Treatments of anaerobic digestate: assessment and effects on the final environmental quality

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PhD Course

Sciences for Civil, Environmental and Architectural Engineering

XXXIII series

Doctoral Thesis

Università degli Studi di Padova

2020

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Abstract

The European biogas sector produces yearly about 180 million tons of digestate, whose management influences the sector's sustainability. In this context, this thesis investigated innovative and conventional digestate treatment options. The work focuses on the influences of the studied treatments on the final environmental quality of digestate derived from the anaerobic digestion (AD) of separately collected organic fraction of municipal solid waste (OFMSW).

Environmental quality of digestates is initially reviewed according to the parameters established in the regulation and in the literature. Average quality profiles are determined for digestates derived from the AD of agro-industrial residues and OFMSW, to identify significant differences, based on a statistical analysis performed on digestates characterization data. Both digestate categories display high degree of variability among the considered quality parameters and full compliance with the Italian regulation limits. The outcomes do not support the current Italian regulation excluding OFMSW digestates from direct agricultural reuse.

Results are then presented from a first experimental activity conducted to assess the effects of the application of woody biochar on dry thermophilic AD of OFMSW in terms of improved process stability and resulting environmental quality of biochar enriched-digestates. Tested biochar shows small particle size, poor adsorption potential and high content of polyaromatic hydrocarbons (PAH). Coming from semi-pilot biomethanization tests operated in different configurations involving different inoculums and biochar presence, the experimental outputs do not show significant increase in methane yield due to biochar addition. Nevertheless, significant changes in the microbial community composition were noted. Digestate quality shows higher biological stability, lower heavy metals concentrations but increased PAH content of biochar-enriched digestates, which could determine nonconformity with regulation limits for agricultural reuse. Conversely, the outcomes from plant bioassays showed decreased phytotoxicity of biochar-enriched digestates.

The outputs are discussed also from the lab-scale application of chemically enhanced solid liquid separation (CES) on OFMSW digestate. Simulating real scale CES through Jar Test apparatus and lab-scale centrifugation, all tested dosages of polyaluminum chloride (PAC), epichlorohydrine-dimethylamine with ethylendiamine (DEED) and polyacrilamides (PAM) significantly increase the suspended solids mitigation in the separated liquid fraction of digestates. Treated solid fractions from the 3 best performing CES trials are characterized by full compliance with regulation limits for agricultural reuse. However, lower P and HMs leachability is reported, together with remarkably higher contents of Al, Cl and organic C and N, these latter likely derived by residual byproducts of tested conditioning agents. Finally, the high values of soluble Al and conductivity measured in the water extracts of treated solid fractions could have determined their assessed increased plant toxicity.

Finally, the thesis presents the outcomes from a human-health risk assessment performed on an agricultural sites undergone application of digestates, these latter characterized by the average environmental quality profiles estimated during previous investigations (i.e., digestates from agricultural residues and OFMSW). The risk values estimated for the indirect exposure to HMs through intake of vegetables grown on the analyzed site do not show situations of concern related with scenarios involving digestates application.

The results, taken together, can provide a scientifically-sound background to the choices made to realize sustainable strategies for digestate management.

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PART I
THESIS DEVELOPMENT

1. Background and objectives

Climate change, chemical pollution phenomena and finiteness of raw materials are currently considered among the main environmental challenges (Rockström et al., 2009). To solve them, waste management plans can play a crucial role by ensuring i) the minimization of related greenhouse gases and contaminants emissions and ii) the recovery maximization of waste-derived secondary raw materials. Besides specific environmental performances, the applied practices and technologies should be economically sustainable to ensure their long-term viability.

The European Union promulgated several regulations to support the establishment in the long term of a consistent and sustainable waste management sector. In particular, the issue of biodegradable residues was faced through the introduction of a progressive ban from landfill disposal and by encouraging the development of a bioeconomy based on separate collection and efficient treatment and recovery of biowaste (European Council, 1999; European Parliament and European Council, 2018). This favorable context led to the flourishing of the anaerobic digestion (AD) sector, which counts more than 18,000 full-scale biogas plants Europe-wide, employing more than 68,000 workforces and determining an economic turnover of more than 7 billion euros (EBA, 2019; EurObserv'ER, 2019). Considering the full-scale installations treating biodegradable residues from the agricultural, agro-industrial and municipal waste sectors (i.e. excluding biogas from landfills and sewage sludge treatment), the European biogas sector contributed with almost 13 Mtoe to the

European energy output, representing a share of 6 % of the total renewable energy production (EurObserv'ER, 2019; Eurostat, 2020).

A recent market survey showed that, alongside bioenergy, the European biogas sector was responsible for the production of more than 180 million tons of digested residues, the so-called "digestate" (Corden et al., 2019). Considering its already known fertilizing and amendment properties, digestate represents a valuable resource to be recovered and marketed as potential substitute for inorganic fertilizers. However, due to current public acceptance issues and low confidence of the agricultural sector with digestate-derived organic fertilizers, digestate management is still considered as an economic burden by biogas plant operators, forcing them to focus on feed-in tariffs from the sale of electricity and biomethane and gate fees from waste treatment (Dahlin et al., 2015). Indeed, collecting revenues from digestate marketing could determine the long-term resilience of the biogas sector even within a possible no-subsidies framework.

To tackle this trend, the updated European regulation on CE-marked fertilizers, (expected to come into force by 2022) includes digested residues as component materials for organic fertilizer products, according to proven compliance with quality standards (i.e., expressed in terms of chemical-physical and biological parameters) and positive list of input biodegradable feedstocks treated by the AD (European Parliament and European Council, 2019). This latter includes not only agricultural substrates, (e.g., manures and energy crops), but also separately collected organic fraction of municipal solid waste (OFMSW).

By determining additional operational or providing revenues from possible marketing within a circular economy framework, the choice behind the performed digestate management alternatives (i.e., treatment, reuse or disposal), can influence both the investment payback time of a biogas plant and its long-term profitability. Therefore, specific digestate treatment options must be properly assessed in terms of both specific efficiency and achieved final digestate quality.

In this context, the PhD research project started by the proposals of multiutility companies located in North-East Italy to improve the management of the digested residues daily produced by the respective biogas plants, with a specific focus on possible agricultural valorization of digestates derived from the AD of OFMSW. Therefore, the principal research objective was to investigate the environmental quality of digestates achieved through the application of both innovative and conventional treatment technologies. In particular, resulting digestate quality was assessed according to reached compliance with single regulated parameters and also by estimating the ecotoxicity of treated digestates, thus considering the real bioavailability, e.g., to plants, of possibly occurring contaminants.

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2. Activities and Outputs

The outcomes presented in this thesis work derived from a stepwise research activity, performed during the 3 years of the PhD program. In this respect, Figure 1 depicts the main steps of the research project, logically developed to achieve the general objective of the thesis.

The main outputs of the performed activities are resumed in the next paragraphs, while deeper discussion of performed experimental procedures and analysis together with the specific outcomes are presented in the following Part II, in form of scientific papers (already published or as submitted to peer-review journals).

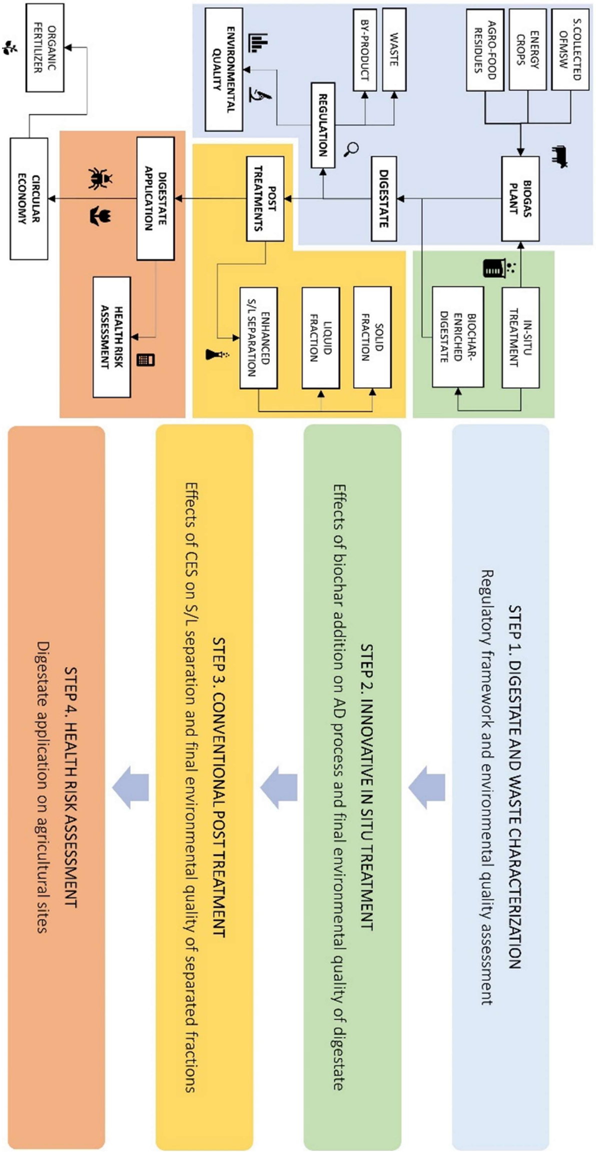

Figure 1. Scheme of the thesis development.

Step 1. Digestate and waste characterization

The first step was crucial to identify how environmental quality of digested residues is defined in the regulatory framework, both at national and European level, and in the existing scientific and technical literature (see Figure 1).

Beside carrying out literature reviews and consulting national and European regulation repositories, the direct exchange occurred with the actors involved in the biogas sector (e.g., at expositions, conferences or directly at the company) allowed to highlight the existing challenges posed by the regulations and on which topics technical research should focus to provide real support to the development of the sector. In this respect, the performed studies permitted to verify whether the requirements for agricultural reuse established in the Italian regulation (i.e., DM 5046/2016) are based or not on scientifically sound and statistical basis.

In particular, the exclusion of digestates derived from the AD of OFMSW from direct land spreading was challenged. To do so, two datasets were built, gathering characterization data of digestates originating respectively from the AD of agro-industrial residues (named AGRO), allowed to be directly applied on agricultural land, and separately collected organic fraction of municipal solid waste (OFMSW). Data were collected from more than 2,000 samples. Several sources were consulted: scientific literature, institutional reports on agricultural use of digestate and unpublished monitoring data provided by private biogas plant operators (the majority, in fact).

Data aggregation and analysis was performed according to the parameters' macro categories defining overall digestate quality, as identified by the literature review: organic amendment properties (pH, Total Solids (TS), Volatile Solids (VS), Total Carbon), fertilizing properties (Total Ammonia Nitrogen (TAN), Total Nitrogen, total Phosphorus, total Potassium and Electroconductivity), and environmental impacts properties (Regulated Heavy Metals (HM), Poly Aromatic Hydrocarbons (PAH), Volatile Fatty Acids (VFA) and

Residual Methane Potential). Descriptive statistics was performed through the use of a statistical software and 95% confidence intervals were calculated for each investigated parameter. Hypothesis testing was performed to assess if enough statistical evidence can claim for differences at population level between the two digestate typologies. Finally, the obtained results were compared with legal limits, where available, to prove compliance with the regulation.

Considering the results, both digestate categories were assessed compliant with current Italian regulation, thus indicating that no statistical ratio seems to support the difference in the legislative approach as proposed by Italian lawmakers. This latter currently forces OFMSW digestates to undergo highly energy demanding post-composting to reach the "End-of-waste" status. Conversely, treatments options should be applied to reach digestate quality criteria, defined for the intended reuse scenario.

OFMSW digestate resulted significantly different from AGRO digestate in terms of lower VS and higher Nitrogen, Phosphorus, Pb, Ni, Cr and Hg. In particular, both categories were characterized by high degree of variability related to all investigated parameters. This is likely due to the wide range of substrates included in both categories and to the different AD plant configurations considered in the analysis.

Furthermore, the average quality profiles for both digestate categories could be defined and considered consistent with the current legislation (see a resume in Table 1). In particular, the calculated confidence limits resulted crucial to interpret the results derived by the following experimental activities. The reported findings need to be strengthened by further analysis on parameters not covered by the regulation (e.g., ecotoxicological features).

Table 1. Calculated confidence intervals (95%) of digestate quality parameters for AGRO and OFMSW digestates.

Parameter	Unit	AGRO	OFMSW
pH	-	7.41-8.03	7.18-8.19
TS	gTS kg^{-1}FM	112.3-131-9	39.9-136.0
VS	gVS kg^{-1}TS	827.6-860.0	600.5-699.6
N-NH$_4$	g kg^{-1}TS	33.26-58.97	59.77-104.03
N-TOT	g kg^{-1}TS	59.64-71.01	90.60-128.70
P	g kg^{-1}TS	19.56-24.24	0-15.70
K	g kg^{-1}TS	27.60-68.70	19.25-52.84
EC	mS cm^{-1}	8.92-26.57	0-16.40
Cd	mg kg^{-1}TS	0.32-0.48	0.33-0.83
Pb	mg kg^{-1}TS	2.98-6.34	13.31-23.85
Cu	mg kg^{-1}TS	55.74-68.75	33.30-73.10
Hg	mg kg^{-1}TS	0.046-0.055	0.059-0.095
Ni	mg kg^{-1}TS	6.85-9.56	6.93-15.13
Zn	mg kg^{-1}TS	246.00-314.00	128.30-336.90
Cr	mg kg^{-1}TS	6.62-10.86	10.48-15.01
PAH$_{16}$	mg kg^{-1}TS	0-6.54	0-4.91

One scientific paper was published on the outcomes of this activity:

- Beggio, G., Schievano, A., Bonato, T., Hennebert, P., Pivato, A., 2019. Statistical analysis for the quality assessment of digestates from separately collected organic fraction of municipal solid waste (OFMSW) and agro-industrial feedstock. Should input feedstock to anaerobic digestion determine the legal status of digestate? Waste Manag. 87, 546–558. https://doi.org/10.1016/J.WASMAN.2019.02.040

The investigative methodology developed during the first phase of the research activity was applied concurrently in the general context of waste ecotoxicological characterization. Performed regulations and literature reviews and analysis resulted in the publication of the following two scientific papers about the design and optimization of experimental methods for ecotoxicity classification of waste, here presented in Part III “Additional Papers”.

- Pivato, A., Beggio, G., Raga, R., Soldera, V., 2019. Forensic assessment of HP14 classification of waste: evaluation of two standards for preparing water extracts from solid waste to be tested in aquatic bioassays. Environ. Forensics 20, 275–285. https://doi.org/10.1080/15275922.2019.1630517
- Pivato, A., Beggio, G., Hennebert, P., Bonato, T., Favarin, M., Raga, R., 2020. Proposal of a Testing Program for the Hp14 (Ecotoxic) Classification of Automotive Shredder Residues (Asr) By a Battery of Ecotoxicological Bioassays. Detritus 13, 12–22. https://doi.org/10.31025/2611-4135/2020.14032

Within the scope of the first step of the PhD research project, a parallel activity was performed for three months (February-April 2020) at Ineris (National Institute for Industrial Environment and Risk Assessment) in Aix-en-Provence (France), under the scientific supervision of Dr. Pierre Hennebert, whose main research topic is related with solid waste characterization for circular economy purposes.

The research project was aimed at reviewing the theory behind the technical standards of representative sampling of granular solid waste to facilitate its use within industry. In particular, the work’s outcomes can be easily transferred to sampling of solid fractions of digestate, to derive reliable characterization data upon which base decision on further agricultural use or disposal.

Particles analyses (i.e., chemical characterization and particle size distributions) performed on different waste streams (solid digestate, electric and electronic waste, bottom ashes and industrial slags, recovered aggregates, etc.) showed that properties of interest, such as contaminants content, are not normally distributed among solid waste particles, but skewed by few rare large values. Consequently, a representative sample must include the same fraction of these “rare” particles of the lot of material to be analyzed. Therefore, the key concept is the number of particles that must be present in a portion of matter to be representative of a larger portion of matter. In this context, “rare” particles must be considered both in terms of their chemical composition and size of particles, especially when lacking knowledge of possible size-composition relationship. Once the number of particles was estimated, a calculation method to simply determine the mass of a representative sample was proposed, based on deep knowledge of the waste constituency and particle size distribution. Two papers are currently under review:

- Hennebert, P. and Beggio, G. Sampling and sub-sampling of granular waste: Part 1 – Size of a representative sample in terms of number of particles and application to waste containing rare particles in concentration. Submitted to Detritus Journal (29/01/2021).
- Beggio G. and Hennebert, P. Sampling and sub-sampling of granular waste: Part 2 – Size of a representative sample in terms of number of particles with different distributions of particle concentration and particle size. Submitted to Detritus Journal (29/02/2021).

