with respect to the controls ($P < 0.01$); in particular, the AD application increased markedly in the first days. The initial WEOM concentrations in AD and PS amended soils were 3.3 and 1.7 times greater, respectively, than the control soil. The WEOM content of the AD treated soil showed a clear decreasing trend throughout the incubation period due to the microbial activity, whereas the other amended soils showed a rather constant trend. The constant trend can be explained considering the dynamic equilibrium that occurred between the consumption of WEOM caused by the microbial mineralizing activity and microorganism release of WEOM for their hydrolytic activity (Said-Pullicino et al., 2007b). At the end of the incubation period, only the AD amended soil showed a WEOM content significantly higher than the control ($P < 0.01$). Stability-dependent respiration rates were reported by previous studies for soils amended with organic materials. Most of them showed CO_2 peak emissions in the first few days after amendment, with an intensity related to the contents of WEOM and microbial biomass (Solé-Bundó et al., 2017; Bustamante et al., 2010; Sánchez-Monedero et al., 2004). It is well known that organic amendment can change the amount and quality of dissolved organic matter in the soil solution with important implications on microbial activity and soil respiration (Pezzolla et al., 2013). Moreover, Said-Pullicino et al. (2007b) have shown that the soluble C fraction of organic amendments tends to decrease with organic matter stabilization.

In this work, this aspect was confirmed when the WEOM added with the organic fertilizers was correlated to the cumulative soil CO_2 emissions at the end of the incubation period (Table 4). A positive linear correlation between these two parameters was found to be significant (R^2=0.9035, n=9, $P < 0.01$).

3.4 Effects on soil enzymatic activities

The potential toxicity towards soil microorganisms after PS, AD and CM application could be achieved through the determination of a set of soil enzymatic activities. Microbial activity measurements appear as good indicators of the degree of stress and pollution of soils (Bastida et al., 2012).

Hydrolysis of fluorescein diacetate (FDA) has been widely used to estimate microbial activity in soil, since FDA is hydrolyzed by all the enzymes involved in the microbial decomposition of organic matter in soil (Araujo et al., 2015). FDA hydrolysis activity curves of PS, AD and CM are shown in Figure 3(A). All amended soils showed a strong increase of this enzymatic activity with respect to the control in the first days after the application. Specifically, all the organic materials added caused a significant increase of FDA activity ($P < 0.01$) from the 4^{th} day of incubation until the end of the experiment (60 days). This behavior could be explained by the microbial metabolic activity, which was increased due to the rapidly degradable source of C added to the soils after the amendment. After the 17th day, the FDA hydrolysis activity decreased in all the amended soils due to the consumption of the easily available C source. Nevertheless, at the end of the incubation, this enzymatic activity was still significantly higher in the amended soils than in the control soil ($P < 0.01$), confirming the absence of toxicity towards soil microorganisms, even after two months of incubation.

Determination of dehydrogenase (DH) activity has been proposed by Ameloot et al. (2014) as a rapid and cost-effective toxicity test for soil microorganisms that can be very useful also for the identification of contaminated and perturbed soils. Moreover, DH activity is considered a good indicator of microbial activity in soil for its mineralizing function and thus for its relation with CO_2 emissions (Araujo et al., 2015). As shown in Figure 3(B), PS and AD addition to the soil caused a strong increase of the DH activity, probably due to the high content of labile C added with the amendment. Specifically, PS and AD measurements of the DH activity in these treated soils were significantly higher than in the control during the entire incubation period ($P < 0.01$). Conversely, CM addition did not cause a significant increase of this enzymatic activity, as expected from the lower respiration rates observed. Although the addition of PS and AD appeared to stimulate positively the soil microflora, the large increase in microbial activity could represent an environmental issue. Indeed, it is well known that the addition of easily degradable C could excessively stimulate microorganisms, leading to anoxic conditions in the soil and, consequently, to phytotoxicity (Wu et al., 2000).

Figure 3(C) shows the results obtained from the determination of the soil urease activity during the incubation period. Whereas Bastida et al. (2008) found positive effects of organic amendment on the urease activity, other authors found negative impacts on soil urease activity when applying organic materials, such as sewage sludge (Gao et al., 2010). The inhibition of urease may be due to heavy metals, to some constituents of the organic matter of the biomass, or to a high concentration of ammonia-N in the soil (Gao et al., 2010). Since a low content of heavy metals was observed in all the biomasses involved in the present experiment, a strong increase in this enzymatic activity was observed when PS and AD were applied, particularly in the first days. The large amount of labile C applied with the sludge and the digestate can justify these results, as already observed for the DH activity. The CM treated soils showed the lowest increase of the urease activity, as expected.

In the present work, short-term variations of the soil phosphomonoesterase activity were observed after the amendment (Fig. 3(D)). Phosphomonoesterase activity resulted significantly increased ($P < 0.01$) after the addition of the PS and AD to the soil, as expected; in fact, soil phosphomonoesterases can be mainly inhibited by heavy metals, which were not abundant in the PS and AD (Gao et al., 2010). Similar results were obtained by Ros et al. (2006), who amended an agricultural soil with bio-solids. They observed that treated soils generally show significantly higher phosphatase activity compared to the activity of control soil due to higher amounts of available nutrients with respect to the untreated controls. Conversely, in the present experiment, CM addition to the soil resulted in non-significant differences with respect to the control, probably due to the high organic matter stabilization of this organic material.

The analyses of soil enzymatic activities demonstrated that PS, AD and CM application to the soil did not show any effects of toxicity on soil microorganisms. Nevertheless, agricultural reuse of a stabilized fertilizer, such as the compost, appears the most suitable strategy to improve soil quality, avoiding environmental issues and perturbations of soil microorganisms.

3.5 Potential phytotoxicity

Organic fertilizers can cause phytotoxicity, mainly due to high contents of soluble salts, ammonia-N and low weight organic compounds such as total volatile fatty acids (Alburquerque et al., 2012). The germination index (GI) and growth index (GrI) of different species were used in the present study to assess the potential phytotoxicity of all materials (Figures 4, 5 and 6).

As expected, CM did not inhibit the germination of the studied plants (Fig. 4). Moreover, CM-diluted extracts induced positive effects on the GI of all plants, confirming the suitability for their agricultural reuse. The PS showed a moderate phytotoxicity, whereas AD produced a strong inhibition of the germination in all plants. However, the lowest performances were obtained with the pure extracts, demonstrating that all the studied materials showed a dose-response phytotoxic effect on germination.

Among plant species used for the germination tests, lettuce appeared to be the most susceptible species, highlighting GI differences among all the materials tested; conversely, both cucumber and barley showed a lower sensitivity to phytotoxicity with respect to the other plant species.

With respect to the GI, the results obtained in the present study confirmed that the inhibition of germination is strictly related to the physico-chemical parameters of the biomass (e.g., soluble salts, ammonia-N, total volatile fatty acids) (Solé-Bundó et al., 2017; Di Maria et al., 2014; Alburquerque et al., 2012). When the GI was related to the soluble salt contents of all the materials studied, a significant negative linear correlation was found ($R^2 = 0.8449$, n = 12, $P < 0.05$). Similar linear negative correlations were found when the GI was correlated to the ammonia-N content and the total volatile fatty acid content ($R^2 = 0.8533$ and $R^2 = 0.8239$, respectively; n=12, $P < 0.05$).

The effects of PS, AD and CM on the growth index (GrI) of *L. minor* are shown in Figure 5. The GrI was 0% for all the dilutions of the AD extract, confirming the phytotoxicity of this material. Conversely, positive results were obtained when the CM extracts were tested (the average GrI was 86.3%), while PS affected the growth index of *L. minor* with a moderate phytotoxicity (the average GrI was 25.7%). As already observed for the GI, a dose-response effect was also found in the growth of aquatic plants for all the studied fertilizers. Cayuela et al. (2007) reported that the *Lemna gibba* growth inhibition bioassay was highly related to maturation indices commonly used to evaluate the toxicity of biomasses during composting. In this study, it was assessed also that the *L. minor* growth inhibition is correlated to the germination index of *L. sativum*, a common maturation index. A highly positive linear correlation was found between the GI of cress and the GrI of *L. minor* ($R^2=0.7848$, n=12, $P < 0.05$).

The assessment of the potential phytoxicity was completed through the growth tests, for which results are shown in Figure 6. The AD produced phytotoxic effects also in the growth tests and the highest phytotoxicity was observed for lettuce and cress, as a demonstration that these two species were more sensitive to phytotoxic compounds than cucumber and barley. Specifically, no plant growth was observed when the AD was applied at 22.5 and 30.0 tons ha^{-1}. With regard to the PS, the GrI determination in all the four species studied showed that this material possessed residual phytotoxicity (the average GrI was 62.3, 20.0, 38.8 and 76.2% for cucumber, lettuce, cress and barley, respectively). As already observed for the GI, the CM did not produce phytotoxic effects on plant growth. Once again, a dose-response effect was observed between the application dose of the fertilizer and the accumulation of biomass in all the species tested.

In the present work, soluble salts, total volatile fatty acids and ammonia-N were found to be significantly and negatively correlated to the GrI, as highlighted in previous studies (Solé-Bundó et al., 2017; Alburquerque et al., 2012). The relationship between the GrI and the ammonia-N content of the pharmaceutical wastes was described as a negative linear correlation (R^2=0.6348, n=12, $P < 0.05$). Conversely, the GrI was found to be positively correlated to stability and maturation parameters, as already reported by Young et al. (2016). The GrI was positively correlated to the C/N ratio of the pharmaceutical organic wastes (R^2=0.6678, n=12, $P < 0.05$) and it was confirmed that the C/N can be used as a maturation index, as observed in Bernal et al. (2009) and in Said-Pullicino et al. (2007b).

The large set of phytotoxicity assessments demonstrated that CM could be considered the best pharmaceutical-derived organic fertilizer for the absence of phytotoxicity. Several extract dilutions and doses used in the bioassays resulted in germination and growth higher than those of the control. Moreover, in some cases (e.g., germination of lettuce and barley, growth of cucumber and lettuce), CM affected positively the plant development, probably due to a hormone-like action, resulting in GI and GrI values higher than the 100% of the control (Alburquerque et al., 2012). However, the agricultural reuse of the AD should be avoided, since it was found to be phytotoxic in most of the bioassays. Within the assays tested in the present work, the *L. minor* growth inhibition test appeared the most suitable. It was demonstrated that this aquatic plant is very sensitive to the phytotoxicity of organic fertilizers and can highlight properly the differences among organic materials.

4. Conclusions

The pharmaceutical-derived organic wastes studied in the present work were characterized by high content of plant macronutrients (N, P and K) and low concentrations of heavy metals, pathogens and daptomycin residues. The sludge and the anaerobic digestate showed a low organic matter stabilization that may affect soil microbial activities, mainly in terms of CO_2 emissions. In contrast, the compost may represent an important source of stabilized organic matter for the soil and nutrients for plants, due to its chemical characteristics.

According to the results, the compost appears to be the most promising organic fertilizer derived from the daptomycin production process. Its agricultural reuse may allow recovery of plant nutrients while avoiding environmental risks of soil contamination and toxicity towards soil microorganisms and plants. Thus, integrated anaerobic-aerobic treatment can represent a suitable strategy to valorize wastewaters derived from antibiotic manufacturing.

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Table 1: Physico-chemical and fertilizing properties of the three pharmaceutical-derived organic wastes.

Parameter	**Units**	**Sludge**	**Anaerobic digestate**	**Compost**
TS	%	14.3 ± 0.8	3.8 ± 0.3	51.9 ± 1.0
VS	%	8.8 ± 0.1	2.7 ± 0.0	31.6 ± 0.2
VS/TS	%	61.9 ± 0.7	70.9 ± 0.2	61.0 ± 0.5
pH	-	8.6 ± 0.0	7.4 ± 0.0	8.4 ± 0.0
EC	$dS \cdot m^{-1}$	7.9 ± 0.1	12.1 ± 0.2	7.0 ± 0.0
TVFA	$g \cdot kg^{-1}$	9.6 ± 0.4	48.0 ± 1.30	3.8 ± 0.4
TOC	%	17.8 ± 0.3	34.0 ± 0.2	28.1 ± 1.6
TKN	%	3.99 ± 0.19	9.42 ± 0.26	2.71 ± 0.04
C/N	-	4.5	3.6	10.4
NH_4^+-N	%	1.03 ± 0.03	4.86 ± 0.24	0.07 ± 0.00
Organic-N	%	2.96	4.56	2.64
Total P	%	2.04 ± 0.14	0.56 ± 0.00	0.62 ± 0.09
Total K	%	0.16 ± 0.01	0.67 ± 0.03	1.78 ± 0.13
Total Na	%	1.30 ± 0.20	0.91 ± 0.04	0.69 ± 0.01
Humification degree	%	41.4 ± 0.1	55.8 ± 0.5	62.3 ± 0.1

Note: TS = total solids, VS = volatile solids, EC = electrical conductivity, TVFA = total volatile fatty acids, TOC = total organic C, TKN = total Kjeldahl N.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Table 2: Heavy metal contents of the three pharmaceutical-derived organic wastes.

Parameter	Units	Sludge	Anaerobic digestate	Compost
Total Cd	mg · kg^{-1}	< 0.20*	< 0.20*	< 0.20*
Total Cr	mg · kg^{-1}	6.2 ± 0.2	< 0.50*	9.3 ± 0.1
Total Ni	mg · kg^{-1}	6.6 ± 1.0	< 0.50*	18.3 ± 1.9
Total Pb	mg · kg^{-1}	11.1 ± 1.8	< 1.00*	26.6 ± 5.7
Total Cu	mg · kg^{-1}	59.7 ± 3.9	23.4 ± 2.5	36.8 ± 4.7
Total Zn	mg · kg^{-1}	92.3 ± 4.5	117.2 ± 5.2	113.8 ± 6.3
Total Hg	mg · kg^{-1}	0.30 ± 0.04	0.41 ± 0.00	0.31 ± 0.02
Total As	mg · kg^{-1}	0.21 ± 0.00	0.19 ± 0.04	0.11 ±0.02

Note: * = detection limit of the method.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Table 3: Hygenization properties and daptomycin residues of the three pharmaceutical derived organic wastes.

Parameter	Units	Sludge	Anaerobic digestate	Compost
Salmonella spp.	MPN g^{-1}	0.90 ± 0.07	Absent	Absent
Escherichia coli	MPN g^{-1}	Absent	Absent	Absent
Daptomycin	mg · kg^{-1}	< 0.10*	4.50 ± 0.24	< 0.10*

Note: MPN = most probable number, * = detection limit of the method.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Table 4: Organic matter turn-over in the amended soils during the microcosm experiment.

Parameter	**Units**	**Sludge**	**Anaerobic digestate**	**Compost**
Application dose	$g \cdot kg^{-1}$	10.7	5.6	6.8
TOC_{added}	$g \cdot kg^{-1}$	1.90	1.91	1.89
WEOM	$g \cdot kg^{-1}$	47.4 ± 0.2	265.0 ± 0.4	30.6 ± 0.4
$WEOM_{added}$	$mg \cdot kg^{-1}$	507.0	1349.0	206.5
Net CO_2 emission	$mg\text{-}C \cdot kg^{-1}$	358.0 ± 5.0	649.0 ± 4.3	47.0 ± 2.8
% TOC_{added} mineralized	%	18.8 ± 0.5	34.1 ± 1.2	2.5 ± 0.4

Note: TOC = total organic C; WEOM = water extractable organic matter.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Figure 1: Diagram of daptomycin production, wastewater treatments and sampling sites. Solid line: actual disposal of wastewater; interrupted line: wastewater valorization proposed by Cucina et al. (2017).

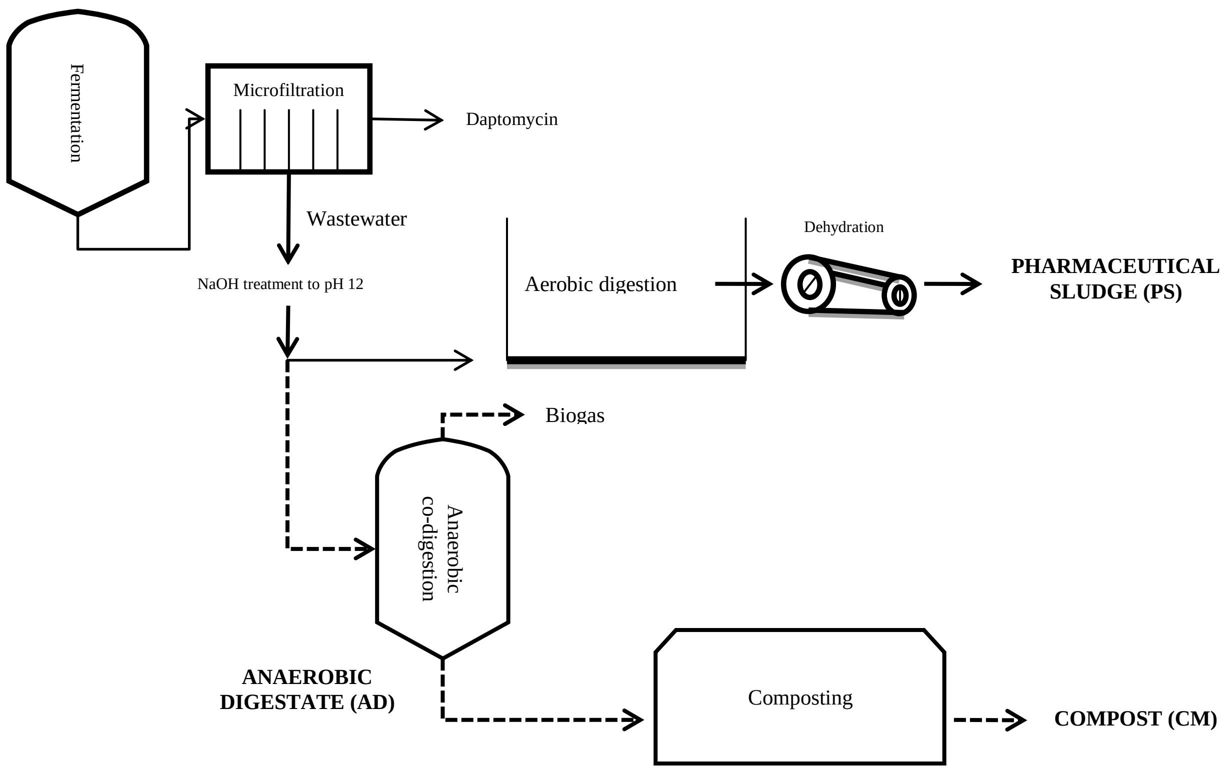
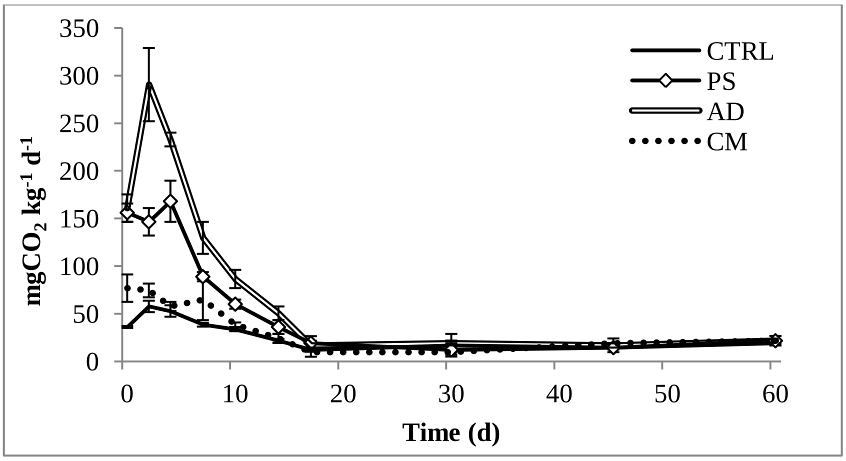


Figure 2: Changes over time in the (A) CO_2 emissions and (B) WEOM content determined in the soils amended in the microcosm experiment (mean value ± SD, n=3). CTRL: non-amended soil; PS: sludge; AD: anaerobic digestate; CM: compost. Data are expressed on dry weight basis.

(A)



(B)

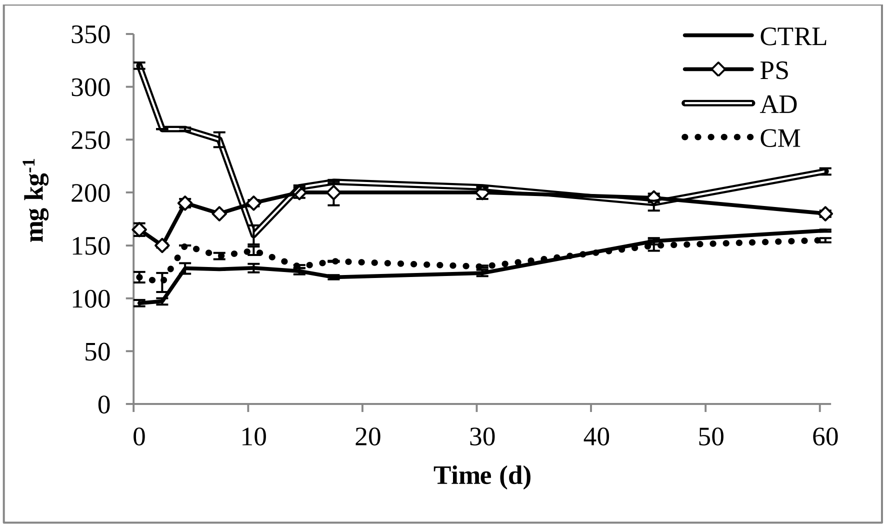


Figure 3: Changes over time in the enzymatic activities determined after PS, AD and CM application to the soils: (A) FDA hydrolysis activity; (B) total dehydrogenase activity; (C) alkaline phosphomonoesterase activity; (D) urease activity (mean value ± SD, n=3). CTRL: non-amended soil; PS: sludge; AD: anaerobic digestate; CM: compost. Data are expressed on dry weight basis.