The following parts of the PhD research project involved experimental activities aimed at reproducing at lab-scale both innovative and conventional treatments, specifically fine-tuned to be applied on digested residues, to assess their efficiency and effects on their resulting environmental quality. The considered treatments were chosen according to the requirements of the proponent industrial partners, willing to evaluate possible up-scaling of the specific technologies or expand their market share in the field of digestate treatment. As a result, an innovative in-situ treatment (i.e., applied directly in the AD reactor) and a conventional post-processing technology were investigated (Figure 1).

Step 2. Innovative in situ treatment

A first experimental activity was conducted together with the Technology Transfer Centre of the Edmund Mach Foundation (San Michele all'Adige, Trento, Italy), to assess possible industrial symbiosis paths between thermochemical and biological Waste-to-Energy techniques. In practice, the research project allowed to investigate the innovative use of waste biochar on dry thermophilic anaerobic digestion of Organic Fraction of Municipal Solid Waste (OFMSW) in terms of process efficiency (i.e., methane production and microbial stability) and influence on the final quality of biochar-enriched digestate, with a view to its possible agricultural reuse. In fact, while the potential improvement of the anaerobic process due to biochar addition is already reported (adsorption of process inhibiting compounds - TAN and VFA - and improved methanogenic bacteria acclimation), the scientific literature highlighted the presence of possible contaminants in biochars (mostly HM and PAHs), which can trigger the non-compliance with established regulation limits for agricultural reuse of biochar-enriched digestates.

The choice to conduct the investigation on the dry thermophilic AD of OFMSW was made according to local availability and possible application scale-up. In particular, the application of waste biochar, produced by a neighboring woodchips gasification plant, was proposed to the local multiutility currently treating separately collected OFMSW with a dry thermophilic anaerobic process.

The experimental procedure consisted on the performance of dry thermophilic biomethanization (BMP) tests conducted on 20 L semi-pilot reactors at E. Mach laboratories. Four different configurations were tested in four test replicates, according to two different inoculums (i.e., digestates from a full-scale dry anaerobic digestion plant and from a previous lab-scale BMP test on OFMSW) and biochar application (dosed to reach 6% on fresh mass basis of the inoculum). Biogas production and methane content were monitored during each test run (which lasted for 21 days on average) and modeled using the modified Gompertz equation. After each BMP replicate, resulting digestates were collected and transported to the laboratory at the University of Padova to conduct chemical-physical and biological characterization: TS, VS, pH, EC, VFA content, Respiration Index (RI) through dynamic respirometry, Total Kjeldahl Nitrogen (TKN), TAN, phosphates (PO_4^{3-}), HMs (Cd, Pb, Cu, Hg, Ni, Zn and Cr) and PAHs. Further, Germination Index of *Lepidium Sativum* seeds was determined for resulting digestates water extracts derived through conventional leaching tests. Digestates have also undergone genomic DNA analysis to assess the achieved microbial community thus estimate occurred bacterial diversity and acclimation. Finally, the TAN adsorption potential of the used biochar was tested through batch adsorption test.

The performed molecular approach showed that biochar determined significantly increased microbial diversity, thus suggesting an achieved higher methanogenic biomass resilience to stressors (mostly VFAs). Here, increasing the retention time of biochar within the AD reactor (achievable through biochar-enriched digestate recirculation) proved to promote the observed beneficial effects to microbial community stability. However, non-significative influence on increased methane production was noted, likely due to the measured poor features in terms of granulometry and adsorption potential, and the dry AD configuration tested.

Biochar-enriched digestates were characterized by higher biological stability (in terms of measured RI), probably determined by the contribution of stable graphite-like structure of the dosed biochar. Further, lower HMs content were recorded but higher PAHs concentrations could, in one case (i.e., 8,9 mg kg^{-1} TS), have triggered incompliance with regulation limits on agricultural reuse. Nevertheless, conducted plant bioassays showed a decreased phytotoxicity related with biochar addition, highlighting both the possible reduced bioavailability of occurring phytotoxic compounds. This could have been determined by the contaminant-binding capacity of biochar particles or to the increased carbon content of biochar-enriched digestates able to complex with dissolved contaminants. Further, the trend reported for phytotoxicity could suggest that PAHs contained in biochar are characterized by low bioavailability, as also discussed in the reviewed literature.

Finally, no increase in fertilizing potential could be estimated in terms of higher PO_4^{3-} and TAN retention in biochar-enriched digestates, likely due to the poor adsorption characteristics shown by the tested gasification residues.

It is important to highlight the novelty behind the performed experimental activity. First, it assesses the effects of biochar addition on dry thermophilic AD at a semi-pilot scale, where similar scientific papers were mostly conducted at mesophilic, wet small-scale conditions (e.g. syringe reactors or serum bottles). Further, the dry AD tests were performed on biochar produced as waste by an industrial full-scale waste-to-energy plant, while most literature studies investigate lab-made biochar or commercial products purposely manufactured as products to act as adsorbents or soil improvers. Finally, it addresses the issue of final environmental quality of biochar-enriched digestates, which is currently scarcely reported in available literature.

The collected data and findings were discussed, and the main considerations were published in a scientific paper:

- Bona, D., Beggio, G., Weil, T., Scholz, M., Bertolini, S., Grandi, L., Baratieri, M., Schievano, A., Silvestri, S., Pivato, A., 2020. Effects of woody biochar on dry thermophilic anaerobic digestion of organic fraction of municipal solid waste. J. Environ. Manage. 267. https://doi.org/10.1016/j.jenvman.2020.110633

Step 3. Conventional post treatment

A second experimental activity consisted on studying the efficiency of a conventional digestate post-processing technology, i.e., chemically enhanced solid-liquid separation (CES).

This latter is commonly applied as a pretreatment to achieve easier handling and transport of separated solid fraction and improve further membrane filterability, implemented towards the complete purification of the liquid fraction. This latter will allow direct discharge or in situ reuse according to a zero-water discharge concept applied on biogas plant design.

So far, the application of coagulant and flocculant products demonstrated to improve the pretreatment performance of solid-liquid separation alone (e.g., through centrifugation) at low operational and investment costs, at least for digestates from manures and agricultural substrates. However, the reported positive results cannot be transferred directly to digestates derived from AD of OFMSW. Further, the environmental quality of resulting conditioned fractions should be investigated with respect to related modifications of chemical content and related possible increase of ecotoxicity, to be assessed according to the expected agricultural reuse.

Here, the research was supported by a private company dealing with chemicals manufacturing for wastewater treatment sector, interested in expanding its business on digestate treatment. A particular focus was given on 3 coagulants products, polyaluminum chloride, epichlorohydrin-dimethylamine with ethylenediamine, and a mixture of them. Coagulants action was integrated with 2 flocculant products, a cationic and an anionic polyacrylamide. The tested chemicals are sold for the industrial and civil wastewater treatment sector, thus not currently available for digestate treatment.

A total of 16 different CES treatments were performed, according to different combination and increased dosages of selected chemicals. CES trials were conducted by using Jar Test apparatus and further lab-scale centrifugation.

The derived liquid fractions were characterized by resulting Total Suspended Solids (TSS) content, which allowed to calculate the achieved TSS removal improvement (%) with respect to a control treatment (i.e., without chemicals application). The liquors from the 3 best performing CES treatments were characterized with the control for pH, TS, EC, TKN, TAN, P, Cl and HMs (Cd, Pb, Cu, Hg, Ni, Zn, Cr). A particular focus was given on the conditioned solid fractions derived from the same treatments, which were fully characterized for fertilizing properties TS, VS, TOC, TKN, TAN, Aluminum content and HMs (Cd, Pb, Cu, Hg, Ni, Zn, Cr). Also, achieved leachability of the same nutrients and contaminants, was determined in solid fractions eluates derived from conventional leaching tests, to assess fertilizing potential and estimate long-term environmental effects. Finally, phytotoxicity testing was perfomed on the same water extracts, through Germination Index bioassay on seeds of *Lepidium Sativum*.

Lab-scale conditioning and centrifugation tests allowed to evaluate the efficiency of selected coagulant and flocculant products in terms of suspended solid mitigation in the treated digestate liquid fractions. Results showed that the applied CES trials allowed to increase removal of suspended solids of liquid fraction up to 90% if compared with untreated digestate. In particular, the highest dosages of polyaluminum chloride (3.2 g/L) and epichlorohydrin-dimethylamine (42 g/L), resulted the best performing treatments. All investigated liquid fractions showed the occurred P and HMs removal due to the addition of conditioning products, with possible consequences on the quality of solid fractions, where these elements likely migrated.

However, no significative variations can be noted in the treated solid fractions in terms of P and HMs content, leading to a general compliance of the treated solid fractions with the requirements established in the regulation for agricultural reuse for solid organic fertilizers. Besides, aluminum concentration, which is not regulated, and organic nitrogen content increased remarkably in the treated solid fractions likely due to the residual byproducts of dosed chemicals. The characterization of related water extracts showed higher contents of soluble Al and chlorides together with lower leachability of HMs and P, this latter likely determined by occurred chemically driven metallic complexation. Here, the reported high contents of soluble Al of the treated eluates could have determined a direct phytotoxic action on the root growth of the cress seeds tested through germination index bioassay. Also, the resulting reported higher salinity, indirectly due to both soluble Al and Cl^-, could have also played a major role in the measured higher plant toxicity of treated fractions. This aspect, together with the suggested lower fertilizing potential could discourage the further agricultural land spreading of conditioned solid fractions.

Under a circular economy perspective, these considerations allow to understand whether the application of CES can help or not to achieve the requirements established by the planned reuse/disposal scenario of the resulting digestate fractions.

These considerations were included in the following paper:

- Beggio, G., Cerasaro, A., Bonato, T., Pivato, A. Chemically enhanced solid-liquid separation of OFMSW digestate: suspended solids removal and effects on environmental quality of separated fractions. Submitted to Journal of Water Process Engineering (07/12/2020).

Step 4. Health risk assessment of digestate application on agricultural soils

Beside the compliance with quality requirements established for digestate agricultural use, several biogas plant operators expressed concern about possible non-conformity situations arising from the application of the law framework protecting the agricultural soil quality when applied on cultivated fields undergone digestate application (i.e., Italian law DM 46/2019). In particular, whether risks to ecosystems or human receptors are proved, criminal conviction for site contamination can be issued and expensive mandatory remediation can be prescribed, ultimately undermining the possible agricultural reuse of digestate.

For this reason, the fourth step of the PhD research project consisted in the performance of a human health risk assessment procedure performed on an agricultural site in North-East Italy assumed to be fertilized with digestate. The approach followed by the assessment was fully consistent with the procedural requirements established by the Italian Regulation and with internationally acknowledged exposure databases.

In practice, it focused on the estimation of the risk related to human intake of carcinogenic and non-carcinogenic Heavy Metals (Cr, Pb, Cd, Ni, Cu, Hg, Zn) present in the crops grown on the agricultural site, after digestate land spreading. Digestate application rate and data for quality characterization of considered digestate types (i.e., deriving from AD of OFMSW and agricultural residues) were determined from the first step of the PhD. Further, location-specific soil background contents were taken from the estimations derived by the regional environmental agency. Two spreading techniques were considered to determine the resulting soil concentrations, i.e., splash-plate spreading and sub-surface application. The US Environmental Protection Agency (USEPA) approach was selected to conduct the assessment and the so called "Baes model" was implemented to simulate the soil-to-plant transfer, based on the contaminant-specific soil-water partition coefficient K_d. Data for contaminants toxicity (oral Reference Doses for non-carcinogenic and oral Slope Factors for carcinogenic contaminants) and exposure assessment were collected from internationally acknowledged risk databases. Finally carcinogenic and non-carcinogenic risk values were calculated for both child and adult receptors.

The outcomes were used to discuss the compliance of identified digestate quality with the Italian regulation on contaminated agricultural soils and how the model's assumptions, made according to the regulation requirements, can influence the reliability of the resulting risk values.

While no risks were estimated related with scenarios concerning digestate application, the performed assessment emphasized that possible situation of concerns can derive by assuming the total background concentrations of considered HMs as available for the soil-plant transfer. In fact, significative risk for both adult and child receptors were determined by the contribution given by the sole soil background concentration value, evidently disagreeing with its definition. This issue could be tackled by assuming a more realistic value to be attributed to the bioavailable fraction of the considered contaminants.

Finally, the study results could also help to draw the attention of the public opinion and lawmakers on the impacts potentially related with performed digestate spreading techniques, i.e. also on the "use" of digestates, instead of focusing exclusively on its environmental quality.

The following paper originated the above described activity:

- Beggio, G., Bonato, T., Schievano, A., Garbo, F., Pivato, A. Agricultural application of digestates derived from agricultural and municipal organic waste: a health risk-assessment for heavy metals. Submitted to Human and Ecological Risk Assessment: An International Journal (30/01/2021).

3. Conclusions and perspectives

In this thesis, innovative and conventional digestate treatment technologies were investigated in terms of resulting efficiency and influence on the achieved environmental quality of digestates originated from the AD of separately collected OFMSW, with a view to agricultural reuse. In particular, the environmental quality of treated digested residues was evaluated according to the parameters established by the relative international and national regulations, but also beyond them, i.e., by integrating conventional characterization with phytotoxicity assessment and risk estimation.

The environmental quality was first defined for two typologies of digestates, namely residues from the AD of agricultural feedstocks and separately collected organic fraction of municipal solid waste (OFMSW), according to the parameters established both in regulations and scientific literature. Average quality profiles for both categories could be estimated based on characterization data from published literature and provided by private biogas plant operators. The two considered digestate types were found significantly different only for a limited set of considered parameters (i.e., OFMSW digestates characterized by lower VS and higher content of some regulated HMs). However, considering each digestate category, all investigated parameters were characterized by similar high degree of variability, confirming the role of the wide range of input feedstocks and AD plant configurations in the determination of digestate quality in general. Besides, both categories were found generically compliant with the established regulation limits for agricultural reuse. This suggested a lack of statistical ratio behind the a priori exclusion of OFMSW digestates from direct land-spreading, as established by the Italian regulation, forcing OFMSW digestates to undergo post-composting to just reach an "End-of-waste" status. Instead, treatments should be applied to decrease plant operational costs while reaching specific digestate quality for the intended management scenario.

An innovative in-situ treatment (i.e., applied directly in the AD reactor) and a conventional post-processing technology were investigated through dedicated experimental activities.

Results from innovative woody biochar application on dry thermophilic anaerobic digestion showed possible efficiency improvement of the sole process stability through the increase in microbial diversity measured in the digestates, leaving methane production almost unaffected. These trends were justified according to the analyzed features of tested biochar, characterized by small particle size and poor adsorption potential, and to the tested dry AD configuration, which could have hindered the interactions of the feedstock-biomass-biochar system. Resulting biochar addition lowered HMs content but increased remarkably the PAHs content of biochar-enriched digestate, possibly triggering noncompliance with regulation for agricultural reuse and consequent need to dispose it as a waste. These results were challenged by the performed plant bioassays, showing decreased phytotoxicity related with biochar-enriched digestates, likely due to contaminant-binding capacity and the non-bioavailable nature of the PAH content of biochar.

The lab-scale application on OFMSW digestate of a conventional Chemically Enhanced solid-liquid Separation (CES) proved to increase centrifugation efficiency in terms of suspended solids removal from the separated liquid fractions, thus suggesting its improved membrane filterability and potential decrease of plant operational costs in full scale applications. In particular, high dosages of polyaluminum chloride and epichlorohydrin-dimethylamine determined suspended solids mitigation up to 90% if compared to untreated liquid fraction.

The resulting conditioned solid fractions were found all compliant with the legal framework regulating digestate agricultural reuse. Nevertheless, according to the outcomes from their characterization, their further agricultural reuse was not suggested. In fact, lower fertilizing potential was noted, due to decreased leachability of P and useful plant micronutrients (i.e., Cu and Zn) related with the decreased solubility of this element caused by chemical fixation. Together, remarkably higher contents of non-regulated Al, chlorides, and organic N were measured, probably derived from residual byproducts of tested conditioning products. These figures could have been the responsible of the increased digestate phytotoxicity, likely determined by the direct effects on root growth acted by the increased soluble Al contents and, indirectly, by the resulting high levels of salinity found in the eluates from treated solid fractions.