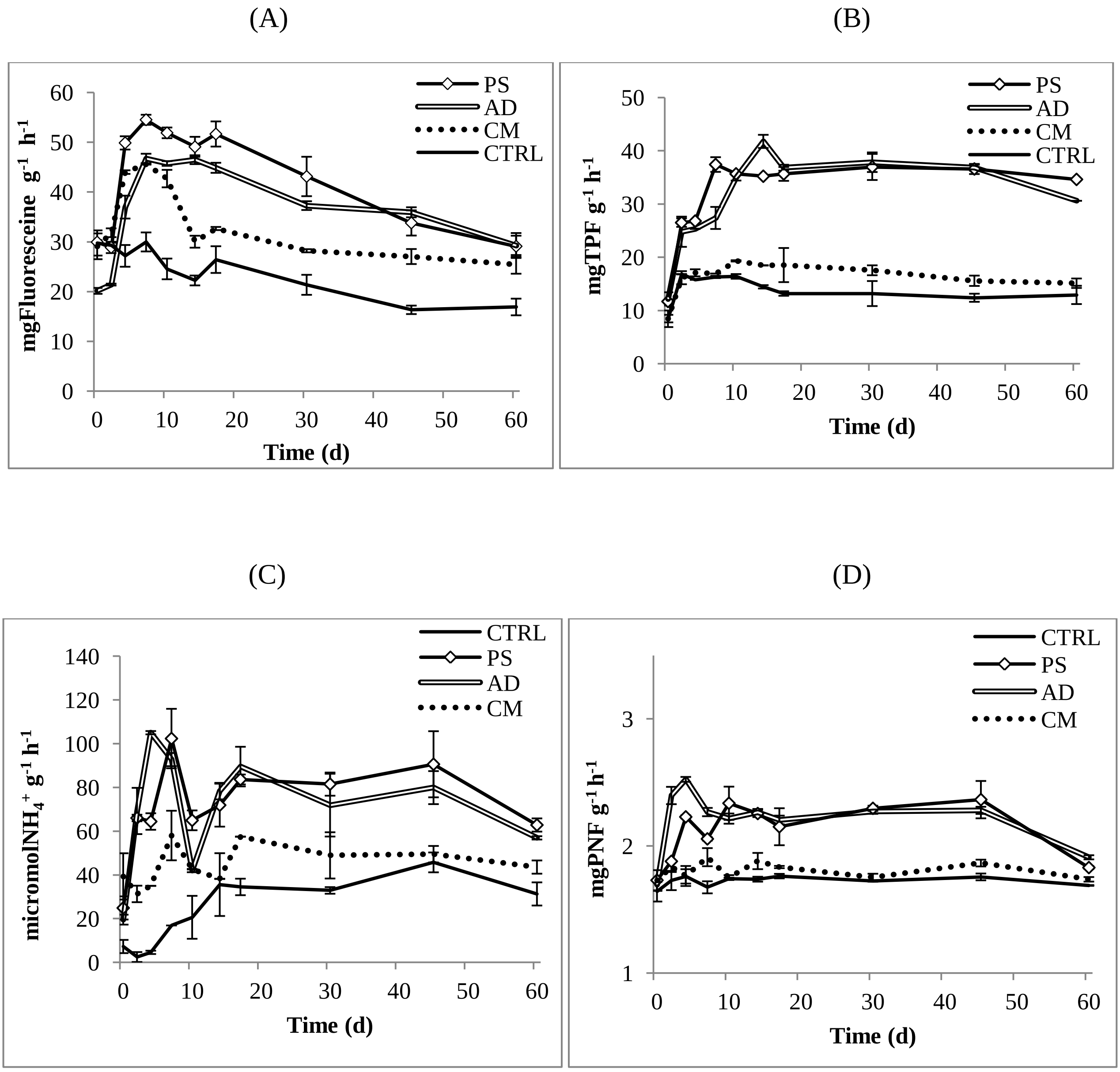


Figure 4: Effects of PS, AD and CM extracts and dilutions on the germination index (GI) of (A) cucumber (*C. sativus*), (B) lettuce (*L. sativa*), (C) cress (*L. sativum*) and (D) barley (*H. vulgare*) (mean ± SD, n=5).

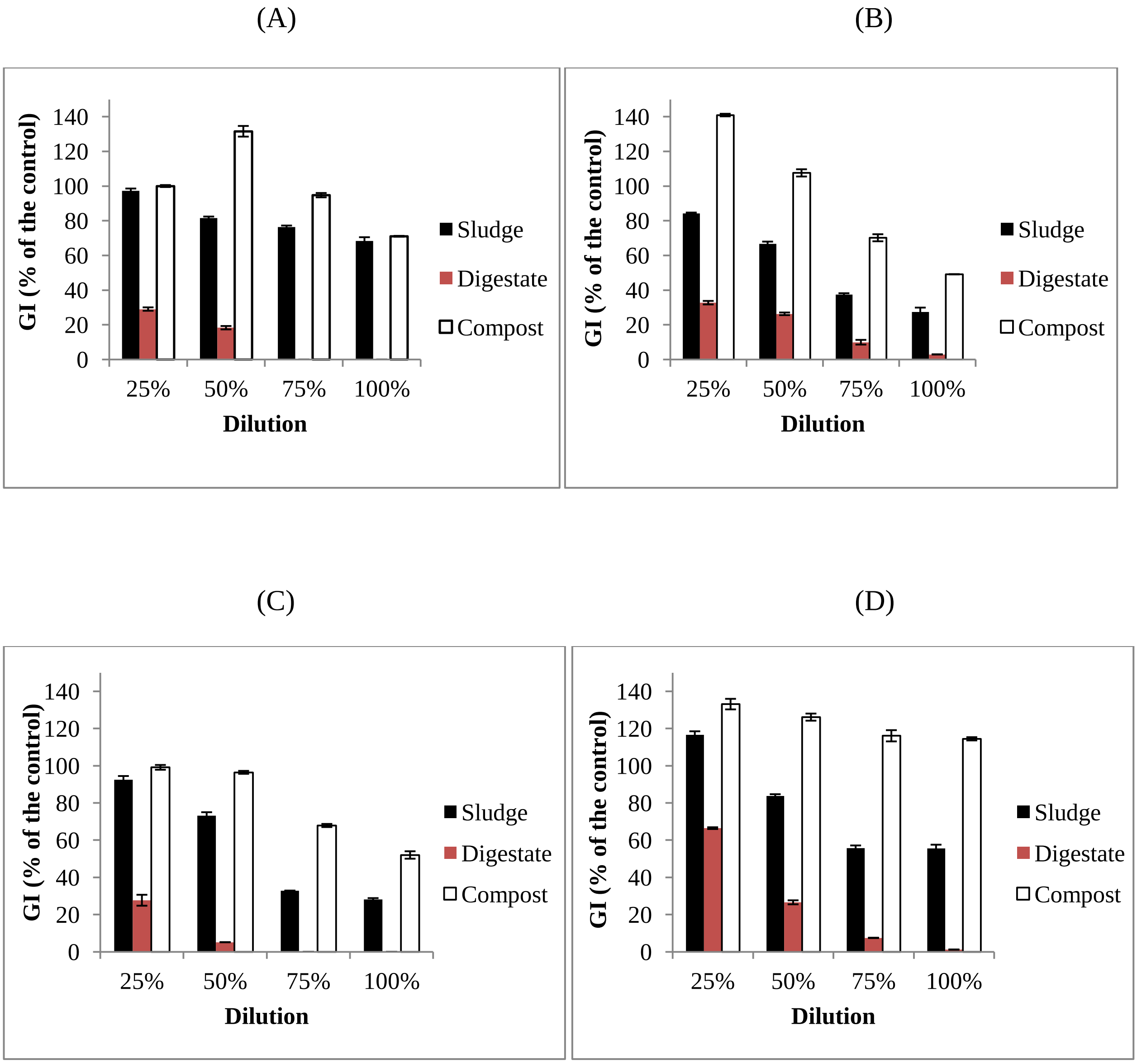


Figure 5: Effects of PS, AD and CM extracts and dilutions on the growth index (GrI) of *L. minor* (mean ± SD, n=6). GrI was 0% for all the dilutions of the digestate extract.

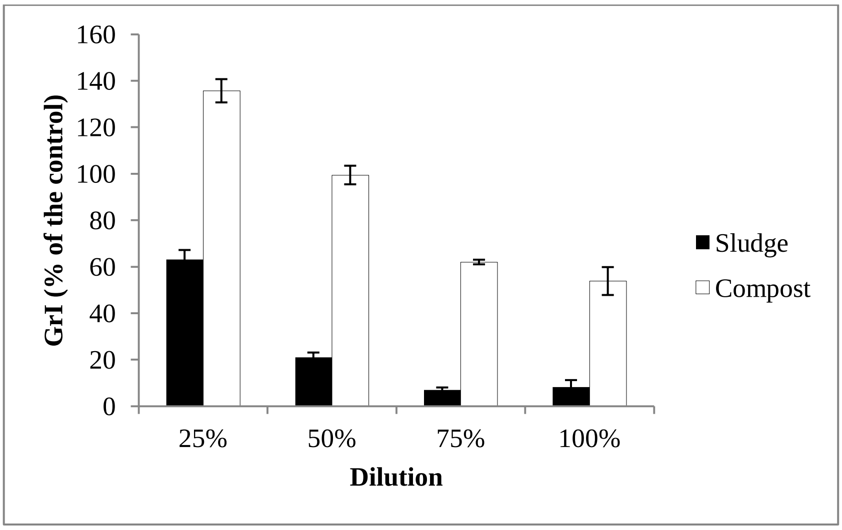
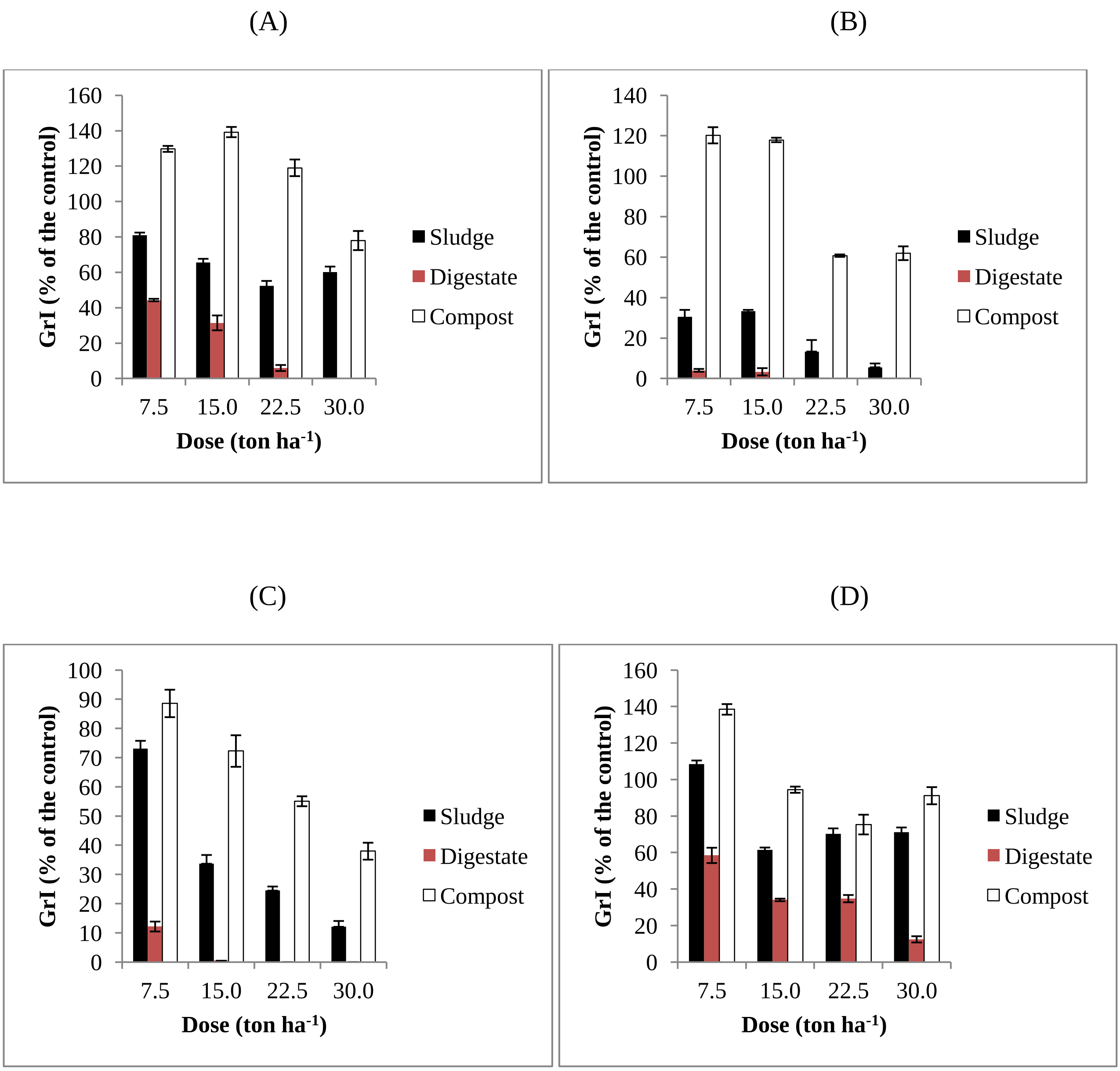


Figure 6: Effects of PS, AD and CM doses on the growth index (GrI) of (A) cucumber (*C. sativus*), (B) lettuce (*L. sativa*), (C) cress (*L. sativum*) and (D) barley (*H. vulgare*) (mean ± SD, n=3).



Evaluation of benefits and risks associated with the agricultural use of organic wastes of pharmaceutical origin

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Abstract

Industrial fermentations for the production of pharmaceuticals generate large volumes of wastewater that can be biologically treated to recover plant nutrients through the application of pharmaceutical-derived wastes to the soil. Nevertheless, benefits and risks associated with their recovery are still unexplored. Thus, the aim of the present work was to characterize three potential organic residues (sludge, anaerobic digestate and compost) derived from the wastewater generated by the daptomycin production process. The main parameters evaluated were the physico-chemical properties, potential contaminants (heavy metals, pathogens and daptomycin residues), organic matter stabilization and the potential toxicity towards soil microorganisms and plants.

The results showed that all the studied materials were characterized by high concentrations of plant macronutrients (N, P and K), making them suitable for agricultural reuse. Heavy metal contents and pathogens were under the limits established by European and Italian legislations, avoiding the risk of soil contamination. The compost showed the highest organic matter stabilization within the studied materials, whereas the sludge and the anaerobic digestate were characterized by large amounts of labile organic compounds. Although the pharmaceutical-derived fertilizers did not negatively affect the soil microorganisms, as demonstrated by the enzymatic activities, the sludge and the anaerobic digestate caused a moderate and strong phytotoxicity, respectively. The compost showed no toxic effect towards plant development and, moreover, it positively affected the germination and growth in lettuce and barley. The results obtained in the present study demonstrate that the valorization of pharmaceutical-derived materials through composting permits their agricultural reuse and also represents a suitable strategy to move towards a zero-waste production process for daptomycin.

Keywords

Sludge, Anaerobic Digestate, Compost, Fertilizer, Phytotoxicity, Recycle

1. Introduction

In the past few decades, the enormous demand for life-saving drugs, such as antibiotics, has led to the development of the pharmaceutical manufacturing industry. Indeed, in 2015, the European Union population-weighted mean consumption of antibiotics for systemic use in the community (i.e., hospitals) was 22.4 defined daily doses per 1000 inhabitants per day (ECDC, 2016). Antibiotic manufacturing can involve a complex series of mainly batch processes in which numerous raw materials are often used and large volumes of wastewater are generated (Tang et al., 2011; Oktem et al., 2008). These processes are characterized by high values of biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) and are usually stabilized through physical and/or chemical or biological processes. In particular, physical and chemical treatments are not always suitable for wastewater treatment due to their low efficiency for dissolved COD removal and high consumption of chemicals (Oktem et al., 2008). Conversely, biological treatments are efficacious systems because they reduce the high COD concentrations. In particular, the aerobic stabilization of pharmaceutical-derived wastewaters is considered one of the most common strategies for the disposal of these wastewaters. At the end of the biological wastewater treatment, the residual sludge is usually disposed by landfill and incineration, although the Council Directive 86/278/EEC (CEC, 1986) encourages their agricultural reuse, preventing "harmful effects on soil, vegetation, animals and man" (Martín et al., 2015). The residual sludge can be reclaimed for agricultural land, producing several benefits to the soil, e.g., improving nutrient and organic matter content. Although some researchers have noted the potential risks of soil contamination by pathogens, heavy metals and emerging contaminants present in the sludge (Alvarenga et al., 2015; Verlicchi and Zambello, 2015), there is still a lack of evidence concerning the suitability of pharmaceutical sludge for agricultural reuse in terms of potential toxicity and soil benefits.

Recently, integrated biological systems combining anaerobic digestion and the composting process have been applied to industrial and high-strength wastewaters. Previous studies have shown that the integrated treatment can be considered operationally and economically advantageous, due to the energy recovery through biogas production and nutrient supply from the digestate (organic residues from biogas plant) and/or through compost agricultural reuse (Cucina et al., 2017; Bustillo-Lecompte and Mehrvar, 2016; Aquilanti et al., 2014). Although digestates and composts are widely considered to have high agricultural qualities (Solé-Bundó et al., 2017; De Bertoldi, 2013), these organic materials derived from pharmaceutical-wastewaters treatment have not been characterized yet. Furthermore, the agronomical and environmental implications derived from their application to the soil should be evaluated using different parameters and indicators. Particular attention should be paid to the macronutrient content, potential toxicity and stabilization of the organic matter (Solé-Bundó et al., 2017). The evaluation of several soil enzymatic activities after amendment and in vivo bioassays are useful tools to assess the potential toxicity towards soil microorganisms and plants, respectively (Solé-Bundó et al., 2017; Alburquerque et al., 2012; Bastida et al., 2012). Organic matter stabilization can be evaluated through the quantification of CO_2 emissions and the water extractable organic matter (WEOM) content in amended soils (Pezzolla et al., 2013). Moreover, bio-accumulative organic contaminants and pathogens need to be assessed, as recommended by the European Directive draft (CEC, 2003).

Hence, the aim of the present study was to evaluate the benefits and risks associated with the agricultural use of three different potential organic fertilizers (sludge, anaerobic digestate and compost), derived from a pharmaceutical manufacturing wastewater. This wastewater could be considered representative of wastewaters derived from antibiotic fermentation processes (Cucina et al., 2017; Coskun et al., 2012; Chen et al., 2011). The effect of these materials on soil organic matter stabilization and their potential toxicity towards soil microorganisms and plants were investigated through a soil microcosm experiment and in vivo bioassays.

2. Material and methods

2.1 Organic materials and sampling

The pharmaceutical sludge (PS) was provided by the ACS Dobfar SpA plant in Anagni (Rome) after the aerobic stabilization of a pharmaceutical wastewater, which was derived from the daptomycin fermentation. The wastewater was experimentally treated through anaerobic co-digestion with some agricultural by-products, and the obtained digestate (AD) was later used as a substrate for the composting in order to produce a high-quality organic amendment (compost, CM). All the processes were described in detail by Cucina et al. (2017). Representative samples of PS, AD and CM were cooled and stored at 4 °C for transport to the laboratory, and the sampling points of each type of material are outlined in Figure 1. Once in the laboratory, the samples were divided into three aliquots: one aliquot was stored at 4 °C for the analytical determination, one was frozen at -18 °C, and the third was freeze dried for the determination of daptomycin residues.

2.2 Characterization of organic materials

Total solids (TS), volatile solids (VS) and total organic carbon (TOC) were analyzed according to Standard Methods (APHA, 2005). The pH and the electrical conductivity (EC) were measured in a solid/water suspension (1:10 w/v) by using a glass electrode and a conductivity probe, respectively. Total volatile fatty acids (TVFA) were determined according to the HACH Lange methodology and expressed as g of acetic acid kg^{-1}. Fresh samples were used for the determination of total Kjeldahl-N and NH_4^+–N by means of macro and micro-Kjeldahl distillation methods, respectively (APHA, 2005). Total organic N was calculated by the difference between total Kjeldahl-N and $NH4^+$–N. Total P was measured spectrophotometrically after digestion of the samples with concentrated H_2SO_4/$HClO_4$ and humification degree was determined, both as described by Massaccesi et al. (2013).

For the metals determination, samples were digested in HNO_3 at 200 °C in a microwave oven (maximum power 800 W, Milestone Inc. ETHOS One, Sorisole, Italy) and then analyzed by flame atomic absorption spectroscopy using a Shimadzu AA-6800 apparatus (Shimadzu Corp., Tokyo, Japan). Total K and total Na were determined through the flame photometric method. Total Hg was determined by a cold-vapor generator coupled with an atomic absorption spectroscopy apparatus. Pathogens (*Salmonella* spp. and *Escherichia coli*) were determined for the fresh samples according to Standard Methods (APHA, 2005). Analysis of daptomycin residues in the organic materials was conducted as described by Cucina et al. (2017). Briefly, 10 mg of freeze-dried samples was dissolved in 50 mL of $CH_3CN/NH_4H_2PO_4$ 0.45 M solution (80/20% v/v). The obtained solutions at different dilution rates (1:2, 1:5 and 1:20) were analyzed in a Perkin-Elmer PE 200 HPLC system, and the results were confirmed using the standard addition method. For the analysis, a daptomycin reference standard (Sigma Aldrich, St. Louis, MO, USA), a column IB-Sil C8-HC (5 mm x 250 mm x 4.6 mm Phenomenex) and a pre-column IB-Sil C8 (5 mm x 30 mm x 4.6 mm Phenomenex) were used.

2.3 Soil incubation experiment

A soil microcosm experiment was conducted to evaluate how the pharmaceutical-derived organic materials affect the soil organic matter processes. Freeze-dried PS was applied to an agricultural soil (sandy-clay texture), according to the maximum dose allowed by the Italian legislation concerning agricultural reuse of sludge (30 tons ha^{-1}, Decree 99/92). The application doses used for AD and CM were then calculated to apply an equivalent quantity of organic C to the soil, and the freeze-dried samples were then used for the amendment. For each treatment, 30 cylindrical glass jars (250 mL) were filled with 200 g (dry weight) of soil to allow for ten destructive samplings after 0, 2, 4, 7, 10, 14, 17, 30, 45 and 60 days of incubation. After amendment, soil samples were incubated aerobically and in non-leached conditions for 60 days at 20 °C and at 80% of the water-holding capacity to ensure good biomass activation, as suggested by Pezzolla et al. (2013). CO_2 emissions were evaluated by using an alkaline trap and subsequent titration. Fresh soil samples were then divided in two portions: (1) air dried for the WEOM determination and (2) frozen at -20 °C for the enzymatic determinations. Water extractable organic C (WEOC) was extracted from the organic fertilizers and from the amended soils as described by Solé-Bundó et al. (2017) and the C concentration in the extracts was determined through a C-analyzer (Analytic Jena-Analyzer multi N/C 2100S). WEOM was calculated by the equation suggested by Pribyl (2010):

WEOM = WEOC · 2

2.4 Soil enzymatic activities

The potential toxicity of PS, AD and CM to soil microorganisms can be evaluated through the determination of several enzymatic activities in the amended soil, as described by Bastida et al. (2012). Fresh soil samples, amended with PS, AD and CM obtained as described in the previous paragraph (2.3), were used for the determination of the total dehydrogenase (expressed as mg of triphenyl formazane g_{soil}^{-1} h^{-1}), fluorescein diacetate (FDA) hydrolysis (expressed as mg of fluorescein g_{soil}^{-1} h^{-1}), urease (expressed as micromole of NH_4^+ g_{soil}^{-1} h^{-1}) and alkaline phosphomonoesterase (expressed as mg of paranitrophenol g_{soil}^{-1} h^{-1}) activities (Torres et al., 2016; Schumacher et al., 2015; Ameloot et al., 2014).

2.5 Plant bioassays

Three dicotyledonous (*Cucumis sativus* L., *Lepidium sativum* L., *Lactuca sativa* L.) and one monocotyledonous (*Hordeum vulgare* L.) plants were used for the seed germination tests. Water extracts were prepared from the freeze-dried samples of PS, AD and CM, as described by Cayuela et al. (2007). Pure extracts together with three dilutions (75%, 50% and 25% v/v in deionized water) were used as germination media, and the germination tests were carried out as described by Solé-Bundó et al. (2017). A control test for each species was carried out using deionized water as germination media (5 replicates for each control). After the incubation period (2 days for *L. sativum*, 5 days for the other species), the germination index (GI) was calculated as a percentage of the control.

The tests with the aquatic plant *Lemna minor* L. were conducted according to the standard ISO SO/WD 20079. The test was performed in triplicate in 100-mL beakers with a working volume of 50 mL. The same dilutions of the seed germination tests were used as growth media. Distilled water was used as control. Ten fronds of *L. minor* were used as inoculums. The test was conducted in a climatic chamber (25 ± 2 °C, light intensity 100 µE s^{-1} m^{-1}) for seven days. Plant dry weights were used to calculate the growth index (GrI) as a percentage of the control.