Finally, the outcomes of a health risk assessment considering indirect exposure to HMs in vegetables grown on an agricultural site, pointed out that no situations of concern could be related with fertilization with digestates characterized by the average quality profiles estimated during this 3-years investigation. However, further studies on the real bioavailability of contaminants (both originated from digestate application or soil background content) will improve the reliability of risk assessment estimations. Also, collected results could help to draw the public attention (i.e., law-makers and controllers) also on the performed agricultural practice behind digestate utilization, other than focusing on the sole achievable digestate quality.

The results, taken together, provided a rigorous scientific grounding on which base the choices regarding digestate management options.

As future developments, the assessment methodology for different treatment options should be integrated by investigating parameters not regulated and not covered by this study. An important role can be played by additional ecotoxicity assessment performed on different organisms (both from the aquatic and soil compartments). Here, the challenge is to develop methods and thresholds capable of being conventional and thus potentially adopted transparently within the EU legislation. Currently, there is no consensus within the scientific community about ecotoxicity testing of organic substrates intended to be used as fertilizers, such as digestates. The bigger challenges include both the adoption of suitable test methods (i.e., which test battery to be performed) and the proposal of reliable limits (i.e., in terms of EC50). These aspects should be established as consistently as possible with other fertilizers and soil amendments (such as inorganic fertilizers, limestone, compost, etc.) and considering the specific features of digestate itself. In this context, these findings will serve as a rigorous background to develop more comprehensive and sustainable criteria for field application rates.

PART II

List of Publications

Paper I

Beggio, G., Schievano, A., Bonato, T., Hennebert, P., Pivato, A., 2019. Statistical analysis for the quality assessment of digestates from separately collected organic fraction of municipal solid waste (OFMSW) and agro-industrial feedstock. Should input feedstock to anaerobic digestion determine the legal status of digestate? Waste Manag. 87, 546–558. https://doi.org/10.1016/j.wasman.2019.02.040

Waste Management 87 (2019) 546–558

Contents lists available at ScienceDirect

Waste Management

journal homepage: www.elsevier.com/locate/wasman

Statistical analysis for the quality assessment of digestates from separately collected organic fraction of municipal solid waste (OFMSW) and agro-industrial feedstock. Should input feedstock to anaerobic digestion determine the legal status of digestate?

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ARTICLE INFO

Article history:
Received 2 July 2018
Revised 19 February 2019
Accepted 20 February 2019

Keywords:
Circular economy
Biogas plant management
Digestate management
OFMSW
Agricultural waste

ABSTRACT

Management options for digestate produced by anaerobic digestion plants influence the environmental and economic sustainability of the biogas sector. Further, digestate can be both used or disposed of according to its legal classification: that is, waste or by-product, or product (by using End of Waste procedure). Currently, legal digestate status is decided by EU member states on a case-by-case basis, according to specific positive lists of input feedstocks and quality requirements in terms of physical properties and chemical concentrations. Biased exclusion of input feedstock can force digestate to a specific waste classification and undergo post-treatment and disposal options that can negatively affect the profitability of biogas installations. This is the case of the Italian regulation, where the positive list of input feedstock excludes a priori separately collected organic fractions of municipal solid waste (OFMSW), while including agro-industrial residues (AGRO). This study determined the differences between the two digestate typologies (OFMSW versus AGRO) through statistical analysis, implemented on a dataset, designed to gather data about digestate's physical-chemical parameters from relevant scientific literature and unpublished private databases. The datasets consisted of 190 entries, derived from more than 2,000 samples. Further, the study provided a compliance assessment between the resulting parameter means and the current regulation limits. Upper confidence limits for the means (level of significance $\alpha = 0.05$) calculated for both digestate typologies were found to be compliant with the legal requirements. Therefore, no statistical ratio seems to support the difference in the legislative approach as proposed by Italian lawmakers. OFMSW resulted significantly different from AGRO for VS (650.1 g/kg TS vs. 843.8 g/kg TS, respectively), N-NH4 (81.9 g/kg TS vs. 46.19 g/kg TS), N-TOT (109.7 g/kg TS vs. 65.32 g/kg TS), P-TOT (7.22 g/kg TS vs. 21.9 g/kg TS), Pb (18.6 mg/kg TS vs. 4.66 mg/kg TS), Ni (11.03 mg/kg TS vs. 8.20 mg/kg TS), Cr-TOT (12.74 mg/kg TS vs. 8.74 mg/kg TS) and Hg (0.08 mg/kg TS vs. 0.05 mg/kg TS). However, the statistical analysis must be implemented on a wider set of parameters not covered by this study (e.g. ecotoxicological features).

1. Introduction

In Europe, the number of biogas plants doubled between 2009 and 2016, reaching 17,662 installations in 2016 (EBA, 2017). As a result, in 2015, the biogas sector contributed with 5.5 Mtoe (megaton of oil equivalent) to the European electricity production, a share of 7% of the total renewable electricity production (European Commission, 2017). Alongside bioenergy, the European biogas sector produces yearly about $56 * 10^6$ tons of digested residues, usually defined as digestate, from the anaerobic treatment of biodegradable waste (i.e. excluding wastewater sludge treatment) (Saveyn and Eder, 2014). Digestate management options thus represent a key determinant of the decision to invest in the construction of anaerobic plants. Therefore, it must be properly considered by plant operators and policy-makers.

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https://doi.org/10.1016/j.wasman.2019.02.040
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From the circular economy perspective, the digestate content of nutrients and stable organic carbon potentially represent valuable resources to be recovered. Currently, the majority of biogas plant operators focus on the profits related to financial subsidies from electricity or biomethane production, while considering digestate as waste and neglecting the market opportunities related to its re-use (Dahlin et al., 2015). Collecting revenues from digestate would be recommended to allow anaerobic plants to be long-term profitable within a no-subsidies framework as well (Gebrezgabher et al., 2010). This approach has been implemented by the European Commission, which ultimately established the principles for sustainable digestate management within the Circular Economy Package, thus defining the use of digestate as organic fertilizer as a major opportunity to realize sustainable development compliant with the model of the circular economy (European Commission, 2016).

Several authors demonstrated the efficiency of digestate land spreading to substituting mineral fertilizers and/or acting as soil amendment (Lukehurst et al., 2010; Tambone et al., 2010, 2009). Conversely, other studies reported potential biological negative effects (e.g. soil-plant toxicity), when digestate is applied using inadequate agricultural practices (e.g. inefficient splash plate application, leading to uneven digestate distribution and uncontrolled N application rate) and without a proper quality assessment (Abdullahi et al., 2008; Alburquerque et al., 2012; Tigini et al., 2016).

The non-agricultural applications of digestate involve several back to earth alternatives (BEAs), including soil remediation, biochar production, landfill cover, and landscape restoration, as recently introduced by Peng and Pivato (2017). The marketing of digestate to third parties from the agricultural and non-agricultural sectors is also a favourable option (Dahlin et al., 2015).

Digestate management and market demand depend on the digestate's legal status as waste or by-product. In the EU, member states can decide for case-by-case End of Waste criteria of digestate to be implemented into national regulations (Fachverband Biogas et al., 2013) according to the general requirements established by the Waste Framework Directive (European Parliament and European Council, 2008). National regulatory approaches framing digestate use establish End of Waste criteria by defining both **quality standards** of digestate characteristics (i.e. pH, TS, VS, nutrients, Persistent Organic Pollutants and Heavy Metals concentrations, physical impurities, hygiene status, biological stability, phytotoxicity) and **positive lists of input feedstocks** (Mannelli et al., 2016). Where already established, End of Waste criteria are solely set for direct application on agricultural land and no other management options (e.g. BEAs).

In this context, defining digestate quality and the role of the positive lists of input feedstock is crucial to ensuring the economic viability and environmental safety of digestate use. Al Seadi and Lukehurst (2012) listed the essential features for defining digestate quality: nutrient content, pH, dry matter and organic dry matter content, unwanted physical and chemical impurities content (i.e. plastic and glass particles, heavy metals, and persistent organic pollutants), and hygiene status (e.g. presence of Salmonella and/or Coliforms). Further, Teglia et al.(2011) included biological stability and phytotoxicity as important aspects to defining digestate quality.

Positive lists of input feedstock are used to control the influence of Anaerobic Digestion (AD) substrates on the variability of digestate characteristics (Al Seadi and Lukehurst, 2012; Nkoa, 2014; Tambone et al., 2010; Teglia et al., 2011). AD plants design is considered mature enough to allow the treatment of nearly all types of biodegradable feedstock, ranging from animal by-products and agro-food residues (Holm-Nielsen et al., 2009) to organic fraction of municipal solid waste (OFMSW) (Mata-Alvarez et al., 2000). Its potential is supported by the European Waste Catalogue and the Annex III of the Waste Framework Directive, which comprehensively list the biodegradable waste that can undergo anaerobic treatment, including animal manure, crops, organic residues from food-processing industries, animal by-products (as defined and regulated by the Animal By-Products Regulation), and OFMSW (European Commission, 2000; European Parliament and European Council, 2009, 2008).

According to the review of parameters (and their specific limit values) and the positive list of input feedstock adopted by single Member States, the different regulatory frameworks in Europe are heterogeneous and rather inconsistent (Dahlin et al., 2015). Given this background, the European Commission announced the delivery of a regulation setting harmonized quality criteria that, if met, will allow digestate to be sold and used on the EU market as organic fertilizer under a CE certification (European Commission, 2016).

In this heterogeneous context, the presence of unrealistic quality standards and/or biased lists of input feedstocks may force digestate towards waste classification. This would result in further expensive legal procedures or mandatory post treatments, solely implemented to achieve legal product status for digestate. An example is the Italian regulation, where a positive list of input feedstocks allows the direct agricultural use of the derived digestate as fertilizer, excluding separately collected OFMSW and including animal manure, energy crops, and agro-food residues (Ministero delle Politiche Agricole, 2016). This limitation is not present within any other national regulatory scheme. UK (England, Wales and Northern Ireland) established End Of Waste Criteria for agricultural and land restoration use of digestates produced from a positive list of waste including source separated OFMSW (WRAP, 2014); Germany regulates land use of digestates derived from source separated OFMSW under the Ordinance on the Utilisation of Bio-wastes on Land used for Agricultural, Silvicultural and Horticultural Purposes (Federal Republic of Germany, 2013). Further, both Sweden and Germany provided voluntary quality certification for digestates, which can be applied also to OFMSW digestates (Siebert, 2007; Sverige Avfall, 2018, 2013). In this latter cases, the certified digestate maintains the legal status of waste but are exempt from major control management required by waste regulations (Saveyn and Eder, 2014). According to Italian Regulation, the digestate produced from separately collected OFMSW (alone or in a co-digestion regime) is classified as waste and must undergo a post-composting process to acquire legal End of Waste status, thus falling under the Fertilizers Regulation Regulating Composted Amendment (Italian Parliament, 2010).

This issue will play a role in increasing the importance of a future no-incentives framework, where AD plants treating agricultural feedstock could be converted to treat separately collected OFMSW to gain profit from gate fees.

From the analysis of the Italian law-making context, Mannelli et al. (2016) reported (at page 38) 'there was not a preliminary evaluation of the permitted materials before the compiling of the list', claiming the reason supporting the exclusion of separately collected OFMSW from the positive list is biased, since it is not supported by any scientific assessment. According to the recent review by Tampio et al.(2016), the literature focused on the agricultural land spreading of digestate from agricultural feedstock (i.e. manure, agro-food residues, and energy crops), while only a minor part deals with digestate originating from urban feedstock (e.g. OFMSW or sewage sludge). They concluded that the literature still lacks in studies highlighting the possible differences between the qualities of digestates originating from agricultural feedstock and separately collected OFMSW. This comparison would be beneficial to objectively clarify cases such as the Italian Regulation approach towards a smarter management of digestate.

This study assesses the possible differences between the digestate originating from agricultural feedstock and separately collected OFMSW in terms of parameters defining digestate quality. The comparison was provided by a statistical analysis on a dataset of 190 observations, obtained from the relevant literature, public institutional data, and private companies working in the agricultural, waste management, and anaerobic plants construction and management sector. As a secondary objective, the possible consistency between the results of the statistical analysis and the regulation limits established in the aforementioned Italian and European law frameworks, regulating the agricultural use of digestates, was assessed.

2. Materials and methods

2.1. Meta-analysis and dataset design

The data sources on digestate quality characterization were obtained from:

- unpublished monitoring data collected by private biogas plant operators;
- unpublished academic scientific databases;
- institutional studies and guidelines on the agricultural use of digestate (ARPAE, 2016; Rossi et al., 2017; Saveyn and Eder, 2014; WRAP, 2011);
- peer-reviewed articles from a meta-analysis of the relevant literature (Alburquerque et al., 2012; Chiew et al., 2015; Jensen et al., 2017; Knoop et al., 2018; Kupper et al., 2014; Peng and Pivato, 2017; Pivato et al., 2016; Schievano et al., 2008; Stoknes et al., 2016; Tambone et al., 2010, 2009, 2017; Tampio et al., 2016)

Two different datasets were built to fulfil the aim of this study. The first dataset, named AGRO, contains the quality features of digestates originating from AD of the so-called agro-industrial feedstocks listed in the Italian Regulation (Ministero delle Politiche Agricole, 2016), which can be applied on agricultural land as by-products (i.e. without post-treatment). The second dataset, named OFMSW, includes the quality features of digestates classified as waste by the Italian regulation, because of being derived from the anaerobic treatment of separately collected OFMSW alone or in a co-digestion regime with different agro-industrial feedstocks. No data characterizing digestates derived from AD of wastewater sludge were included in the datasets. Initially, both datasets included both unseparated digestate and its solid and liquid fractions from mechanical separation.

Data sources not clearly describing units and AD input feedstocks were excluded from the datasets. Only digestates originating from full-scale anaerobic plants were considered. The typology of the AD plant design of each data source was omitted (e.g. wet/semi-dry/dry regime, CSTR/PFR/batch reactors, single/two-stage configuration, meso/thermophilic thermic regime, organic loading rate), since this information was not always available. Each data source provided one or more dataset entries, according to the data-source-specific study design (e.g. assessing different substrates mixtures, plant configurations, or parameters in the same study). Further, some data sources provided only the arithmetic mean calculated on a specified number of samples for each analysed parameter, while some others also reported variability parameters (i.e. standard deviation and/or coefficient of variation). The two datasets consisted in 193 entries (160 for AGRO dataset and 33 for OFMSW) derived from more than 2,000 samples (919 for AGRO and 1397 for OFMSW dataset), referring either to unseparated, liquid or solid fractions of digestate.

Since variability parameters cannot be reported for all sources, every dataset entry included only the mean values of the investigated parameters calculated on the sample size involved in the respective data source.

2.2. Criteria for selecting comparison parameters

Given the above design assumptions, the datasets were intended to record the totality of available digestate quality data from each data source to allow a comparison based on the largest possible set of parameters. However, most data sources presented incomplete quality characterization: that is, assessing either amendment and fertilizing properties or innocuousness parameters. Further, the selected data sources provided an extremely heterogeneous set of data in terms of the studied parameters and units of measurement. Therefore, only a minor set of parameters, present in both datasets, could be exploited for dataset comparison.

The selected parameters were then organized in the following macro-categories, as suggested by Teglia et al.(2011) and Nkoa (2014):

- organic amendment properties: physical parameters assessing the potential of digestates to improve the physical characteristics of soils;
- fertilizing properties: macro and micro-nutrient content of digestates for assessing the potential of digestates to improve soil fertility and crop production yield; and
- environmental impacts properties: biological stability, physical and chemical contaminant concentrations and pathogen presence for assessing the potential risks on general ecosystems (soil, water, air) and receptors (human and non-human) derived by digestate use.

Data characterizing solid and liquid fractions of digestate were excluded from the comparison because of both the scarce number of available data sources and relative entries and their different intrinsic features compared to unseparated fractions of digestate. Including all fractions in the comparison could have increased the variability of the results to a biased degree, thus influencing the possibility of identifying significant differences between datasets. Therefore, the comparison involved only data concerning the non-separated fraction of digestates.

Table 1 lists, for both datasets, the selected parameters, related macro-categories and selected units of measurement. It is important to note that the comparison does not include hygiene features (e.g. Salmonella or Escherichia Coli possible presence), phytotoxicity and ecotoxicological parameters, and physical impurities content. In fact, these types of data were not present in significant number for the OFMSW dataset to allow the further statistical analysis and comparison (i.e. 1 data source for Salmonella presence and E.coli concentration and no data source for ecotoxicological parameters).

2.3. Data reporting

Where case studies reported parameters values under the quantification limit, these values were included in the datasets as equal to the quantification limits under a conservative assumption.

To allow the comparison between dataset entries, the values of the selected parameters were homogenized in terms of units of measurement.