A modification of the method described by Pivato et al. (2016) was used to evaluate the influence of the pharmaceutical-derived organic fertilizers on the biomass accumulation of three dicotyledonous (*C.sativus*, *L.sativum*, *L.sativa*) and one monocotyledonous (*H. vulgare*) species. Decreasing doses of PS, AD and CM were mixed with an artificial soil (sand/sphagnum peat/expanded clay in the ratio 80/10/10 w/w) to obtain four different concentrations (10, 7.5, 5 and 2.5 g 100 g^{-1} of artificial soil, corresponding to agricultural doses of 30, 22.5, 15, 7.5 tons ha^{-1}, respectively). Non-amended soils were used as control. A total of 10, 7, 5 and 5 seeds of cress, lettuce, cucumber and barley were sown on the substrate, respectively. The test was conducted in a climatic chamber (25 ± 2 °C, light intensity 100 µE s^{-1} m^{-1}) for 15 days with a photoperiod of 16 hours of light and 8 hours of darkness. Plants were collected and dry weights were used to calculate the growth index (GrI) for each species as a percentage of the control.

2.6 Statistical analysis

All the reported data are the arithmetic means of three replicates. Two-way analysis of variance (ANOVA) was done to determine significant differences among the parameters analyzed at a level of significance of $P < 0.05$, whereas linear regression analysis was done to determine significant correlations between selected parameters at a level of significance of $P < 0.05$.

3. Results and discussion

3.1 Physico-chemical and fertilizing properties of pharmaceutical-derived organic materials

Physico-chemical characteristics of organic fertilizers depend strictly on the raw materials used for their production. Moreover, these properties are related to the biological process performed to obtain the fertilizer (aerobic digestion, anaerobic digestion, composting). Physico-chemical properties of the studied materials are reported in Table 1.

In the present study, all the organic wastes exhibited dry matter contents compatible with their origin (Table 1). In particular, PS and CM showed high TS content that made them solid products (14.3±0.8 and 51.9±1.0%, respectively), unlike AD, which can be considered a liquid material (3.8±0.3%). The management of liquid products such as AD entails technical issues due to the high cost of transportation and distribution; thus, the agricultural reuse of PS and CM may appear more appropriate (Alvarenga et al., 2015). pH values of all the samples were slightly alkaline (> 7.0). In particular, AD showed the lowest pH (7.4±0.0), probably due to the high content of TVFA in this material (48.0±1.30 g kg^{-1}). As expected, PS was characterized by the highest pH value (8.6±0.0) due to the addition of NaOH before the aerobic stabilization of wastewater. In fact, the wastewater is treated with NaOH to pH 12 in order to ensure microorganism inactivation and degradation of daptomycin residues. CM showed a pH value of 8.4±0.0, which was typical of mature compost and in the range established by the Italian law concerning fertilizers (Alvarenga et al., 2015; Gigliotti et al., 2012; Italian Decree 75/2010). However, pH values observed in all the materials (PS, AD and CM) allow their agricultural reuse without any negative effect on soil pH. Both salinity, estimated through the EC, and TVFA contents of the organic fertilizers may affect soil properties due to their phytotoxicity (Solé-Bundó et al., 2017; Di Maria et al., 2014; Alburquerque et al., 2012). EC was moderate in all the studied materials and ranged from 7.0 dS m^{-1} in CM to 12.1 dS m^{-1} in AD; these values cannot represent a potential risk for soil secondary salinization. Of the three materials studied, AD showed the highest TVFA content. High TVFA content may result in a phytotoxic effect, since it was demonstrated that these low weight organic acids are responsible for seed germination inhibition (Di Maria et al., 2014; Alburquerque et al., 2012). As expected, both PS and CM showed low contents of TVFA due to their mineralization during aerobic treatments (aerobic digestion and composting) (Said-Pullicino et al., 2007a). A moderate content of organic matter (as deduced from the volatile solids content) was found in the three organic fertilizers (Table 1). The VS/TS ratio ranged from 61.0% in the CM to 70.9% in the AD. The TOC content differed significantly among the studied materials (17.8, 34.0 and 28.1% for PS, AD and CM, respectively) due to the different biological treatments from which they originated. As expected, PS and CM, both derived from aerobic treatments, showed the highest degree of OM mineralization, leading to materials with an OM content similar to agro-industrial sludges and composts (Alvarenga et al., 2015; Tambone et al., 2010).

The OM content of the studied organic fertilizer was affected not only by the treatment conditions (aerobic, anaerobic or their combination) but also by the initial substrate composition. This could explain the TOC content observed in AD that was higher than TOC values commonly found for digested sludge (Solé-Bundó et al., 2017). The AD was obtained from the anaerobic co-digestion of the pharmaceutical wastewater and other agricultural by-products, characterized by high contents of organic matter such us corn silage, olive husk, bovine serum milk and pig slurry (Cucina et al., 2017).

Although the main plant nutrient present in the studied materials was nitrogen, TN content differed among the biomasses studied, following this order: AD > PS > CM (Table 1), as expected from the composition of the starting organic matrix and the biological processes that the matrices underwent (Cucina et. al, 2017; Solé-Bundó et al., 2017; Alvarenga et al., 2015; Tambone et al., 2010). As a consequence of the TOC and TN contents, PS and AD showed low C/N ratios relative to the CM (4.5, 3.6, and 10.4 for PS, AD and CM, respectively). These C/N values can be considered ideal for land application, avoiding the risk of soil N immobilization. It is well known that biomasses characterized by high C/N value may affect negatively the soil N-cycle through the immobilization of this important nutrient in the cell constituents of soil microorganisms (Nelson et al., 2011). Data obtained from the repartition of TN into ammonia and organic N gave interesting results. The high pH of the matrices and the aerobic stabilization caused ammonia losses during the aerobic digestion of the wastewater and the composting of the anaerobic digestate, resulting in low ammonia-N contents in PS and CM. Conversely, anaerobic digestion, causing the transformation of the organic N into ammonia-N, led to its increase in the AD (Tambone et al., 2010). Since organic N contributes to the medium and long-term N turnover in soil, PS and CM could act as more effective N sources for the crops in a long-term perspective. In contrast, the application of the AD may raise environmental issues due to its high ammonia-N content producing ammonia volatilization in the air or nitrate leaching into the soil.

The studied materials presented interesting contents of the other plant macronutrients (P and K). The highest P content was observed in the PS (2.0±0.1%), which was expected because of its tendency to combine with the solid fraction during the wastewater treatment process (Alvarenga et al., 2015). Conversely, the highest K content was observed in the CM (1.8±0.1%), due to the OM matter mineralization and the sequential concentration that occurred during composting. All the studied materials presented rather high sodium contents; as expected, the highest value of total Na was observed for the PS (1.30±0.20%) followed by AD and CM (0.91±0.04 and 0.69 ±0.01%, respectively). The Na content should be carefully considered when the pharmaceutical-derived organic fertilizers are applied to the soils to avoid salinization or other negative effects, e.g., colloid dispersion, loss of soil structure, or the inhibition of plant growth (Daliakopoulos et al., 2016).

From an agronomic point of view, humic-like substances are considered key indicators to evaluate the quality of fertilizers (Bernal et al., 2009). As expected, the humification degree increased from 41.4% in the PS to 62.3% in the CM, mainly due to the production of humic-like substances during the biological treatments. Thus, the application of the pharmaceutical organic wastes may represent an effective strategy to reclaim high quality organic matter to the soil. Nevertheless, CM application should be more recommended due to the lower salinity and ammonia-N content with respect to the other studied materials.

3.2 Environmental risks: heavy metals, pathogens and daptomycin residues

The potential risks of soil contamination related to the agricultural use of the pharmaceutical wastes were assessed through the determination of heavy metals, pathogens and the occurrence of daptomycin residues.

Heavy metal concentrations in the PS, AD and CM are reported in Table 2. Heavy metal contents were low if compared to typical values for biomasses usually applied in agriculture (Alvarenga et al., 2015; Chen et al., 2008) and even the total Cd content was lower than the detection limit of the method used for all the samples. These values were expected, since the raw materials used in the daptomycin fermentation process were always checked for their chemical quality, such as the presence of heavy metals that could interfere with the daptomycin production process. Thus, it can be assumed that the low heavy metal contents of the studied materials may be due to their low concentrations in the wastewater from which they originated. Moreover, the addition of the agricultural by-products in the biological treatments, as described in Cucina et al. (2017), may be responsible for a dilution effect of heavy metals in the organic residues. Thus, PS, AD and CM were within the legal limits established by European and Italian authorities for the agricultural reuse of sludge, digestate and compost (CEC, 2003; CEC, 1986; Italian Decree 75/2010; Italian Decree 99/92).

Since the absence/low content of pathogens (*E. coli* and *Salmonella* spp.) was observed in almost all the samples, it is possible to state that the pharmaceutical materials studied in the present work are well sanitized biomasses (Table 3). The raw materials used in the daptomycin fermentation process must be devoid of contaminant microorganisms.

Regarding daptomycin residues, the concentrations obtained in the PS and in the CM were lower than the detection limit of the method used. However, antibiotic residues were still detectable in the AD (4.50±0.24 mg kg^{-1}), despite the concentration of daptomycin being diluted in the anaerobic bioreactor as a result of the addition of the agricultural by-products. This result suggests that the anaerobic process may not be effective for the complete degradation of daptomycin. Thus, to avoid any possible risk of soil contamination, the agricultural reuse of PS and CM should be preferred over AD, although the daptomycin concentration in AD was low. The absence of daptomycin residues in PS and in CM may be due to the aerobic microorganisms that are able to mineralize the daptomycin residues through protease-mediated hydrolysis mechanisms, as described by Cucina et al. (2017). Thus, the comparison of PS, AD and CM showed that composting of the digestate resulted in increased agronomic properties and an absence of organic contaminants in the mature compost.

3.3 Effect of organic matter stabilization on CO_2 emissions

Variations in CO_2 emissions with time after the application of PS, AD and CM to the soil are shown in Figure 2(A). Whereas control soils showed relatively constant emission rates throughout the incubation period, the addition of PS and AD generally resulted in greater CO_2 fluxes, particularly in the first days after amendment. Similar results were obtained by other authors after amending soils with anaerobic digestate and compost (Solé-Bundó et al., 2017; Pezzolla et al., 2013; Köster et al., 2011; Alluvione et al., 2010). The highest emission rates were observed for soil treated with the AD within 2 days after amendment, and the daily respiration rate was significantly higher for both PS and AD amended soils, with respect to the control, until the 14th day of incubation ($P < 0.01$). During the experiment, CO_2 emissions tended to decrease steadily, reaching relatively constant values similar to those obtained for the unamended controls within 18 days. Conversely, CM-treated soils did not show significant differences in emission rates with respect to the unamended controls throughout the incubation period.

Cumulative CO_2 emissions at the end of the incubation period increased in the order CM < PS < AD (Table 4). The application of the PS and AD to the soil induced a remarkable effect on the cumulative mineralized C ($P < 0.01$). The total amounts of CO_2 released after 60 days of incubation for the PS and AD amended soils were 71.6 and 129.8 mg-C, respectively. Considering that the application doses were designed to yield the same TOC addition to the soil for all the samples, CO_2 emission was probably related to the different quantity and quality of labile organic C added with the amendment (Pezzolla et al., 2013). This idea is consistent with the higher biodegradability of PS and AD compared to CM. This observation can be demonstrated by the values of C-mineralization, expressed as the percentage of the added TOC that was mineralized at the end of the incubation (Table 4). When the PS and the AD were applied to the soil, 18.8% and 34.1%, respectively, of the organic C added with the amendment was mineralized and lost as CO_2 at the end of the incubation period (60 d). Conversely, when CM was added to the soil, this value was 2.5%, which is significantly lower than the values obtained with the PS and the AD ($P < 0.01$). The high values of mineralized C in the PS and AD amended soils was probably due to the so-called *priming effect*, defined as a strong short-term change in the turnover of soil organic matter caused by the amendment (Kuzyakov et al., 2000). Confirming this hypothesis, a high linear correlation was found between the mineralized C in the 60 days of the incubation experiment and the C/N ratio, a key biomass stability parameter (Bernal et al., 2009). Indeed, a significant linear correlation was found between the C/N ratio of the pharmaceutical organic wastes and the mineralized C at the end of the incubation period (R^2=0.7769, n=9, $P < 0.05$). Specifically, the higher the organic matter stabilization of the fertilizer, the lower the percentage of added TOC that was mineralized.

Figure 2(B) shows the WEOM evolution in the soils amended during the microcosm experiment. The application of the PS and the AD significantly enhanced the concentrations of WEOM with respect to the controls ($P < 0.01$); in particular, the AD application increased markedly in the first days. The initial WEOM concentrations in AD and PS amended soils were 3.3 and 1.7 times greater, respectively, than the control soil. The WEOM content of the AD treated soil showed a clear decreasing trend throughout the incubation period due to the microbial activity, whereas the other amended soils showed a rather constant trend. The constant trend can be explained considering the dynamic equilibrium that occurred between the consumption of WEOM caused by the microbial mineralizing activity and microorganism release of WEOM for their hydrolytic activity (Said-Pullicino et al., 2007b). At the end of the incubation period, only the AD amended soil showed a WEOM content significantly higher than the control ($P < 0.01$). Stability-dependent respiration rates were reported by previous studies for soils amended with organic materials. Most of them showed CO_2 peak emissions in the first few days after amendment, with an intensity related to the contents of WEOM and microbial biomass (Solé-Bundó et al., 2017; Bustamante et al., 2010; Sánchez-Monedero et al., 2004). It is well known that organic amendment can change the amount and quality of dissolved organic matter in the soil solution with important implications on microbial activity and soil respiration (Pezzolla et al., 2013). Moreover, Said-Pullicino et al. (2007b) have shown that the soluble C fraction of organic amendments tends to decrease with organic matter stabilization.

In this work, this aspect was confirmed when the WEOM added with the organic fertilizers was correlated to the cumulative soil CO_2 emissions at the end of the incubation period (Table 4). A positive linear correlation between these two parameters was found to be significant (R^2=0.9035, n=9, $P < 0.01$).

3.4 Effects on soil enzymatic activities

The potential toxicity towards soil microorganisms after PS, AD and CM application could be achieved through the determination of a set of soil enzymatic activities. Microbial activity measurements appear as good indicators of the degree of stress and pollution of soils (Bastida et al., 2012).

Hydrolysis of fluorescein diacetate (FDA) has been widely used to estimate microbial activity in soil, since FDA is hydrolyzed by all the enzymes involved in the microbial decomposition of organic matter in soil (Araujo et al., 2015). FDA hydrolysis activity curves of PS, AD and CM are shown in Figure 3(A). All amended soils showed a strong increase of this enzymatic activity with respect to the control in the first days after the application. Specifically, all the organic materials added caused a significant increase of FDA activity ($P < 0.01$) from the 4th day of incubation until the end of the experiment (60 days). This behavior could be explained by the microbial metabolic activity, which was increased due to the rapidly degradable source of C added to the soils after the amendment. After the 17th day, the FDA hydrolysis activity decreased in all the amended soils due to the consumption of the easily available C source. Nevertheless, at the end of the incubation, this enzymatic activity was still significantly higher in the amended soils than in the control soil ($P < 0.01$), confirming the absence of toxicity towards soil microorganisms, even after two months of incubation.

Determination of dehydrogenase (DH) activity has been proposed by Ameloot et al. (2014) as a rapid and cost-effective toxicity test for soil microorganisms that can be very useful also for the identification of contaminated and perturbed soils. Moreover, DH activity is considered a good indicator of microbial activity in soil for its mineralizing function and thus for its relation with CO_2 emissions (Araujo et al., 2015). As shown in Figure 3(B), PS and AD addition to the soil caused a strong increase of the DH activity, probably due to the high content of labile C added with the amendment. Specifically, PS and AD measurements of the DH activity in these treated soils were significantly higher than in the control during the entire incubation period ($P < 0.01$). Conversely, CM addition did not cause a significant increase of this enzymatic activity, as expected from the lower respiration rates observed. Although the addition of PS and AD appeared to stimulate positively the soil microflora, the large increase in microbial activity could represent an environmental issue. Indeed, it is well known that the addition of easily degradable C could excessively stimulate microorganisms, leading to anoxic conditions in the soil and, consequently, to phytotoxicity (Wu et al., 2000).

Figure 3(C) shows the results obtained from the determination of the soil urease activity during the incubation period. Whereas Bastida et al. (2008) found positive effects of organic amendment on the urease activity, other authors found negative impacts on soil urease activity when applying organic materials, such as sewage sludge (Gao et al., 2010). The inhibition of urease may be due to heavy metals, to some constituents of the organic matter of the biomass, or to a high concentration of ammonia-N in the soil (Gao et al., 2010). Since a low content of heavy metals was observed in all the biomasses involved in the present experiment, a strong increase in this enzymatic activity was observed when PS and AD were applied, particularly in the first days. The large amount of labile C applied with the sludge and the digestate can justify these results, as already observed for the DH activity. The CM treated soils showed the lowest increase of the urease activity, as expected.

In the present work, short-term variations of the soil phosphomonoesterase activity were observed after the amendment (Fig. 3(D)). Phosphomonoesterase activity resulted significantly increased ($P < 0.01$) after the addition of the PS and AD to the soil, as expected; in fact, soil phosphomonoesterases can be mainly inhibited by heavy metals, which were not abundant in the PS and AD (Gao et al., 2010). Similar results were obtained by Ros et al. (2006), who amended an agricultural soil with bio-solids. They observed that treated soils generally show significantly higher phosphatase activity compared to the activity of control soil due to higher amounts of available nutrients with respect to the untreated controls. Conversely, in the present experiment, CM addition to the soil resulted in non-significant differences with respect to the control, probably due to the high organic matter stabilization of this organic material.

The analyses of soil enzymatic activities demonstrated that PS, AD and CM application to the soil did not show any effects of toxicity on soil microorganisms. Nevertheless, agricultural reuse of a stabilized fertilizer, such as the compost, appears the most suitable strategy to improve soil quality, avoiding environmental issues and perturbations of soil microorganisms.

3.5 Potential phytotoxicity

Organic fertilizers can cause phytotoxicity, mainly due to high contents of soluble salts, ammonia-N and low weight organic compounds such as total volatile fatty acids (Alburquerque et al., 2012). The germination index (GI) and growth index (GrI) of different species were used in the present study to assess the potential phytotoxicity of all materials (Figures 4, 5 and 6).

As expected, CM did not inhibit the germination of the studied plants (Fig. 4). Moreover, CM-diluted extracts induced positive effects on the GI of all plants, confirming the suitability for their agricultural reuse. The PS showed a moderate phytotoxicity, whereas AD produced a strong inhibition of the germination in all plants. However, the lowest performances were obtained with the pure extracts, demonstrating that all the studied materials showed a dose-response phytotoxic effect on germination.

Among plant species used for the germination tests, lettuce appeared to be the most susceptible species, highlighting GI differences among all the materials tested; conversely, both cucumber and barley showed a lower sensitivity to phytotoxicity with respect to the other plant species.

With respect to the GI, the results obtained in the present study confirmed that the inhibition of germination is strictly related to the physico-chemical parameters of the biomass (e.g., soluble salts, ammonia-N, total volatile fatty acids) (Solé-Bundó et al., 2017; Di Maria et al., 2014; Alburquerque et al., 2012). When the GI was related to the soluble salt contents of all the materials studied, a significant negative linear correlation was found ($R^2 = 0.8449$, $n = 12$, $P < 0.05$). Similar linear negative correlations were found when the GI was correlated to the ammonia-N content and the total volatile fatty acid content ($R^2 = 0.8533$ and $R^2 = 0.8239$, respectively; $n=12$, $P < 0.05$).

The effects of PS, AD and CM on the growth index (GrI) of *L. minor* are shown in Figure 5. The GrI was 0% for all the dilutions of the AD extract, confirming the phytotoxicity of this material. Conversely, positive results were obtained when the CM extracts were tested (the average GrI was 86.3%), while PS affected the growth index of *L. minor* with a moderate phytotoxicity (the average GrI was 25.7%). As already observed for the GI, a dose-response effect was also found in the growth of aquatic plants for all the studied fertilizers. Cayuela et al. (2007) reported that the *Lemna gibba* growth inhibition bioassay was highly related to maturation indices commonly used to evaluate the toxicity of biomasses during composting. In this study, it was assessed also that the *L. minor* growth inhibition is correlated to the germination index of *L. sativum*, a common maturation index. A highly positive linear correlation was found between the GI of cress and the GrI of *L. minor* ($R^2=0.7848$, $n=12$, $P < 0.05$).

The assessment of the potential phytoxicity was completed through the growth tests, for which results are shown in Figure 6. The AD produced phytotoxic effects also in the growth tests and the highest phytotoxicity was observed for lettuce and cress, as a demonstration that these two species were more sensitive to phytotoxic compounds than cucumber and barley. Specifically, no plant growth was observed when the AD was applied at 22.5 and 30.0 tons ha^{-1}. With regard to the PS, the GrI determination in all the four species studied showed that this material possessed residual phytotoxicity (the average GrI was 62.3, 20.0, 38.8 and 76.2% for cucumber, lettuce, cress and barley, respectively). As already observed for the GI, the CM did not produce phytotoxic effects on plant growth. Once again, a dose-response effect was observed between the application dose of the fertilizer and the accumulation of biomass in all the species tested.

In the present work, soluble salts, total volatile fatty acids and ammonia-N were found to be significantly and negatively correlated to the GrI, as highlighted in previous studies (Solé-Bundó et al., 2017; Alburquerque et al., 2012). The relationship between the GrI and the ammonia-N content of the pharmaceutical wastes was described as a negative linear correlation (R^2=0.6348, n=12, $P < 0.05$). Conversely, the GrI was found to be positively correlated to stability and maturation parameters, as already reported by Young et al. (2016). The GrI was positively correlated to the C/N ratio of the pharmaceutical organic wastes (R^2=0.6678, n=12, $P < 0.05$) and it was confirmed that the C/N can be used as a maturation index, as observed in Bernal et al. (2009) and in Said-Pullicino et al. (2007b).

The large set of phytotoxicity assessments demonstrated that CM could be considered the best pharmaceutical-derived organic fertilizer for the absence of phytotoxicity. Several extract dilutions and doses used in the bioassays resulted in germination and growth higher than those of the control. Moreover, in some cases (e.g., germination of lettuce and barley, growth of cucumber and lettuce), CM affected positively the plant development, probably due to a hormone-like action, resulting in GI and GrI values higher than the 100% of the control (Alburquerque et al., 2012). However, the agricultural reuse of the AD should be avoided, since it was found to be phytotoxic in most of the bioassays. Within the assays tested in the present work, the *L. minor* growth inhibition test appeared the most suitable. It was demonstrated that this aquatic plant is very sensitive to the phytotoxicity of organic fertilizers and can highlight properly the differences among organic materials.

4. Conclusions

The pharmaceutical-derived organic wastes studied in the present work were characterized by high content of plant macronutrients (N, P and K) and low concentrations of heavy metals, pathogens and daptomycin residues. The sludge and the anaerobic digestate showed a low organic matter stabilization that may affect soil microbial activities, mainly in terms of CO_2 emissions. In contrast, the compost may represent an important source of stabilized organic matter for the soil and nutrients for plants, due to its chemical characteristics.

According to the results, the compost appears to be the most promising organic fertilizer derived from the daptomycin production process. Its agricultural reuse may allow recovery of plant nutrients while avoiding environmental risks of soil contamination and toxicity towards soil microorganisms and plants. Thus, integrated anaerobic-aerobic treatment can represent a suitable strategy to valorize wastewaters derived from antibiotic manufacturing.

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Table 1: Physico-chemical and fertilizing properties of the three pharmaceutical-derived organic wastes.