Data referring to a fresh mass (FM) basis were converted to dry mass (DM) basis, where total solids (TS) content was provided. When the quality of whole fraction of digestate is assessed, the concentrations reported on a volumetric basis (e.g. mg/l) were con-

Table 1
Parameters selected for datasets comparison and Regulation limits used for comparison with statistical analysis results. AGRO = Digestate derived from agricultural feedstocks as listed in Ministero delle Politiche Agricole (2016). AGRO + OFMSW = Digestate derived from AD of agricultural feedstocks and separate collection of OFMSW.

Macrocategory	Parameter	Units	Limits	Application	Source regulation
Amendment properties	pH	–	–	–	–
	TS	g TS/kg FM	–	–	–
	VS	g/kg TS	≥200	AGRO	Ministero delle Politiche Agricole (2016)
	C-TOT	g/kg TS	–	–	–
Fertilizing properties	N-TOT	g/kg TS	≥15	AGRO	Ministero delle Politiche Agricole (2016)
	P-TOT	g/kg TS	≥4	AGRO	Ministero delle Politiche Agricole (2016)
	K-TOT	g/kg TS	–	–	–
	EC	mS/cm	–	–	–
Environmental Impact properties: Chemical contaminants	Cd	mg/kg TS	1.5	AGRO	Ministero delle Politiche Agricole (2016)
	Pb	mg/kg TS	140	AGRO	Ministero delle Politiche Agricole (2016)
	Cu	mg/kg TS	230	AGRO	Ministero delle Politiche Agricole (2016)
	Hg	mg/kg TS	1.5	AGRO	Ministero delle Politiche Agricole (2016)
	Ni	mg/kg TS	100	AGRO	Ministero delle Politiche Agricole (2016)
	Zn	mg/kg TS	600	AGRO	Ministero delle Politiche Agricole (2016)
	Cr-TOT	mg/kg TS	100	AGRO + OFMSW	Siebert (2007)
	PAH16	mg/kg TS	6	AGRO + OFMSW	European Commission (2016)
Environmental Impact properties: Biological Stability	VFA	mg/l	4000	AGRO + OFMSW	Siebert, 2007
	BMP	l Biogas/kg VS	450	AGRO + OFMSW	European Commission, 2016

verted to weight basis through the application of a density factor. Digestate density is comparable to manure with regard to its consistence and TS content range (Gerber and Schneider, 2015). Therefore, digestate density was calculated as an average between manure densities using the models of Chen and Hruska (1983) and Achkari-Begdouri and Goodrich (1992). Assuming an average TS concentration for the entire digestate of 10% (kg TS/kg FM) and a temperature of 293.15 K, the considered density was 1.037 kg FM/l FM.

Further, where the sources reported nutrient contents in mineral form equivalents (i.e. P_2O_5 or K_2O), nutrient concentrations were converted to total P and total K by applying specific molecular conversion factors, that is, 0.4364 and 0.8301, respectively. Finally, PAH_{10} concentrations were converted to PAH_{16} using a conversion factor of 1.284, as suggested by Saveyn and Eder (2014).

2.4. Statistical analysis

Statistical analysis and consequent graphical representation were performed using Minitab®18. Within the two datasets, data corresponding to each parameter selected for comparison represented single samples.

The normality of samples was verified by the Anderson Darling test (significance p-value < 0.05). Where not confirmed, normality was assumed according to significant sample sizes. The descriptive statistics of the parameters chosen for comparison (mean, standard deviation, coefficient of variance, minimum, maximum, median, and first and third quartile) was performed on each resulting sample, whose size was determined by the number of dataset entries. The resulting descriptive statistics were further summarized graphically through using box plots. For each dataset, the 95% confidence intervals were calculated for each parameter's mean (the pooled standard deviation is used to calculate the intervals). Finally, to compare the same parameter's means derived from the two datasets, hypothesis testing was performed with two-sample t-testing or one-way ANOVA to determine whether there is enough statistical evidence to claim the calculated parameter's means are equal at the population level.

2.5. Comparison with regulation limits

The results of the statistical analysis were compared with the regulation limits currently established by the Italian and European law frameworks. The chosen limits regulate the direct land application of digestate, considered not post-treated. Table 1 shows the used limits, source regulations, and analysed digestate typologies. As per Table 1, the Italian regulation on the agricultural land use of digestate does not cover all parameters available for comparison. Therefore, other significant legislative requirements were chosen from the available European legal frameworks regulating this field. However, the selected regulations do not refer to a specific digestate fractions (i.e. unseparated, liquid, or solid fractions).

3. Results and discussion

3.1. Organic amendment properties: pH, total solids, volatile solids, total carbon

Table 2 and Fig. 1 present the outcomes of the statistical analysis on the chosen quality parameters included in the macrocategory of organic amendment properties.

Table 2
Results of statistical analysis on amendment properties of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset (OFMSW). IQR = Interquartile range (Q3-Q1), it describes the range of values covered by the 50% of collected values. CV = Coefficient of Variation (St.Dev/Mean). 1 Null Hypothesis (H0) = population means are equal; Alternative Hypothesis (H1): population means are not equal. H0 is rejected with P-values lower than confidence limit (α = 0.05).

Parameter	Dataset	Dataset entries	Samples	Mean	St.Dev	CV (%)	Min	Max	IQR	Confidence interval (95%)	p-value[1]
pH (–)	AGRO	11	159	7.72	0.25	3	7.3	8.2	0.3	7.41–8.03	0.916
	OFMSW	4	68	7.69	0.87	11	6.4	8.3	1.46	7.18–8.19	
TS (g TS/kg FM)	AGRO	137	501	127.1	86.6	68	18.8	440	119	112.3–141.9	0.126
	OFMSW	13	135	87.9	99.5	113	24	311	66	39.9 – 136.0	
VS (g/kg TS)	AGRO	122	427	843.8	90.9	11	620	980	170	827.6 – 860.0	0.000
	OFMSW	13	135	650.1	84.6	13	420	738	59	600.5–699.6	
C-tot (g/kg TS)	AGRO	6	92	383.1	42.5	11	302.6	421	56.3	339.4–426.3	0.345
	OFMSW	9	102	357.8	52.6	15	230	397	39.9	322.6–393.1	

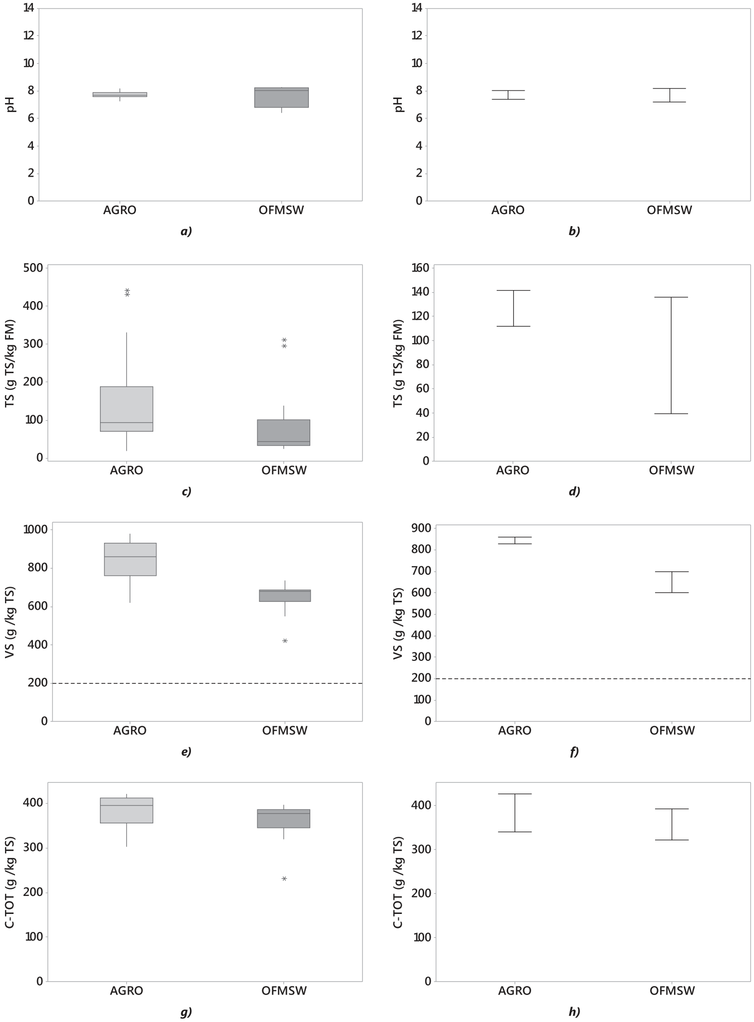


Fig. 1. Amendment properties of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset(OFMSW). On the left, Boxplots resume the results of descriptive statistics of the two datasets. The line within the box shows the median value, the box denotes the range of 50% of data, whiskers range from the lower to the higher value within 1.5 interquartile ranges and asterisks stand for outliers. On the right, the 95% confidence limits of the mean are presented for each parameter chosen for the comparison. Occurring dashed lines highlight the chosen Regulation Limit.

An important aspect of Table 2 is the different number of observations between the two datasets. In fact, the descriptive statistics of the AGRO dataset were calculated on a double, or even higher, number of entries than for the OFMSW dataset. This feature influences the different degrees of variability between datasets, where the OFMSW dataset shows a higher coefficient of variation (standard deviation relative to the mean). For total solids (Fig. 1a), the calculated standard deviation values of the AGRO and OFMSW datasets are 86.56 g TS/kg FM and 99.5 g TS/kg FM, respectively. Total carbon was the only exception, being characterized by a comparable number of observations (92 and 102, respectively) and, consequently, similar variability degrees (i.e. standard deviations of 42.5 g/kg TS and 52.6 g/kg TS). On the other hand, the aforementioned trend is not confirmed by the relative box plots (Fig. 1c, e, g), which generally show a higher variability degree for the AGRO dataset in terms of the interquartile range (IQR). For instance, the total solids in both the AGRO and OFMSW were characterized by IQRs of 119 and 66.3, respectively. The higher variability in the OFMSW dataset resulted in wider 95% confidence intervals compared with the AGRO dataset (see Fig. 1d). Again, for total solids, the confidence limits for the mean are 112.3–141.9 g TS/kg FM and 39.9–136 g TS/kg FM for the AGRO and OFMSW datasets, respectively. However, the mean pH, TS and TC of the two datasets are not different ($p > 0.05$).

The volatile solids (VS), considered as a measure of OM content, represents an exception (p-value = 0.000). According to the 95% confidence intervals, the means of the VS content for the OFMSW dataset is lower than for the same parameter from the AGRO dataset (i.e. 600.5–699.6 g/kg TS against 827.6–860 g/kg TS). However, OFMSW respects the considered minimum requirement for VS, thus it can be considered suitable for agricultural reuse (Fig. 1e and f).

3.2. Fertilizing properties: Ammonium, total nitrogen, total phosphorus, total potassium and conductivity

The descriptive statistics and results from the ANOVA on the parameters referring to the macro category fertilizing properties are presented in Table 3 and Fig. 2. The OFMSW dataset shows a mean for nitrogen content almost twice the size of the same parameter for the AGRO dataset, both in terms of ammonium (i.e. 81 g NH_4/kg TS and 109.7 g NH_4/kg TS, respectively for AGRO and OFMSW) and total nitrogen (i.e. 46.19 g N/kg TS and 65.32 g N/kg TS, respectively). This feature is confirmed by the calculated 95% confidence intervals (i.e. 33.26–58.97 g NH_4/kg TS for AGRO versus 59.77–104.03 g NH_4/kg TS for OFMSW, and 59.64–71.01 g N/kg TS versus 90.6–128.7 g N/kg TS). However, the fractions of ammonium content on the total nitrogen content are comparable, resulting in 70% (w/w) for the AGRO dataset and 74% for the OFMSW dataset. The ammonium content derived from the AGRO dataset is characterized by a higher variability than for the OFMSW dataset, reflecting the influence of the minimum and maximum values recorded for this dataset (i.e. 21.79 g NH_4/kg TS and 179.49 g NH_4/kg TS). This trend is not confirmed by the box plots depicted in Fig. 2a, where variability is not influenced by extreme values and the recorded maximum is considered an outlier, resulting in comparable IQRs between the two datasets. The ANOVA results demonstrate significant differences (p-value < 0.05) between the means of the ammonium content and total nitrogen, and admit a higher nitrogen content for digestates derived from the OFMSW dataset.

Moreover, according to Fig. 2c and d, both digestate types are consistent with the specific requirements for direct agricultural use. However, total nitrogen content alone cannot estimate the fertilization potential of digestate. In fact, soluble mineral nitrogen (ammonium) represents the potential content of nitrogen readily available for plant growth (Möller and Müller, 2012). By assuming a fixed nutrient necessity for crops, the claimed higher content of ammonium nitrogen would influence the doses of digestate from OFMSW to be applied on soil.

The AGRO dataset is characterized by a higher phosphorus content, with an arithmetic mean three times the mean calculated for the OFMSW dataset (21.9 g P/kg TS vs 7.22 g P/kg TS). This can be due to the significant presence of outliers recorded for the AGRO dataset (Fig. 2e and f). The ranges of the 95% confidence intervals of the mean P concentration are different, showing a range for AGRO digestates (19.56–24.24 g P/kg TS) narrower than the range calculated for OFMSW digestates. The calculated range for the OFMSW dataset is uncertain and unrealistic (i.e. −1.25–15.7 g P/kg TS), partly due to the small sample size and number of entries involved in the analysis of OFMSW. As for the nitrogen content, the two types of digestate can be considered statistically different in terms of phosphorus content, with the AGRO dataset being characterized by a higher concentration. Further, from Fig. 2e and f, while the AGRO dataset respects specific regulation requirements, while the calculated range for the mean of OFMSW dataset does not provide enough evidence to this end. However, due to the calculation process, the need to improve this analysis by assessing a larger number of observations is evident. These results suggest the use of post-treatment digestate from OFMSW aimed at increasing phosphorus concentration, such as solid/liquid separation (Möller and Müller, 2012; Tambone et al., 2017). Further, the plant availability of P content should be investigated through a speciation analysis able to determine soluble P fractions for both digestate typologies (Tampio et al., 2016).

The box plots in Fig. 2g show that the potassium content for the AGRO dataset is characterized by a higher variability compared to the K content of the OFMSW dataset, even if calculated on a comparable number of observations. This is also reflected by the higher value of the standard deviation and ranges of the 95% confidence intervals (27.6–68.7 g K/kg TS for the AGRO dataset versus 19.25–52.84 g K/kg TS for OFMSW digestates). Beyond the reported variability, the conducted *t*-test (p-value = 0.343) does not demonstrate the difference between the two types of digestates in terms of mean potassium content.

Finally, Table 3 demonstrate that AGRO dataset present (insignificantly different, $p > 0.05$) higher values of average EC than OFMSW digestates (17.75 mS/cm for AGRO vs. 6.54 mS/cm for OFMSW) (Fig. 2i, l). The high variability for both datasets results in large confidence limit for OFMSW (8.92–26.57 mS/cmfor AGRO and −3.33 – 16.40 mS/cm, for OFMSW). EC is acknowledged for being an indirect measurement of the salts content of a solution. Excess salinity characterizing undiluted digestates as fertilizers has been reported to be detrimental both for crops growth (Alburquerque et al., 2012; Möller and Müller, 2012) and terrestrial organisms (Pivato et al., 2016; Tigini et al., 2016). For these rea-

Table 3
Results of statistical analysis fertilizing properties of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset (OFMSW). IQR = Interquartile range (Q3-Q1), it describes the range of values covered by the 50% of collected values. CV = Coefficient of Variation (St.Dev/Mean). [1]Null Hypothesis (H0) = population means are equal. Alternative Hypothesis (H1): population means are not equal. H0 is rejected with P-values lower than confidence limit (α = 0.05).