Parameter	Units	Sludge	Anaerobic digestate	Compost
TS	%	14.3 ± 0.8	3.8 ± 0.3	51.9 ± 1.0
VS	%	8.8 ± 0.1	2.7 ± 0.0	31.6 ± 0.2
VS/TS	%	61.9 ± 0.7	70.9 ± 0.2	61.0 ± 0.5
pH	-	8.6 ± 0.0	7.4 ± 0.0	8.4 ± 0.0
EC	$dS \cdot m^{-1}$	7.9 ± 0.1	12.1 ± 0.2	7.0 ± 0.0
TVFA	$g \cdot kg^{-1}$	9.6 ± 0.4	48.0 ± 1.30	3.8 ± 0.4
TOC	%	17.8 ± 0.3	34.0 ± 0.2	28.1 ± 1.6
TKN	%	3.99 ± 0.19	9.42 ± 0.26	2.71 ± 0.04
C/N	-	4.5	3.6	10.4
NH_4^+-N	%	1.03 ± 0.03	4.86 ± 0.24	0.07 ± 0.00
Organic-N	%	2.96	4.56	2.64
Total P	%	2.04 ± 0.14	0.56 ± 0.00	0.62 ± 0.09
Total K	%	0.16 ± 0.01	0.67 ± 0.03	1.78 ± 0.13
Total Na	%	1.30 ± 0.20	0.91 ± 0.04	0.69 ± 0.01
Humification degree	%	41.4 ± 0.1	55.8 ± 0.5	62.3 ± 0.1

Note: TS = total solids, VS = volatile solids, EC = electrical conductivity, TVFA = total volatile fatty acids, TOC = total organic C, TKN = total Kjeldahl N.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Table 2: Heavy metal contents of the three pharmaceutical-derived organic wastes.

Parameter	Units	Sludge	Anaerobic digestate	Compost
Total Cd	$mg \cdot kg^{-1}$	< 0.20*	< 0.20*	< 0.20*
Total Cr	$mg \cdot kg^{-1}$	6.2 ± 0.2	< 0.50*	9.3 ± 0.1
Total Ni	$mg \cdot kg^{-1}$	6.6 ± 1.0	< 0.50*	18.3 ± 1.9
Total Pb	$mg \cdot kg^{-1}$	11.1 ± 1.8	< 1.00*	26.6 ± 5.7
Total Cu	$mg \cdot kg^{-1}$	59.7 ± 3.9	23.4 ± 2.5	36.8 ± 4.7
Total Zn	$mg \cdot kg^{-1}$	92.3 ± 4.5	117.2 ± 5.2	113.8 ± 6.3
Total Hg	$mg \cdot kg^{-1}$	0.30 ± 0.04	0.41 ± 0.00	0.31 ± 0.02
Total As	$mg \cdot kg^{-1}$	0.21 ± 0.00	0.19 ± 0.04	0.11 ±0.02

Note: * = detection limit of the method.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Table 3: Hygenization properties and daptomycin residues of the three pharmaceutical derived organic wastes.

Parameter	Units	Sludge	Anaerobic digestate	Compost
Salmonella spp.	MPN g^{-1}	0.90 ± 0.07	Absent	Absent
Escherichia coli	MPN g^{-1}	Absent	Absent	Absent
Daptomycin	$mg \cdot kg^{-1}$	< 0.10*	4.50 ± 0.24	< 0.10*

Note: MPN = most probable number, * = detection limit of the method.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Table 4: Organic matter turn-over in the amended soils during the microcosm experiment.

Parameter	**Units**	**Sludge**	**Anaerobic digestate**	**Compost**
Application dose	$g \cdot kg^{-1}$	10.7	5.6	6.8
TOC_{added}	$g \cdot kg^{-1}$	1.90	1.91	1.89
WEOM	$g \cdot kg^{-1}$	47.4 ± 0.2	265.0 ± 0.4	30.6 ± 0.4
$WEOM_{added}$	$mg \cdot kg^{-1}$	507.0	1349.0	206.5
Net CO_2 emission	$mg\text{-}C \cdot kg^{-1}$	358.0 ± 5.0	649.0 ± 4.3	47.0 ± 2.8
% TOC_{added} mineralized	%	18.8 ± 0.5	34.1 ± 1.2	2.5 ± 0.4

Note: TOC = total organic C; WEOM = water extractable organic matter.
Data are expressed on dry weight basis.
Mean value ± SD; n = 3.

Figure 1: Diagram of daptomycin production, wastewater treatments and sampling sites. Solid line: actual disposal of wastewater; interrupted line: wastewater valorization proposed by Cucina et al. (2017).

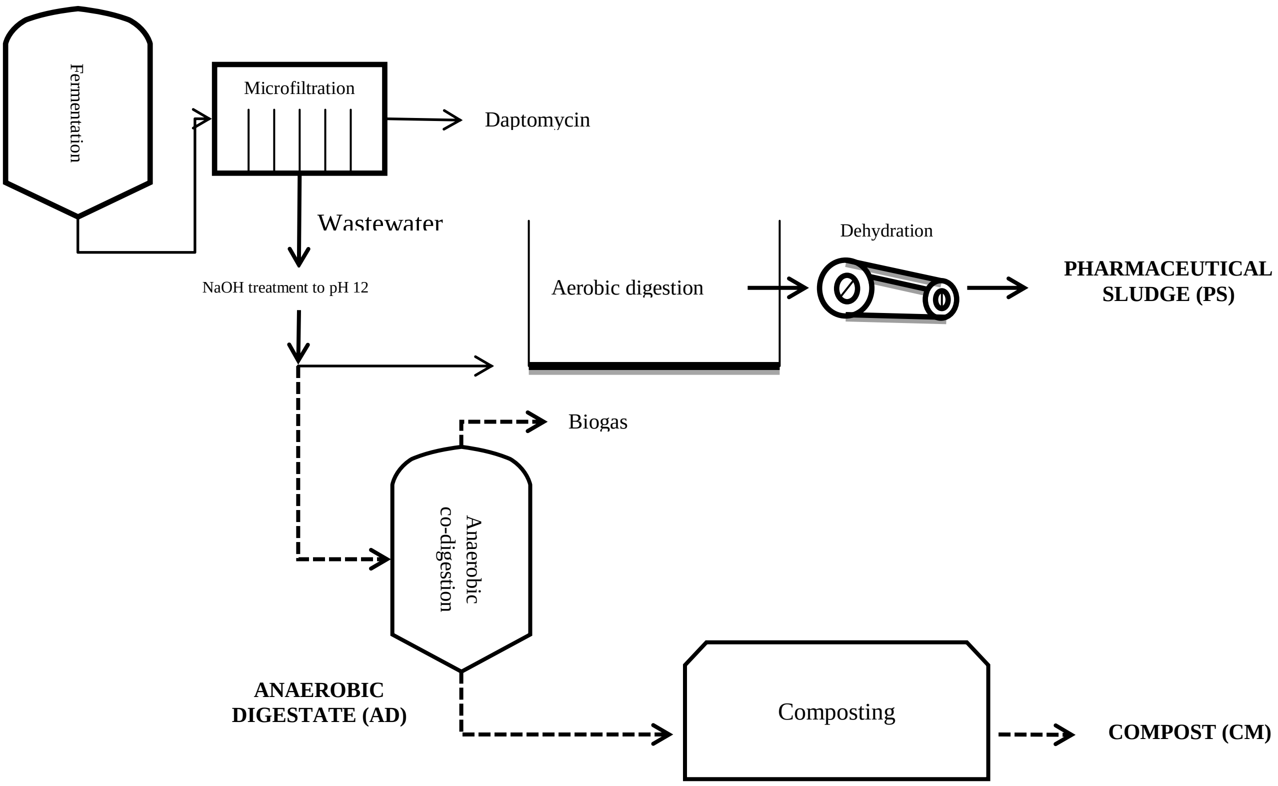
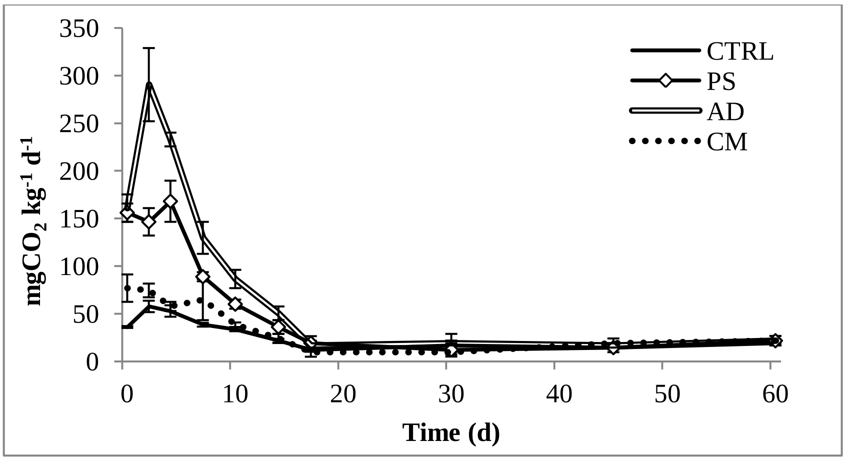


Figure 2: Changes over time in the (A) CO_2 emissions and (B) WEOM content determined in the soils amended in the microcosm experiment (mean value ± SD, n=3). CTRL: non-amended soil; PS: sludge; AD: anaerobic digestate; CM: compost. Data are expressed on dry weight basis.

(A)



(B)

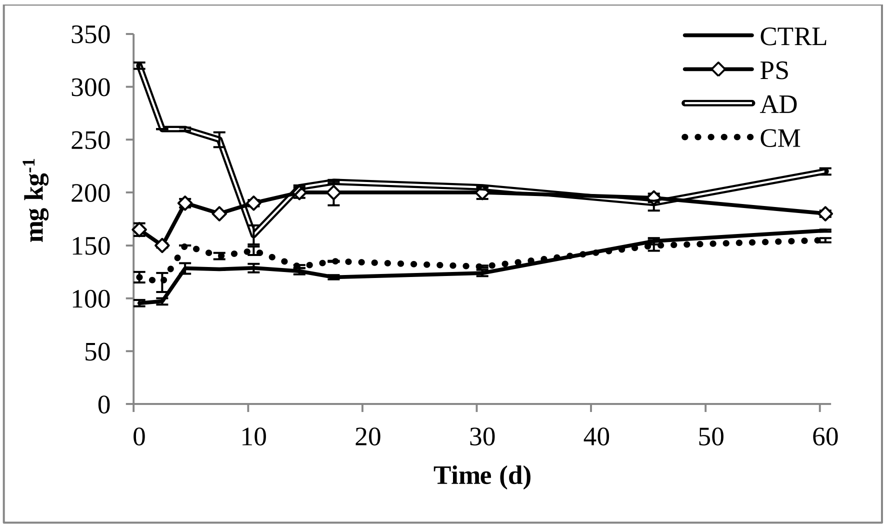
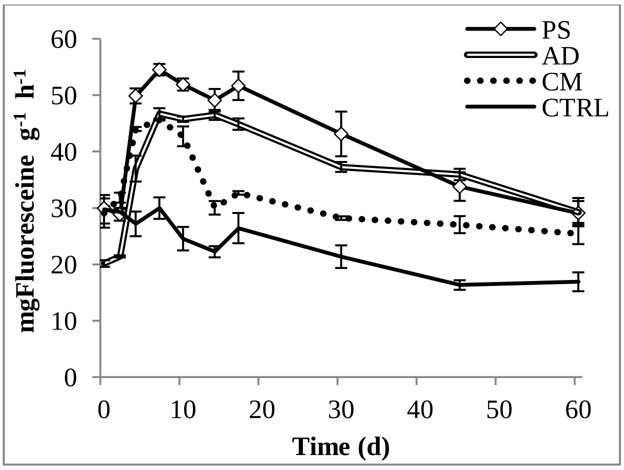
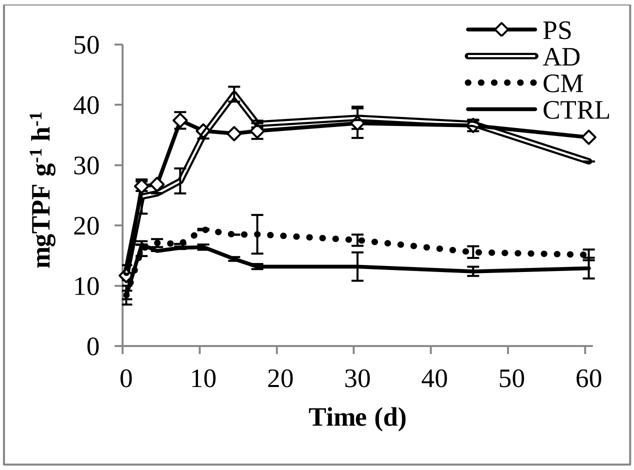


Figure 3: Changes over time in the enzymatic activities determined after PS, AD and CM application to the soils: (A) FDA hydrolysis activity; (B) total dehydrogenase activity; (C) alkaline phosphomonoesterase activity; (D) urease activity (mean value ± SD, n=3). CTRL: non-amended soil; PS: sludge; AD: anaerobic digestate; CM: compost. Data are expressed on dry weight basis.