Parameter	Dataset	Dataset entries	Samples	Mean	St.Dev	CV (%)	Min	Max	IQR	Confidence interval	p-value[1]
N-NH4 (g/kg TS)	AGRO	24	231	46.2	33.3	72%	21.8	179.5	28.8	33.26–58.97	0.008
	OFMSW	8	81	81.9	19.8	24%	40.4	99.4	26.4	59.77–104.03	
N-TOT (g/kg TS)	AGRO	135	337	65.3	31.5	48%	21.0	67.4	54.0	59.64–71.01	0.000
	OFMSW	12	115	109.7	51.4	47%	11.0	121.5	70.3	90.60–128.70	
P-TOT (g/kg TS)	AGRO	131	411	21.9	13.9	64%	2.3	76.3	12.0	19.56–24.24	0.001
	OFMSW	10	79	7.2	4.0	56%	2.8	15.3	6.8	−1.25–15.70	
K-TOT (g/kg TS)	AGRO	6	75	48.1	30.0	62%	15.6	102.6	39.1	27.60–68.70	0.343
	OFMSW	9	45	36.0	17.9	50%	9.7	59.0	32.9	19.25–52.84	
EC (mS/cm)	AGRO	5	41	17.75	10.36	58.40	4.36	30.30	19.45	8.92–26.57	0.085
	OFMSW	4	34	6.54	4.38	67.06	1.00	11.70	8.21	−3.33 to 16.40	

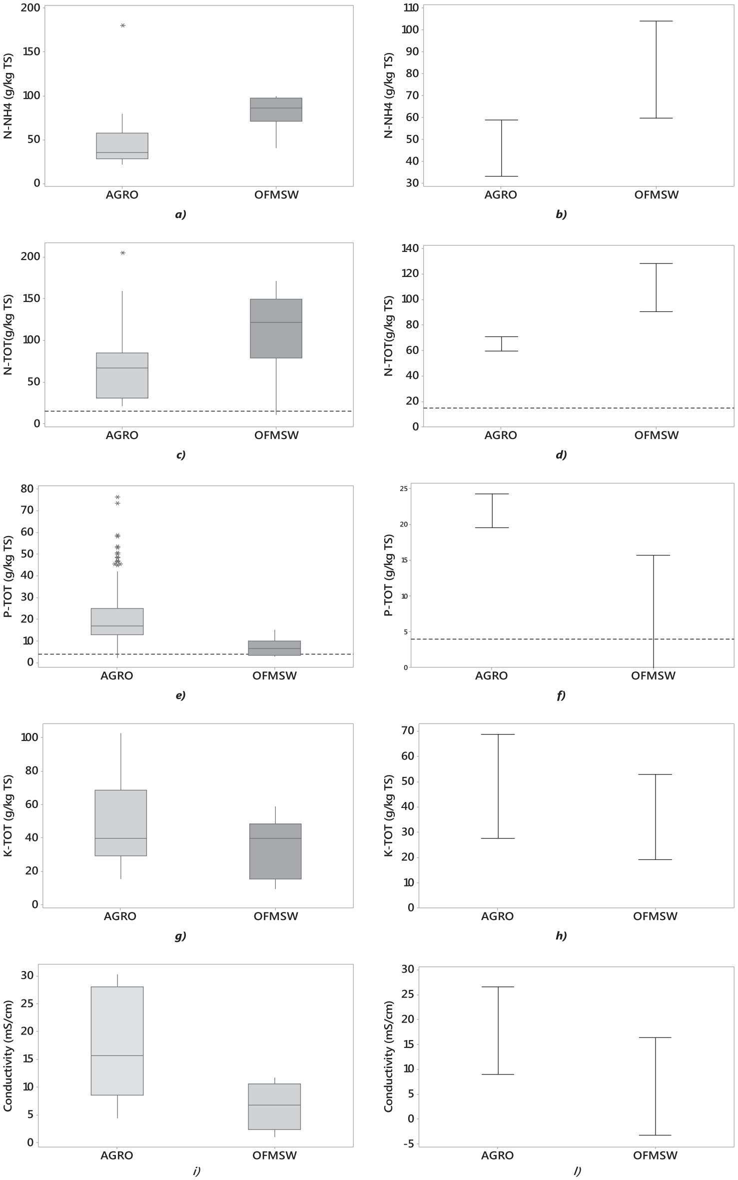


Fig. 2. Fertilizing properties of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset(OFMSW). On the left, Boxplots resume the results of descriptive statistics of the two datasets. The line within the box shows the median value, the box denotes the range of 50% of data, whiskers range from the lower to the higher value within 1.5 interquartile ranges and asterisks stand for outliers. On the right, the 95% confidence limits of the mean are presented for each parameter chosen for the comparison. Occurring dashed lines highlight the chosen Regulation Limit.

sons, EC of digestates is usually asked to be measured and declared, even if no maximum requirements are set by Regulations or certification schemes (Siebert, 2007; Sverige Avfall, 2013; WRAP, 2014).

3.3. Environmental impact properties

Table 4, Fig. 3, and Fig. 4 present descriptive statistics and results from ANOVA analysis on heavy metal and PAH16 contents of the two types of investigated digestates.

According to the descriptive statistics, the mean values obtained from the OFMSW dataset range from 0.08 mg/kg TS (Hg) to 232.6 mg/kg TS (Zn), while the concentration means from the AGRO dataset range from 0.05 mg/kg TS (Hg) to 280.1 mg/kg TS (Zn). The OFMSW dataset shows mean concentrations higher than the AGRO dataset by 45% for Cd, 400% for Pb, 60% for Hg, and 34% for Ni. This trend is confirmed by the calculated upper 95% confidence limits (UCL95). The calculated arithmetic means and UCL95s of the means for heavy metals from both datasets do not exceed legal threshold requirements. Interestingly, the resulting UCL95s of the means for OFMSW digestates were lower by 44% (Zn) to 94% (Hg) than the chosen legal limits for heavy metals, thus showing, on average, full consistency with regulations. From the box plots in Figs. 3a and 4c, it is possible to exceed the requirements due to outliers from the AGRO dataset. The degree of variability for Heavy Metals concentrations covers a wide range in terms of coefficient of variations: on average, the data from the OFMSW dataset show higher variability than in the AGRO dataset for Pb, Hg, and Cr-TOT.

ANOVA reports that only Pb, Hg, and Cr-TOT can be considered significantly different at the 95% confidence level. However, for Pb and Hg, these results should be carefully considered to avoid sampling bias. This could be due to almost all data in the AGRO dataset for Pb and Hg concentrations being recorded under the limit of quantification and thus considered equal to it (5 mg/kg TS for Pb and 0.05 mg/kg TS for Hg). Therefore, no degree of variation can be obtained. This is graphically notable in Fig. 3c and g, the box plots summarizing the data for the AGRO dataset Pb and Hg concentrations, which are reduced only to the resulting median value.

Only one observation for PAH16 concentration could be recorded in the AGRO dataset, that is, a single entry. Therefore, a standard deviation could not be calculated. However, comparatively, PAH16 mean concentrations show similar values for both datasets and, consequently, no significant difference between the PAH16 concentration means of the two types of digestate can be claimed from the performed *t*-test. Further, both UCL95s for the mean do not exceed the chosen legal threshold. As depicted in Fig. 4g and h, the 95% confidence intervals for the mean show unrealistic ranges for values due to small sample sizes for both datasets. For these reasons, the authors suggest using the above considerations with caution and to deepen the knowledge on the presence of PAH16 and persistent organic pollutants in digestates.

The biological stability features of the involved digestate typologies are displayed in Table 4 and Fig. 5. The volatile fatty acids (VFA, Fig. 5a) mean concentrations calculated from the OFMSW dataset are twice higher than the mean values of the AGRO dataset (6991 mg/l versus 2905 mg/l for OFMSW and AGRO, respectively). However, the OFMSW mean value could be strongly influenced by the involved smaller sample size and observation number and by the maximum value, which is two times higher than the correspondent value from the AGRO dataset (15,148 mg/l for OFMSW and 7,286 mg/l for AGRO). The variability of both datasets is high and comparable in terms of the coefficient of variability, while the OFMSW dataset is characterized by a higher variability in terms of IQR. However, the differences in the descriptive statistics involving VFA content cannot be considered significant at the 5% confidence level (p-value = 0.255). The same features can be deduced by statistical analysis on the residual methane potential (RMP). This is probably because, assuming the same biogas plant configuration, input feedstock to the AD characterizing the AGRO dataset is mainly composed by animal manure that can be considered already 'digested', thus generating more biologically stable digestate. However, it is significant that, while the arithmetic mean values and UCL95 for RMP from the two datasets are consistent with the considered limit, the UCL95s for the VFA concentration from both typologies are not respecting the chosen requirements. In this case, higher performance in organic matter degradation should be reached by modifying AD plant configuration options (e.g. higher residence time, increased thermic regime).

3.4. Overview on differences between AGRO and OFMSW digestates and on consistency with Regulation limits

According to the results, some general aspects can be identified. Almost all parameters analysed in this study and derived from both

Table 4

Results of Statistical Analysis on Environmental impacts parameters of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset(OFMSW). [1]Null Hypothesis (H0) = population means are equal. Alternative Hypothesis (H1): population means are not equal. H0 is rejected with P-values lower than confidence limit ($\alpha = 0.05$).

Parameter	Dataset	Dataset entries	Samples	Mean	St.Dev	CV (%)	Minimum	Maximum	IQR	Confidence interval (95%)	p-value[1]
Cd (mg/kg TS)	AGRO	129	316	0.40	0.47	117%	0.10	5.00	0.30	0.32–0.48	0.174
	OFMSW	14	465	0.58	0.51	87%	0.14	1.58	0.45	0.33–0.83	
Pb (mg/kg TS)	AGRO	128	256	4.66	1.23	26%	0.62	11.30	0.00	2.98–6.34	0.000
	OFMSW	13	464	18.6	32.5	175%	2.00	123.60	17.16	13.31–23.85	
Cu (mg/kg TS)	AGRO	131	318	62.2	38.6	62%	2.78	219.00	56.00	55.74–68.75	0.394
	OFMSW	14	465	53.2	26.4	50%	19.40	111.00	39.50	33.3–73.1	
Hg (mg/kg TS)	AGRO	113	177	0.05	0.005	9%	0.05	0.05	0.000	0.046–0.055	0.000
	OFMSW	12	443	0.08	0.073	92%	0.03	0.10	0.058	0.069–0.095	
Ni (mg/kg TS)	AGRO	129	316	8.20	8.01	98%	1.00	65.00	6.75	6.85–9.56	0.198
	OFMSW	14	465	11.03	4.74	43%	4.90	24.50	4.17	6.93–15.13	
Zn (mg/kg TS)	AGRO	131	318	280.00	204.00	73%	31.7	1780	238.0	246–314	0.394
	OFMSW	14	465	233.00	112.00	48%	87.4	427	204.2	128.3–336.9	
Cr-TOT (mg/kg TS)	AGRO	16	134	8.74	2.18	25%	5.41	12.51	3.33	6.62–10.86	0.013
	OFMSW	14	465	12.74	5.60	44%	6.81	26.60	9.25	10.48–15.01	
PAH16 (mg/kg TS)	AGRO	1	6	1.57	–	–	1.57	1.57	–	−3.40 to −6.54	0.776
	OFMSW	2	12	1.39	0.39	28%	1.12	1.67	–	−2.12–4.91	
VFA (mg/l)	AGRO	6	133	2905	2779	96%	672	7286	4,010	−1,593–7,403	0.255
	OFMSW	3	47	6991	7528	108%	311	15,148	14,837	630–13,352	
BMP (l Biogas/kg VS)	AGRO	3	39	161	51.5	32%	108	210.8	102.8	3.1–319.1	0.191
	OFMSW	7	65	278	133.8	48%	77	399	282	174.7–381.5	

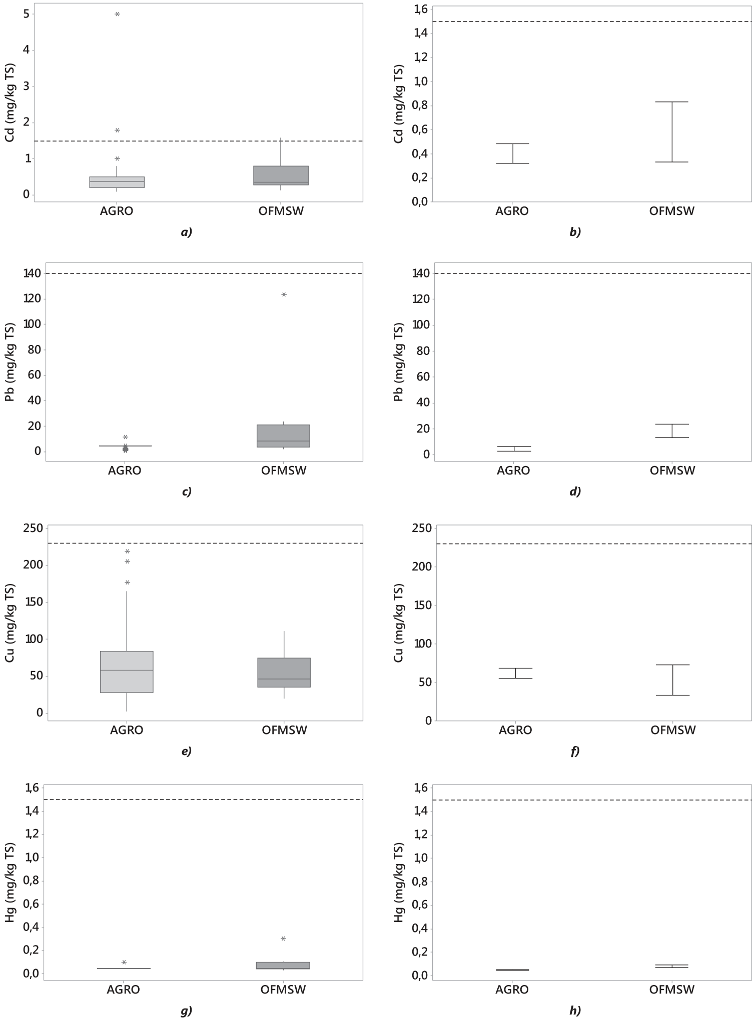


Fig. 3. Environmental Impacts (Chemical Contaminants) parameters of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset(OFMSW). On the left, Boxplots resume the results of descriptive statistics of the two datasets. On the left, Boxplots resume the results of descriptive statistics of the two datasets. The line within the box shows the median value, the box denotes the range of 50% of data, whiskers range from the lower to the higher value within 1.5 interquartile ranges and asterisks stand for outliers. On the right, the 95% confidence limits of the mean are presented for each parameter chosen for the comparison. Occurring dashed lines highlight the chosen Regulation Limit.

digestate typologies and selected for comparison cover a wide range of values. This is reflected in the coefficients of variability (which ranged from 9% to 175%), IQR, and width of calculated 95% confidence limits for the means. This could be in part due to the wide range of AD configurations applied on both input feedstock categories and considered by this study. Also, each feedstock category includes several subtypes of biodegradable materials, which are also subject to composition variability.

Beyond variability, the statistical analysis highlighted the parameters that can be considered significantly different between the two digestate typologies. The results are summarized in Table 5. Despite these significant differences, all derived UCL95s

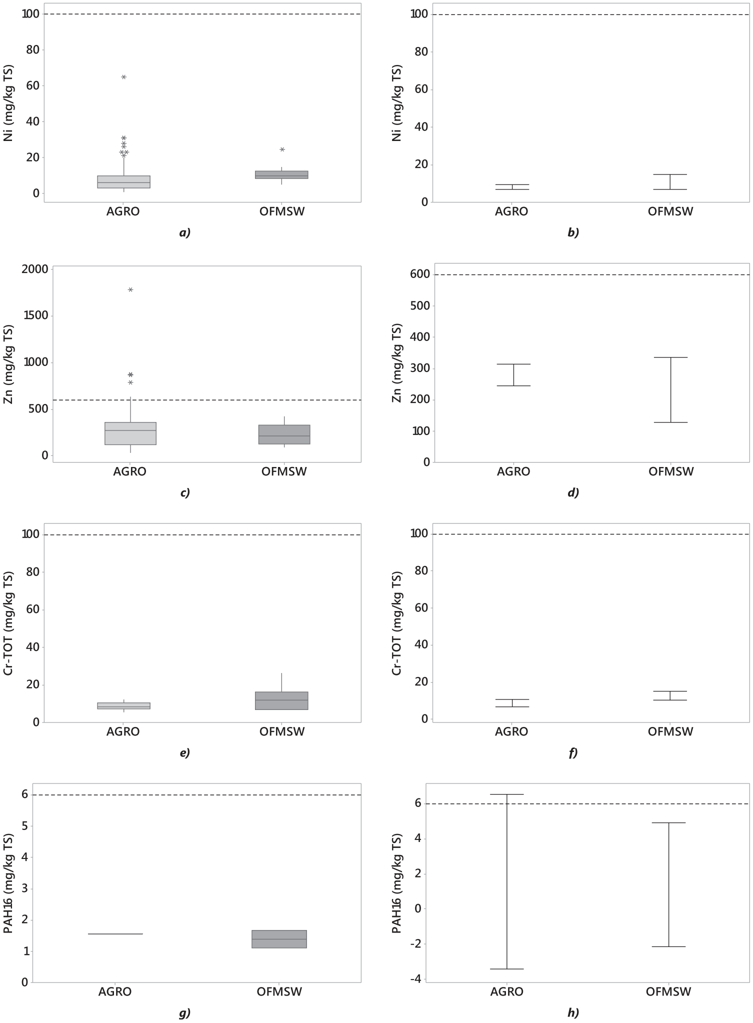
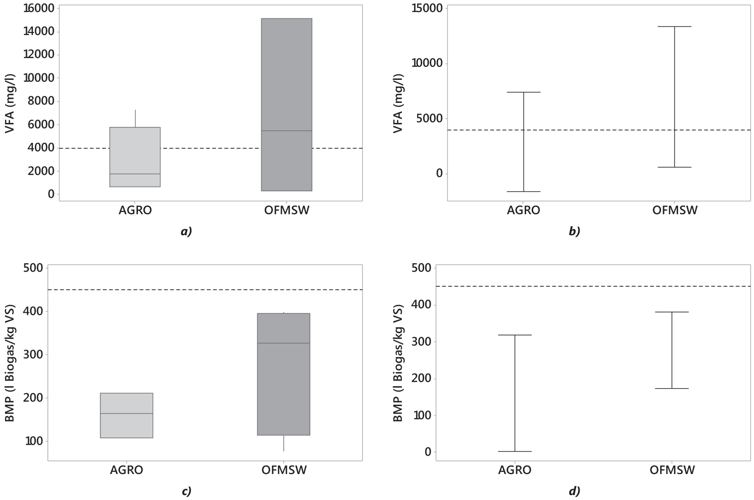


Fig. 4. Environmental Impacts (Chemical Contaminants) parameters of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset(OFMSW). On the left, Boxplots resume the results of descriptive statistics of the two datasets. On the left, Boxplots resume the results of descriptive statistics of the two datasets. The line within the box shows the median value, the box denotes the range of 50% of data, whiskers range from the lower to the higher value within 1.5 interquartile ranges and asterisks stand for outliers. On the right, the 95% confidence limits of the mean are presented for each parameter chosen for the comparison. Occurring dashed lines highlight the chosen Regulation Limit.

for both digestate typologies were consistent with Italian regulation limits, which do not allow the direct agricultural use (i.e. without mandatory post-treatments) of the category of digestates originating from the separate collection of OFMSW.