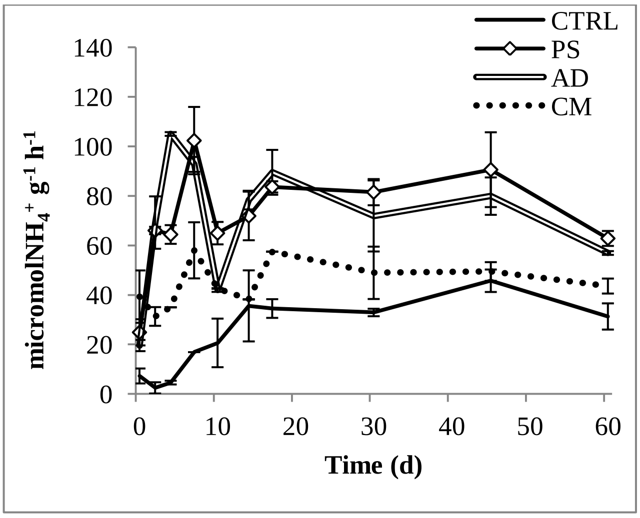
(A)



(B)



(C)



(D)

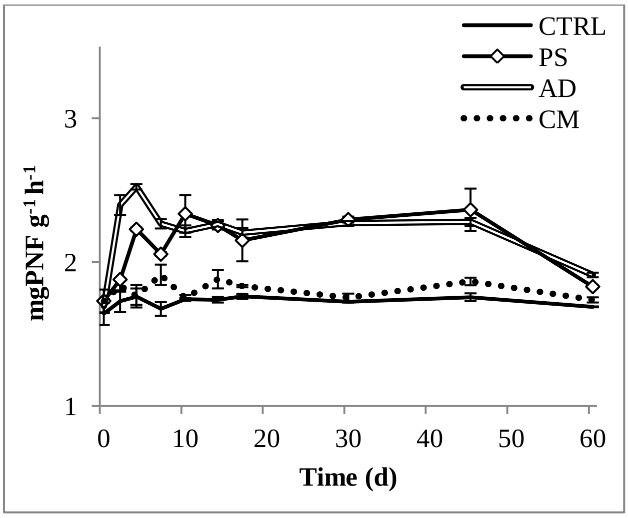


Figure 4: Effects of PS, AD and CM extracts and dilutions on the germination index (GI) of (A) cucumber (*C. sativus*), (B) lettuce (*L. sativa*), (C) cress (*L. sativum*) and (D) barley (*H. vulgare*) (mean ± SD, n=5).

(A) (B)

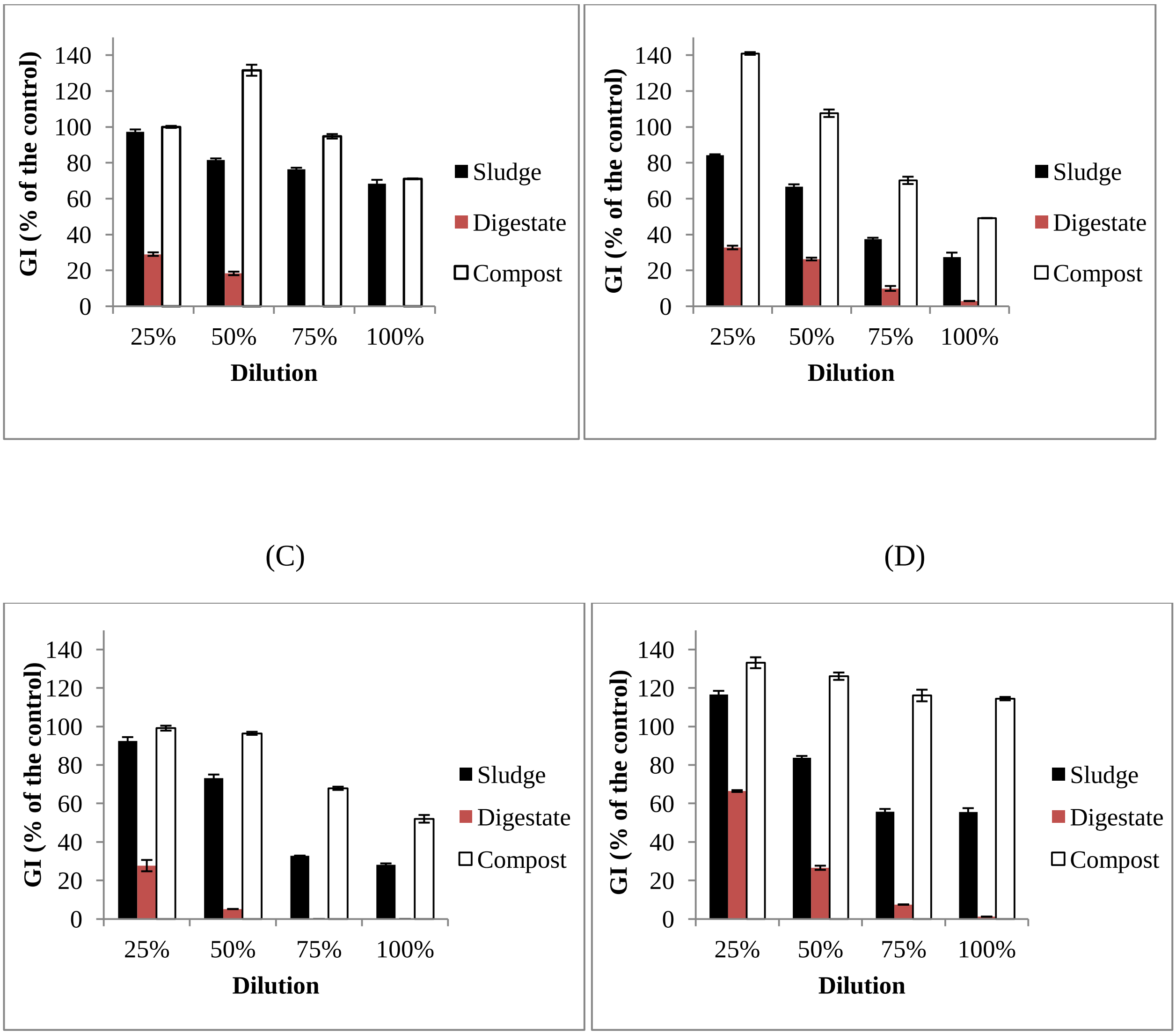


Figure 5: Effects of PS, AD and CM extracts and dilutions on the growth index (GrI) of *L. minor* (mean ± SD, n=6). GrI was 0% for all the dilutions of the digestate extract.

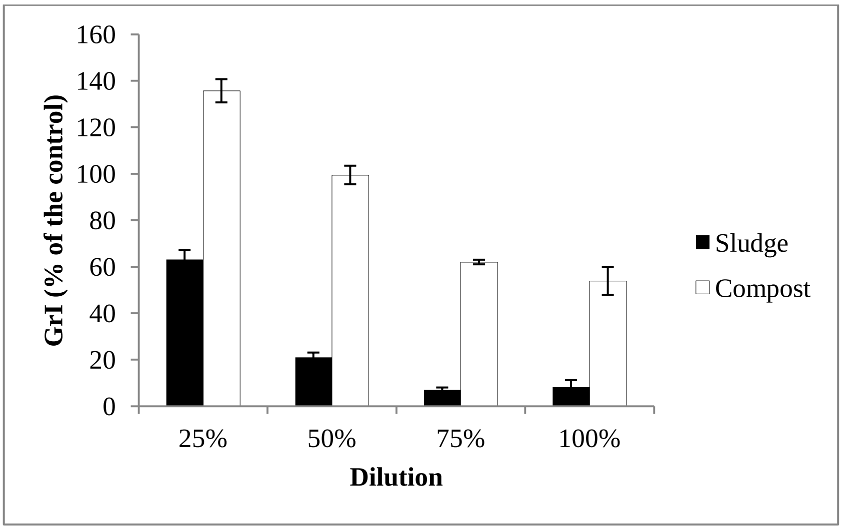
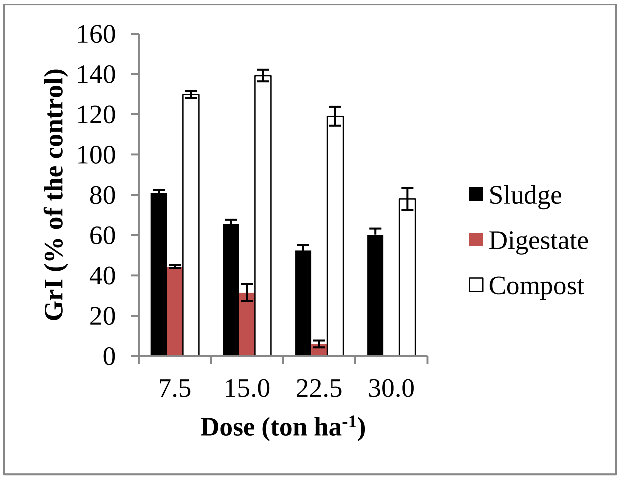
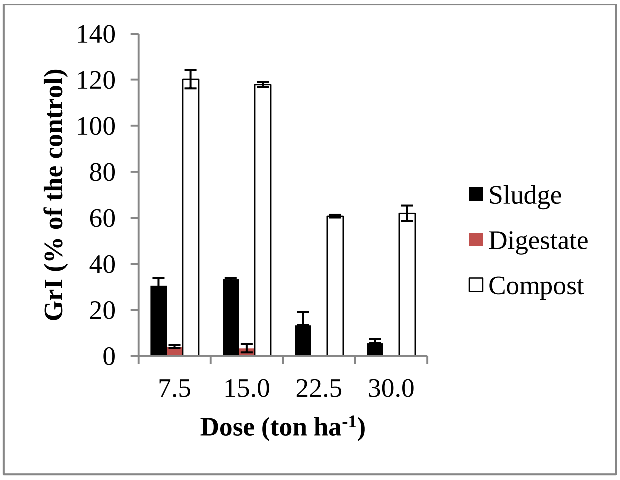


Figure 6: Effects of PS, AD and CM doses on the growth index (GrI) of (A) cucumber (*C. sativus*), (B) lettuce (*L. sativa*), (C) cress (*L. sativum*) and (D) barley (*H. vulgare*) (mean ± SD, n=3).

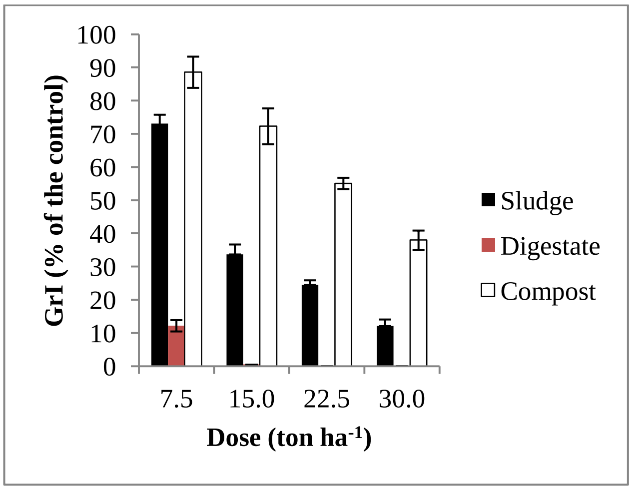
(A)



(B)



(C)



(D)