The VFA concentration represents the only exception, showing both UCL95s derived from the datasets above the reference limit (Siebert, 2007). However, the new upcoming Regulation on CE certified fertilizers (which considers digestates as organic fertilizers) would not include a limit for the VFA concentration, since the biological stability should be assessed according to RMP and oxygen uptake rate (OUR) (European Commission, 2016). On the other hand, the higher VFA concentration would probably lead to an uncompliant degree of biological stability, even if this parameter is not considered by the regulations. Further, requiring a low



a) *b)*

c) *d)*

Fig. 5. Environmental Impacts (Biological Stability) parameters of digestates derived from agro-industrial feedstocks dataset (AGRO) and from organic fraction of municipal solid waste dataset(OFMSW). On the left, Boxplots resume the results of descriptive statistics of the two datasets. On the left, Boxplots resume the results of descriptive statistics of the two datasets. The line within the box shows the median value, the box denotes the range of 50% of data, whiskers range from the lower to the higher value within 1.5 interquartile ranges and asterisks stand for outliers. On the right, the 95% confidence limits of the mean are presented for each parameter chosen for the comparison. Occurring dashed lines highlight the chosen Regulation Limit.

Table 5
Resume for significantly different parameters characterizing digestates from AGRO and OFMSW datasets.

Macrocategory	Significant difference ($\alpha = 0{,}05$)	Non significant difference ($\alpha = 0{,}05$)
Amendment properties	VS	pH, TS, C-TOT
Fertilizing properties	N-NH4, N-TOT, P-TOT	K-TOT, EC
Environmental impacts	Pb, Ni, Cr-TOT, Hg	Cd, Cu, Hg, Zn, PAH16, VFA, BMP

degree of biological stability (in terms of both RMP and OUR) can interfere with the minimum requirement of organic matter content (in terms of VS) of fertilizers.

These findings suggest that no statistical ratio supports the difference in the legislative approach as proposed by Italian law-makers, which consider mandatory to implement post-treatment to OFMSW digestate prior to land application in agricultural fields. As a consequence, Italian law-makers should acknowledge that post-treatment should be implemented as a tool to reach a certain degree of digestate quality when needed, instead of a compelling process to solely allow its legal reuse or disposal.

It should be further supported by a wider statistical analysis based on a comparison of parameters not covered by this study, such as hygiene/microbiological features, ecotoxicological assessment, and physical impurities.

The sanitary issue related to digestate land application is addressed by investigated regulations through requirements on either, or both, absence of Salmonella and limits concentration (i.e. 1000 CFU/g FM) of Coliforms (Saveyn and Eder, 2014; Siebert, 2007; Sverige Avfall, 2013; WRAP, 2014).

The non-applicability of Regulation requirements on OFMSW digestates caused a lack of hygiene data that can be provided by biogas plant operators. According to Italian Regulation, microbiological analyses are not mandatory for OFMSW digestates. Further, selected scientific papers do not include microbiological assessment within study aims. Only the report of WRAP (2011) provided findings detecting no Salmonella presence in 12 samples of OFMSW digestates and 1 positive sample on 6 of AGRO digestates. Further, data sources of AGRO dataset show an average presence of Salmonella in the 1% of analysed samples (ARPAE, 2016; Rossi et al., 2017).

Specific AD configurations proved to increase the hygienization of substrates by bacterial inactivation thanks to the influence of thermal regime and retention time (Al Seadi and Lukehurst, 2012). According to the scientific literature, the risk of sanitary issues of digestates are mainly related with a lack of quality control on AD input feedstock originated from manure and sewage sludge (Al Seadi and Lukehurst, 2012; Nkoa, 2014). Further, the possibility to detect pathogens on digestates are more related to bad sampling practices than to the type of feedstock (Rossi et al., 2017).

With this study, the authors emphasize the importance of setting unbiased scientifically-sound criteria regulating sustainable reuse of digestate. For this reason, regulation limit for digestate land application should be determined integrating the current substance-based approach with a matrix-based approach. This proposal follows the principle that establishing limits for quality assessment including ecotoxicological parameters helps determine more realistically the risk posed to ecosystems by complex and highly variable matrices such as digestates, thus promoting their sustainable reuse as by-products. In this context, the approach proposed by Hennebert (2018), who suggested a battery of six ecotoxicological tests and concentration limits to assess the possible ecotoxic property of waste, provides a good starting point. The six tests (or even other ones) should be performed with widely used (supposed non-ecotoxic) amendments and fertilizing products (e.g. compost from separately collected biowaste, agro-industrial digestate, limestone, animal manure uncomposted and composted, green manure, crop residues, cork, shredded wood, mulching material, and mineral fertilisers). For each test, a limit concentration should be determined from the highest inhibitory effect (lowest EC_{50}) recorded within all supposed non-ecotoxic tested products. Where a digestate sample tested for limit concentrations would present one or more EC_{50} lower than threshold EC_{50} of each of the six tests of non-ecotoxic products, it could be classified as more ecotoxic than the products. Further, so-determined concentrations can also serve as a base point for further development of sustainable dosage criteria (ideally, the application rates when used in the fields should be lower than the rate used in the tests producing 50% of detrimental biological effect, namely the EC_{50}).

4. Conclusions

Data were collected on a dataset from scientific literature and unpublished monitoring data provided by biogas plant operators. This study assessed some differences between the parameters characterizing the quality of the digestate originating from agricultural feedstocks and separately collected OFMSW. Further, the results of the statistical analysis derived from two datasets were compared regarding the limits regulating the agricultural use of digestates derived from agricultural feedstock (European Commission, 2016; Ministero delle Politiche Agricole, 2016).

All investigated parameters are characterized by a comparable degree of variability within the two categories. This could be in part due to the wide range of input feedstock involved in both digestate typologies and to the several AD plant configurations considered by this study.

Overall, digestate from separately collected OFMSW resulted significantly different from agro-industrial digestate (95% confidence) only for a limited number of investigated features, namely parameters describing amendment and fertilizing potential (lower VS, higher N-NH4, N-TOT, P-TOT) and environmental impacts properties (higher Pb, Ni, Cr-TOT and Hg concentrations).

Beside differences, no calculated 95% UCL from both typologies resulted non-compliant with chosen regulation limits, except for VFA concentration, which will not be included in the incoming EU Regulation. For these reasons, these findings do not support the current approach of Italian Regulation, which exclude OFMSW digestate from direct reuse on agricultural land forcing it to undercome mandatory post-composting. Instead, digestate treatment should be implemented to reach digestate quality criteria, defined for a sustainable reuse scenario.

These results should be further confirmed by statistical analysis on parameters non-investigated by this study (e.g. hygiene features) and by consistency assessment based on ecotoxicological thresholds to be derived following the methodology proposed by Hennebert (2018).

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Paper II

Bona, D., Beggio, G., Weil, T., Scholz, M., Bertolini, S., Grandi, L., Baratieri, M., Schievano, A., Silvestri, S., Pivato, A., 2020. Effects of woody biochar on dry thermophilic anaerobic digestion of organic fraction of municipal solid waste. J. Environ. Manage. 267. https://doi.org/10.1016/j.jenvman.2020.110633

Journal of Environmental Management 267 (2020) 110633

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: http://www.elsevier.com/locate/jenvman

Research article

Effects of woody biochar on dry thermophilic anaerobic digestion of organic fraction of municipal solid waste

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ABSTRACT

This study presents the results of semi-pilot scale anaerobic digestion tests conducted under dry thermophilic conditions with the addition of biochar (6% on fresh mass basis of inoculum), derived from an industrial gasification plant, for determining biogas and biomethane production from organic fraction of municipal solid waste. By using two types of inocula (from a full-scale dry anaerobic digestion plant and from lab-scale biomethanation tests), the obtained experimental results did not show significant increase in methane yield related to the presence of biochar (330.40 NL CH_4 $kgVS^{-1}$ using plant inoculum; 335.41 NL CH_4 $kgVS^{-1}$ using plant inoculum with biochar, 311.78 NL CH_4 $kgVS^{-1}$ using lab-inoculum and 366.43 NL CH_4 $kgVS^{-1}$ using lab-inoculum with biochar), but led to significant changes in the microbial community composition. These results are likely related with the specific biochar physical-chemical features and low adsorption potential.

Resulting digestate quality was also investigated: biochar-enriched digestates were characterized by increased biological stability (809 ± 264 mg O_2 $kgVS^{-1}$ h^{-1} vs. 554 ± 76 mg O_2 $kgVS^{-1}$ h^{-1} for biochar-free and biochar-enriched digestates, respectively), lower heavy metals concentrations (with the exception of Cd), but higher polycyclic aromatic hydrocarbons content, with a reported maximum concentration of 8.9 mgPAH $kgTS^{-1}$ for biochar-enriched digestate derived from AD test with lab-inoculum, which could trigger non-compliance with regulation limits for agricultural reuse of digestates. However, phytotoxicity assessments showed a decreased toxicity of biochar-containing digestates when compared to biochar-free digestates.

1. Introduction

Waste-to-energy (WtE) processes are capable of both reducing environmental impacts related to the waste management sector and improving energy and material recovery of waste materials, where reuse or recycling are not suitable (Mayer et al., 2019).

Among the involved processes, anaerobic digestion (AD) is considered to be a consolidated biological WtE technology for the treatment and recycling of biodegradable waste (Van Fan et al., 2018), yielding energy-intensive biogas and a nutrient and carbon-rich final product that can efficiently substitute mineral fertilizers (Lukehurst et al., 2010; Tambone et al., 2009, 2010). In different AD operating conditions, dry processes (i.e. characterized by a TS concentration higher than 20% on a weight basis inside the reactor vessels) are characterized by lower energy requirements for heating and stirring, a reduced need of digestate dewatering efforts and the provision of higher biogas production rates (Luning et al., 2003; Kothari et al., 2014; Cho et al., 2013).

Within a circular economy context, research activity in the field of AD is currently focusing on its possible integration with thermochemical WtE and biomass conversion processes, by investigating the utilization of derived biochar (Shen et al., 2016). The use of biochar could increase overall AD system efficiency in terms of process stability and biogas production (Fagbohungbe et al., 2017; Codignole Luz et al., 2018; Masebinu et al., 2019).

Currently, biochar utilization patterns include its reuse as an agricultural soil improver or as a tool for soil remediation, mainly exploiting its potential of acting as adsorbent material, which is determined by specific chemical-physical features, i.e. elemental composition, ion exchange capacity and surface properties of particles (Fagbohungbe et al., 2017; Masebinu et al., 2019). Biochar quality parameters, and thus adsorption potential, can vary according to input feedstocks and process operating conditions (Oliveira et al., 2017; Benedetti et al., 2018). Further, scientific literature reported the presence of inorganic and organic contaminants (Heavy Metals [HM] and polycyclic aromatic hydrocarbons [PAH]), which can trigger non-compliance with established Regulation limits for agricultural reuse (Oleszczuk et al., 2013;

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https://doi.org/10.1016/j.jenvman.2020.110633
Received 29 August 2019; Received in revised form 17 April 2020; Accepted 19 April 2020

Kołtowski and Oleszczuk, 2015; European Parliament and European Council, 2019). Therefore, the alternative use of biochar as an additive in AD processes can improve the sustainability of industrial gasification plants by reducing costs related to its disposal.

Biochar positive effects on an AD process were reported on (i) controlling substrate-induced inhibition both by limiting the availability of stressors through adsorption mechanism, e.g. of Total Ammonia Nitrogen (TAN) or by promoting direct interspecies electron transfer and on (ii) improving methanogenic bacteria acclimation through the colonization of biochar microporous structures, thus increasing methane yield (Mumme et al., 2014; Luo et al., 2015; Zhao et al., 2015; Fagbohungbe et al., 2017; Lü et al., 2016; Shen et al., 2017; Wang et al., 2020). Here, shifts in microbial community composition can lead to an improvement in the efficiency and stability of the AD process (Sun et al., 2019; Paritosh and Vivekanand, 2019).

However, cited scientific studies were conducted at mesophilic, wet and small lab-scale AD conditions (e.g. syringe reactors or serum bottles), using predominantly synthetic substrates or wastewater sludge. To the authors knowledge, only few papers discuss the occurred beneficial effects of adding biochar on dry thermophilic AD process efficiency (Jang et al., 2018; Paritosh and Vivekanand, 2019; Sun et al., 2019): all cited studies were conducted at small scale (i.e. small volumes glass bottles) by exploiting lab-made biochar (Jang et al., 2018; Sun et al., 2019) or a biochar product specifically manufactured to be sold as soil improver (Paritosh and Vivekanand, 2019). The effects on dry AD of waste biochars derived from full-scale WtE gasification plant still has to be investigated.

Finally, the investigation of final quality of biochar-enriched digestate, in terms of improved nutrients retention potential, achieved stability of organic matter and possible ecotoxicity due to the presence of contaminants (HMs, PAHs, etc.), has been scarcely reported, even if representing a key factor determining digestate agricultural reuse (European Parliament and European Council, 2019). In this context, phytotoxicity testing was already performed efficiently to assess how biochar can affect the toxicity of other organic matrices potentially suitable for agricultural reuse (Oleszczuk et al., 2012; Kołtowski and Oleszczuk, 2015; Ravindran et al., 2019).

To fill the aforementioned gaps, this paper presents the results of AD semi-pilot scale tests conducted under dry thermophilic conditions with the addition of biochar, derived from a full-scale industrial gasification plant, to the organic fraction of municipal solid waste (OFMSW). The choice of studied substrates was driven by their local availability and possible application scale-up. The objective of this study was to evaluate (i) the effects of biochar addition on AD process, in terms of methane production and microbial community composition, and (ii) its influence on the final quality of biochar-enriched digestate, both in terms of fertilizing features and phytotoxicity.

2. Materials and methods

2.1. Origin and properties of input feedstocks

Separately-collected Organic Fraction of Municipal Solid Waste (OFMSW) was sampled from a waste treatment plant located in Northern Italy, downward of the bag-opening and sieving treatment units. Collected samples were representative of the undersieve, constituting the input feedstock to a full-scale AD plug-flow reactor, operating in dry thermophilic conditions (≈20 %TS, 55 °C) with an HRT of approximately 21 days and treating about 33,000 ton/y of separately collected OFMSW and 11,000 ton/y of green waste. Due to seasonal variability in composition, OFMSW samples were collected once and stored at −25 °C until the commencement of specific lab-scale AD test replicates.

The biochar used for this study was the same woody pyrolytic biochar analyzed as "sample F′′" by previous research reported in Benedetti et al. (2018). Biochar was derived from the dual stage pyrolysis-gasification process of wood chip carried out in an industrial gasification plant in Northern Italy. The double-stage gasification plant operates according to an input flowrate of 140 kg/h of spruce woodchips, classified by ÖNORM M7133 with G30 and G50 size. The thermochemical conversion section is represented by a pyrolysis reactor followed by a floating bed gasifier, operating at 500 °C and 850 °C, respectively.

Different inoculums were tested during the performed experimental activity. Digestate, collected at the output of the aforementioned full-scale AD reactor, was used as "plant-made" inoculum. Two different plant-made digestates were used, with and without biochar addition. Furthermore, two "lab-made" inoculums were prepared. The first "lab-made" inoculum was sampled from digestate derived from lab-scale AD tests on OFMSW alone. A second biochar-enriched "lab-made" inoculum was obtained from the digested residues of an equivalent AD test on OFMSW with the addition of biochar. Detailed information on performed test configurations are described in the next paragraph. In accordance with UNI/TS, 2018, digestates used as inoculum were sampled before each AD test run and stored for a maximum of 24 h at 4 °C.

2.2. AD test system and experimental design

The batch tests performed were based on the well-established Biochemical Methane Potential (BMP) procedure (VDI, 2006).

Four different configurations were tested in triplicate, according to the different composition of input mixtures (Fig. 1):

- Test A: plant-inoculum + OFMSW;
- Test B: plant-inoculum + biochar (6% on fresh mass basis of the inoculum) + OFMSW;
- Test C: lab-made inoculum + OFMSW;
- Test D: lab-made biochar-enriched inoculum + biochar (calculated amount to reach a final biochar concentration of 6% on the total fresh mass basis of inoculum) + OFMSW.

Biochar dosage were chosen according to the findings of a previous study conducted by Mumme et al. (2014), which determined good results in AD process enhancement.

OFMSW was tested in Test A and Test B, respectively, after mixing with plant-inoculum. Test C and Test D were performed with the same feedstock configurations as Test A and Test B, respectively, but making use of different inoculums. For Test D configuration, lab-made biochar-enriched inoculum was obtained from digestate derived from a previous biomethanation test of sampled OFMSW with biochar addition (6% on the total fresh mass basis of inoculum). Test D configuration was performed in order to investigate the occurrence of a possible colonization of biochar by functional microorganisms together with the eventual beneficial effects on AD process. Equivalently, the lab-made inoculum for Test C consisted of digestate derived from a previous biomethanation of sampled OFMSW test but without biochar addition. In particular, the biochar dosage for Test D was adjusted to ensure 6% biochar concentration (on total fresh mass basis of inoculum) inside the test vessel, having considered the remaining biochar content in the lab-made biochar-enriched inoculum.

Tests were performed on semi-pilot reactors consisting of three 20 L air-tight sealed vessels, equipped with a thermostatic-system (i.e. a hot water driven heat exchanger operated by control devices) able to ensure established thermal conditions during the entire testing period and a gas analyzer for the continuous monitoring of produced biogas. Each AD batch tested a total amount of about 4 kg of fresh mass (FM) of total mixture (combined total of OFMSW and inoculum). Thermophilic conditions (i.e. 51-53 °C) were maintained throughout the entire testing time.

In accordance with VDI (2006), the mixtures loaded into each reactor were characterized by a substrate/inoculum ratio of 0.7 ± 0.06 on a VS basis. Testing time was set at 21 d, according to the designed HRT of the

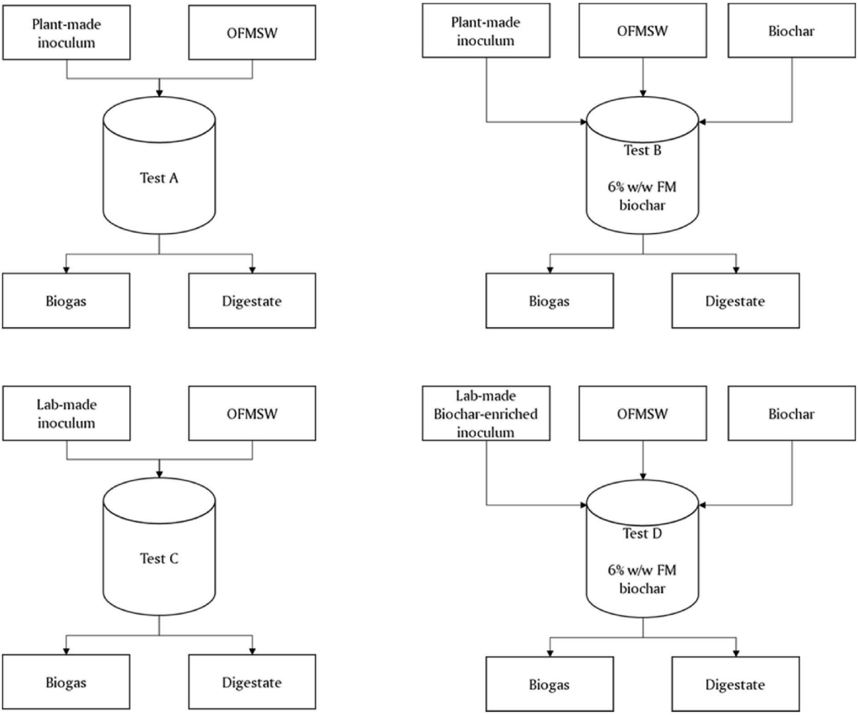

Fig. 1. Graphical representation of performed experimental design. Biochar concentrations are expressed as percentage of fresh mass of inoculum.

full-scale plant (where the used feedstocks originated) and after having verified that daily biogas production was lower than 1% of the ultimate biogas production.

2.3. *Analytical methods*

2.3.1. *TAN adsorption test*

Similarly to Takaya et al. (2016), batch adsorption tests were performed on biochar to investigate the ammonium adsorption potential of used biochar. 1 g/L of biochar was added to solutions characterized by varying TAN concentrations, ranging from 490 to 6720 mg NH4+/L, prepared from ammonium chloride (PanReach AppliChem) to mimic real-case digestate concentrations. A volume of 250 mL of prepared biochar-ammonium solution was poured into 500 mL plastic containers, tightly sealed and shaken at 30 rpm with an overhead mixer for 24 h at room temperature. A 50 mL test sample was collected after 24 h, filtered through 0.45 μm syringe filter and analyzed for TAN resulting concentration through UV spectrophotometry (IRSA-CNR, 2003).

TAN adsorbed concentration at equilibrium was calculated with the following eq. (1):

$$q_e = \frac{(C_0 - C_e)}{C_b} \tag{1}$$

Where q_e is the TAN sorbed in biochar at equilibrium (mg NH4+/g), C_0 and C_e are the starting and final TAN concentrations respectively (mg NH4+/L) and C_b is biochar concentration in solutions (i.e. 1 g/L).

Both Freundlich and Langmuir isotherm adsorption models were used to describe collected adsorption data and to investigate which one was characterized by better fit.

2.3.2. *Biogas measurements and modeling*

Specific volumetric biogas and methane production (NL kgVS^{-1}; NL CH_4 kgVS^{-1}, respectively) during each AD test replicate were quantified by using a manometric measurement system pre-installed with the reactor vessels. The percentage of methane content in biogas was quantified with an OPTIMA7 BIOGAS gas analyzer model after biogas sampling into 6 L Nalophan® bags.

Furthermore, biogas and methane production data, averaged between the performed test runs, were modeled using the modified-Gompertz equation

$$M = M_0 \cdot exp\left\{ - exp\left[\frac{R_{max} \cdot e}{M_0} \cdot (\lambda - t) + 1\right]\right\}$$

where M is the actual biogas/methane production (NL kgVS^{-1}), M_0 is the potential biogas/methane production (NL kgVS^{-1}), R_{max} is the maximum biogas/methane production rate (NL kgVS^{-1} d^{-1}) and λ is the lag phase time (d). Solver Add-in for Microsoft Excel 2016 was used to perform model regression.

2.3.3. *Chemical-physical characterization of input feedstocks, input mixtures and derived digestates*

Sampled and lab-made substrates (OFMSW, biochar, plant and “lab-made" inoculums), input mixtures and digestates derived from AD tests were characterized for TS, VS, pH and EC according to standard methods (UNI, 1998).

Table 1 illustrates data on the basic characterizations of input feedstocks used in the experimental phase. Elemental composition (C 91.5%, H 0.7%, N = 0.3%, O = 3.4%, Ash = 4.2%, on a fresh mass basis), surface area, pore volume, pore size and the morphology of used biochar were previously also investigated and can be found under sample F in Benedetti et al. (2018).

Total Volatile Fatty Acids (VFA) and Total Alkalinity (TA) were determined on input mixtures and resulting digestates using TIM 840 HACH-Lange tool. In addition, Heavy Metals (Cd, Pb, Cu, Hg, Ni, Zn and Cr) were measured once on composite solid samples obtained by mixing a fixed increment from the resulting digestates of the three AD test runs, according to (USEPA, 1994; 1996a, 2014). Finally, the biochar and digestate contents of PAHs were determined according to the appropriate method (UNI EN, 2018).

TAN and phosphate (PO_4^{3-}) concentrations were measured on digestate water extracts using HACK-Lange LCK 303 and LCK 049 kits. The total amount of TAN and phosphate, respectively, were measured by reading the absorbance at 690 nm and 435 nm with a DR3800 spectrophotometer (HachLange). Furthermore, TKN was assessed on the same digestate eluates (IRSA-CNR, 2003). Water extracts were derived from digestates by applying a Liquid-to-Solid ratio of 10 L/kgTS, according to the leaching test guideline EN 12457-2 (UNI EN, 2004).

Finally, dynamic respirometry (oxygen uptake measurement) was performed on digestates resulting from Test A and Test B to assess the effect of biochar addition on biological stability. Maximum dynamic respiration index (DRI_{max}, $mgO_2\ kgVS^{-1}\ h^{-1}$) was determined with an AIR-NL dynamic respirometer, through the prior mixing of analyzed digestates with shredded green waste acting as a bulking agent, according to a 1:1 ratio (on a fresh weight basis). Unfortunately, not enough fresh material from Test C and Test D was available for respirometric analysis.

2.3.4. *Microbial community analysis of derived digestates*

Genomic DNA of digested substrates was extracted from three biological replicates for each tested configuration using the PowerSoil DNA Isolation Kit (Qiagen). DNA quality was assessed by gel electrophoresis and UV–Vis spectroscopy.

Amplicon libraries of the variable V3-V4 region (approximately 460bp length) of the bacterial 16S rRNA gene were prepared using the KAPA HiFi HotStart ReadyMix (Roche Diagnostics). Bacterial primer set 341F (5' -CCTACGGGNGGCWGCAG 3-') and 806R (5' -GACTACNVGGGTWTCTAATCC 3-') was used with overhang Illumina adapters. PCR was performed at an initial denaturation temperature of 94 °C for 5min, followed by 22 cycles of 95 °C for 30sec, annealing at 55 °C for 30sec, elongation at 72 °C for 30sec. A final elongation step was run for 5min. Sequencing library preparation, quality control and quantification of pooled libraries and high throughput sequencing by Illumina technology were performed at the Sequencing Platform of the Edmund Mach Foundation (San Michele all'Adige, Italy).

Paired-end sequencing reads were quality filtered, trimmed, denoised, and merged using version 1.14.0 of DADA2 software (Benjamin et al., 2016). To reject low quality bases 14 bases were cropped from start. Forward reads were truncated to 285 bases and reverse reads were truncated to 250 bases. Taxonomy was assigned to all identified ribosomal sequence variants based on the SILVA rRNA reference database (version 132, Quast et al., 2013). Raw Illumina sequences are available at the European Nucleotide Archive (www.ebi.ac.uk/ena/) under the study accession number PRJEB37451.

2.3.5. *Phytotoxicity testing*

Water extracts of digestates were derived equivalently (UNI EN, 2004) and prepared starting from composite solid samples constituted by fixed aliquots of digestates derived from the three AD test runs (equivalently tested for phytotoxicity, according to APAT (2004), ISO (2005) and USEPA, (1996a, 1996b). Five mL of prepared water extracts, or a dilution with distilled water, were pipetted onto a plastic Petri dish and covered with filter paper. Ten seeds per Petri dish of monocotyledon cress (Lepidium sativum) were placed on filter paper and incubated for 72 h at 25 °C in dark conditions. After incubation time, the number of germinated seeds and root elongation were recorded for each Petri dish and combined to determine the specific Germination Index (GI%) (APAT, 2004). 6 dilutions (% vol/vol) and 4 replicates for each dilution were tested, including the control (i.e. 100% vol/vol distilled water).

2.3.6. *Statistical analysis*

The calculation of arithmetic mean values and standard deviations between replicates was performed using Microsoft Excel 2016. One-way ANOVA and multiple pairwise comparison with Tukey formulation (level of significance $\alpha = 0.05$) were tested to investigate significant differences possibly occurring between tested configurations in analyzed parameters from biogas measurements, and the chemical-physical characterization of digestates, with the exception of metals and PAHs concentrations. Inferential statistics were performed through STATISTICA 9.0 software provided by Statsoft, USA.

Logistic and linear-logistic models were used to extrapolate median effect concentrations (EC50) from tested digestates, which respectively showed sigmoidal-shaped or hormetic dose-response curves (Da Ros et al., 2018). Standard deviation (S) of the distances between data values and fitted values was calculated in terms of the GI% response variable in order to describe the goodness of fit for the chosen regression model.

Alpha diversity estimates were computed using the phyloseq R package (McMurdie and Holmes, 2013). Principal Coordinates Analysis (PCoA) plot for beta diversity is based on cumulative-sum scaling (CSS) normalized data (Paulson et al., 2013) Metagenomic biomarker discovery was performed using LEfSe version 1.0 (Segata et al., 2011), with an all-against-all multi-class analysis strategy and LDA threshold was set to 2.0 ($p = 0.05$ for the factorial Kruskal-Wallis and the pairwise Wilcoxon test).

3. Results and discussion

3.1. *Effects of biochar addition on AD process*

3.1.1. *Biogas and methane production*

Biogas and methane production derived from Test A, Test B, Test C and Test D are described in Figs. 2 and 3, respectively. The residual biogas and methane production of used inoculums were detracted for each test to define the specific production derived from the analyzed feedstocks.

Biogas and methane specific yields measured in Test D are slightly higher, albeit not significantly, than in other tests. Biogas and methane

Table 1
Chemical-physical characterization of input feedstocks.

	OFMSW	Biochar	Plant-made inoculum	Lab-made inoculum	Lab-made biochar enriched inoculum
pH (–)	5.4 ± 0.4	10.5 ± 0.2	8.8 ± 0.1	8.6 ± 0.1	8.7 ± 0.1
EC ($\mu S\ cm^{-1}$)	3324 ± 623	n.a.	3810 ± 496	4522 ± 594	3654 ± 726
TS ($g\ kgFM^{-1}$)	333 ± 34	956	288 ± 37	228 ± 15	281 ± 30
VS ($g\ kgFM^{-1}$)	235 ± 27	924	159 ± 21	136 ± 12	146 ± 23

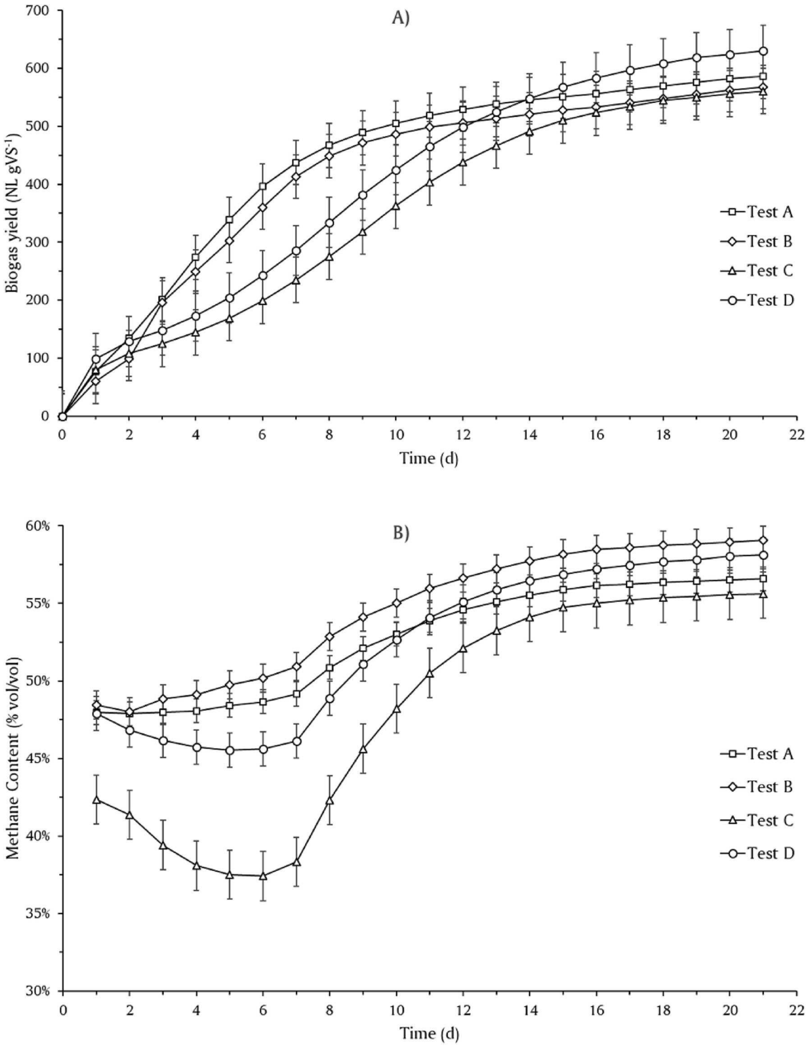

Fig. 2. Biogas production from performed tests: A) Mean cumulative biogas production for all performed test-configurations; B) Methane content of produced biogas. Vertical bars indicate standard errors (n = 3).

production in Test A, Test B and Test C are comparable and not significantly different (Table 2). Methane content (vol/vol) in Test A and Test B showed an initial increasing and thereafter steady pattern during the test, from below 50% to almost 60% (Fig. 2B). This trend, both for Test A and Test B, resembles the behavior of AD in biochar-free configurations. Instead, Shen et al. (2015, 2016) showed that presence of biochar in AD reactors (at any tested dosage) leaded to a trend of CH_4 concentration of biogas characterized by an initial decreasing phase followed by a steady final stage. In fact, Shen et al. (2015, 2016) suggested that this feature is likely due to biochar contribute to CO_2 removal, which decreases within the testing time with increasing rate of total biogas production exceeding sorption and uptake rate. Further, generally increasing trend can be seen in Fig. 2B also for Test C and Test D (from 42% to 56% and from 48% to 58%, respectively), but characterized by an initial decrease in methane concentration followed by a rapid increase. The reported behavior is likely due to initial high concentration of VFA, which could have inhibited methanogenic microflora and increased lag phases (see Tables 2 and 3, as discussed later). However, Test D configuration leaded

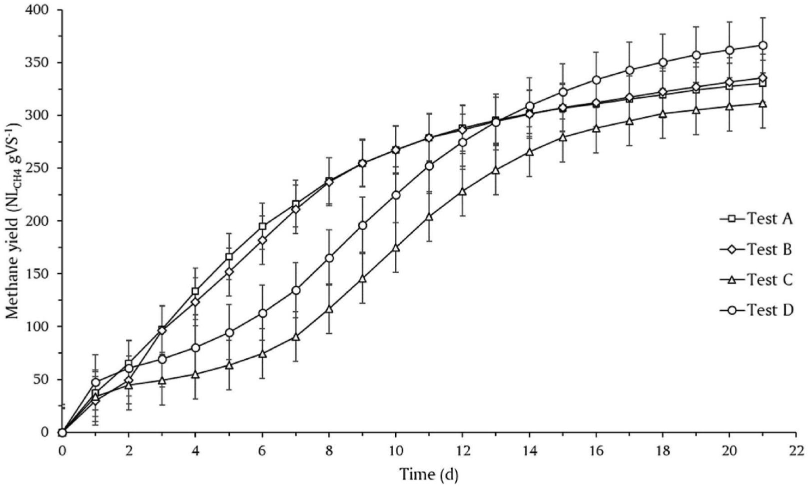

Fig. 3. Mean cumulative methane production for all performed test-configurations. Vertical bars indicate standard errors (n = 3).

Table 2
Measured specific production of biogas (Y_{BG}) and methane (Y_{CH4}), calculated VS reduction (VS%) and modeled values for potential methane production ($M_{0,CH4}$), maximum methane production rate ($R_{max,CH4}$) and lag phase (λ). Values followed by an asterisk are significantly different (α = 0.05).^{a} = Calculated VS reduction (%) does not consider biochar VS content, according to the assumption of poor (absent) biodegradability of biochar.

	Experimental Results			Modeling Results			
	Y_{BG} (NL kg VS^{-1})	Y_{CH4} (NL kg VS^{-1})	VS%^{a} (–)	$M_{0,CH4}$ (NL kg VS^{-1})	$R_{max,CH4}$ (NL kgVS^{-1} d^{-1})	λ (d)	R^2 (–)
Test A	628.02 ± 96.07	330.40 ± 9.51	39.72 ± 5.93	324.20	33.04	0.12	0.9967
Test B	567.91 ± 76.60	335.41 ± 68.75	37.68 ± 4.81	328.13	33.01	0.41	0.9966
Test C	624.86 ± 22.53	311.78 ± 3.57	36.61 ± 0.24	381.94	25.52	2.27	0.9877
Test D	643.61 ± 27.19	366.43 ± 25.22	40.98 ± 12.79	414.49	24.53	1.22	0.9927

Table 3
Chemical-physical characterization of input mixtures. Values followed by an asterisk are significantly different (α = 0.05).

	Test A	Test B	Test C	Test D
pH (–)	8.1 ± 0.3	8.3 ± 0.2	8.3 ± 0.1	8.4 ± 0.1
EC (μS cm^{-1})	3490 ± 1075	3653 ± 169	3910 ± 693	3397 ± 964
TS (g kgFM^{-1})	273 ± 11	318 ± 9 *	260 ± 16	329 ± 28 *
VS (g kgFM^{-1})	164 ± 1	197 ± 10 *	161 ± 1	205 ± 25 *
Total VFA (mgCH3COOH L^{-1})	17,377 ± 554	15,387 ± 2633	22,294 ± 422	21,022 ± 2261

to a lower decrease rate of CH_4 content at the beginning of the test (Fig. 2B). Here, as reported in scientific literature, biochar addition could have alleviated VFA inhibition and related acid stress affecting methanogens (Luo et al., 2015, Fagbohungbe et al., 2017). Further, the calculated values for 21-days average of methane concentration in biogas were not significantly different between tested configurations.

The calculated VS removal percentages related to Test B and Test D did not result significantly different from biochar-free configurations (Table 2). Here, VS content of biochar was not considered for the calculation of VS reduction efficiencies of Test B and Test D. In fact, the volatile carbon content of used biochar can be assumed not to have contributed to the increase of biomethanation yields or increased VS reduction efficiencies, due to its specific low concentration of easily degradable fraction (Mumme et al., 2014). In particular, the low Oxygen-to-Carbon ratio (O/C) characterizing the used woody pyrolytic biochar may indicate that carbon atoms are arranged in very stable graphite-like structures, i.e. not easily biodegradable (Yang et al., 2007; Spokas, 2010; Khodadad et al., 2011; Wu et al., 2012; Dong et al., 2013; Benedetti et al., 2018).

The application of modified Gompertz equation leaded to the potentials of methane production with the highest values for Test D configuration, thus confirming the trend described by experimental results (Table 2). Moreover, the modified-Gompertz model fitted well with the experimental data (i.e. $R^2 \geq 0.987$).

Therefore, these data suggest that maximizing yields in the dry thermophilic AD can likely be achieved by increasing the retention time of biochar inside the reactor, e.g. through recirculation of biochar-enriched digestate (i.e. achieving Test D configuration). However, no clear evidence is given on which fraction of biochar contributed to the observed trend in Test D configuration. According to the outcomes of

Test B, it is likely that the amount of "fresh" biochar, added to biochar-enriched lab-inoculum in Test D configuration at the start of the test to reach the fixed dosage, did not contribute to the reported trend. Conversely, the retained portion of biochar likely represented the responsible for eventually occurred acclimation of the biomass consortium. Nevertheless, Test B and Test D are characterized by different inocula, thus suggesting that further research is needed to clarify this aspect.

Figs. 2A and 3 show that biogas and methane-specific production reached the maximum value faster in Tests A and B than in Tests C and D. According to Table 2, this feature is reflected in the higher values modeled for the lag phase and lower maximum methane production rates of configurations involving "lab-made" inoculums. Longer lag phases and smaller methane production rates likely stem from the significantly higher concentration of Total VFA characterizing input mixtures of Test C and Test D, which could have initially inhibited the methanogenic microflora (Table 3).

Furthermore, Test C configuration almost doubled the lag phase when compared to Test D: these values can likely be influenced by the obtained functional biomass consortium enrichment of biochar-rich inoculum (see further Paragraph 3.1.2) and relieved acid stress due to organic loading rate (Luo et al., 2015, Fagbohungbe et al., 2017). Nevertheless, an inverse trend is noted between the modeled lag phases for Tests A and B, suggesting that the occurrence of bacterial acclimation in biochar-containing substrates could be achieved through longer retention times inside the reactor (e.g. recirculation). However, this specific behavior is not reflected in the calculated maximum methane production rates, which result similar between the configurations involving similar type of inoculums (respectively 33.04 and 33.01 NL kgVS-1 d-1 for Test A and Test B and 25.52 and 24.53 NL kgVS-1 d-1 for Test C and Test D), suggesting the fact that this parameter could have been more influenced by the type of used inoculums than the action of the used biochar.

The results of this study differ from those reported in previous works, which recorded significant positive effects on the methane production rate of biochar addition (Inthapanya et al., 2012; Mumme et al., 2014; Luo et al., 2015). A possible explanation may be due to the specific type of biochar used. The specific high temperature of the biochar production process involved may have caused a breakdown of the pore walls and the consequent sintering of the material reducing the external surface area's porosity (272 m^2g^{-1}), thus affecting the adsorption potential of biochar responsible for positive effects on the AD process and CO_2 removal (Jindo et al., 2014; Benedetti et al., 2018). Another reason could be the particle size of the biochar used, considering that methanogens showed better acclimation on biochar characterized by a coarser particle size than the one used in this work (Luo et al., 2015). Moreover, the used biochar, derived from a dual-stage gasification process, is characterized by a recalcitrant aromatic carbon structure (Sanchez-Monedero et al., 2018) and consequently by labile-carbon content lower than hydrochar used in previous works (Mumme et al., 2014): for these reasons, it probably did not contribute to increasing biogas/methane production. Similarly, not clearly identified effect on CO_2 removal could be related with low content of alkaline earth metals in used biochar, e.g. Calcite Ca $(OH)_2$, which were reported to promote shift of gaseous CO_2 towards bioavailable soluble bicarbonate/carbonate forms, ultimately enhancing CO_2 reduction by hydrogenotrophic methanogens (Benedetti et al., 2018; Shen et al., 2015, 2016).

Also, it seems possible that the dry AD configuration involved could have limited the beneficial action of biochar on the process. In fact, it has been demonstrated that interactions in terms of solid-liquid transfer between the feedstock, biochar and microbial consortium could be more limited in dry conditions than in wet AD processes (García-Bernet et al., 2011).

Finally, the tested biochar concentration could not have represented an optimum condition and effect of higher dosages should be further investigated. However, the semi-pilot experimental configuration did not allow to test higher dosages of used biochar, due to its specific fine granulometry and consequent powdery nature. In fact, higher dosages resulted in difficult mixing of input substrates and leaded to inefficient loading and to the occurrence of an unmixed "free phase" of biochar which could have determined malfunctioning of the used reactors. In this context, material handling issues (e.g. local suspension of biochar fine particles possibly endangering plant operators safety and inefficient mixing with other co-substrates) should be considered when evaluating the opportunity to integrate biochar addition in full-scale biogas plants.

3.1.2. Composition of microbial community

The 16S rRNA amplicon sequencing produced a total of 781,628 reads region from the 12 investigated samples (average read length 412 nt). Quality filtering by DADA2 software resulted in an average read count of 42,763 ± 9685 per sample.

Calculated alpha diversity indexes showed that biochar-enriched samples from Test D configuration were characterized by higher bacterial diversity than all other investigated digestates (Fig. 4). Assessed microbiome compositions at genus level can be found in Supplementary Material, (Fig. S1). This difference was reported significant for the lab-inoculum configuration (Test C vs. Test D; Shannon index (p value $=$ 0.017) and Simpson index (p-value $= 0.006$)).

Principal Coordinates Analysis (PCoA) was performed, based on Bray Curtis dissimilarities, to further investigate between-samples diversity (Fig. 5).

In Fig. 5, component 1 and component 2 describe respectively the 50% and 15% of the data variance. Therefore, the difference in microbial community richness between the two used inocula (Test A and Test B vs. Test C and Test D) can be easily recognized by component 1. Further, according to component 2, influence of biochar addition can only be clearly detected between Test C and Test D samples, thus likely supporting the results derived from the calculated alpha diversity estimates.

The results obtained for species and between samples (Figs. 4 and 5) suggested that biochar addition could promote a higher microbial community diversity in dry AD process (Test A vs. Test B; Test C vs. Test D), thus confirming previous results on microbial colonization of biochar (Mumme et al., 2014; Luo et al., 2015; Wang et al., 2020). However, this difference was significant only when comparing Test C and Test D.

This effect can be related with the reported effects of biochar addition on biogas and methane production (Fig. 2A and B and 3). Here, significantly increased microbial richness in Test D configuration could have led to shorter lag phase (Table 2), which is likely related to the action of biochar in reducing the washout of active microorganisms (Wang et al., 2020; Masebinu et al., 2019).

Finally, LDA Effect Size (LEfSe) for high dimensional biomarker discovery was performed on digestate samples from Test C and Test D, in order to identify bacterial taxa that are statistically different abundant as a function of biochar presence. Results showed that Test D samples were characterized by the significant abundance of *Ruminiclostridium*, *Hydrogenispora*, *Tepidimicrobium* and *Clostridia M55-D21*, while Test C samples by *Defluviitoga*, *Acetomicrobium* and *Lentimicrobiaceae* (Fig. 6). Especially *Clostridia* seem to be characteristic for biochar-enriched samples from Test D. *Clostridia* are involved in degradation of cellulosic and highly complex organic compounds, and *Ruminoclostridium* in particular, devoted to VFAs metabolism, could have played a key role in relieving acid stress during first days of dry AD, ultimately reducing the lag phase (Luo et al., 2015; Sun et al., 2016; Maus et al., 2016; Speda et al., 2017).

In summary, these results suggest that increasing retention time of the investigate biochar (e.g. through recirculation of biochar-enriched digestate) potentially promotes microbial diversity and thereby lead to an enhanced stability of the microbial community. This observation was confirmed by the detected significant difference on lag-phase (λ) detected comparing Test C and Test D (Table 2, Figs. 2 and 3), while, by

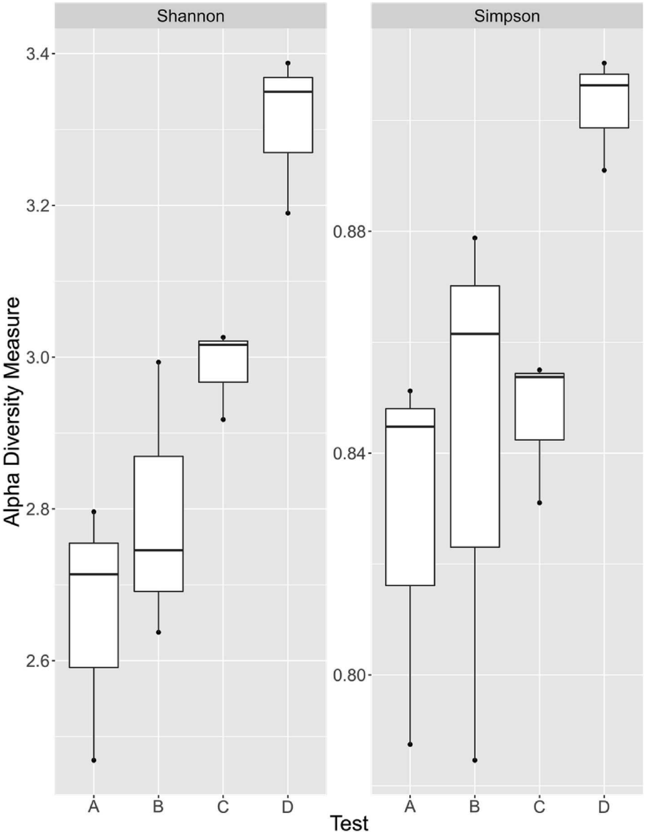

Fig. 4. Comparison of microbiome diversity: Box-plot representation of alpha diversity indices (Shannon index -left- and Simpson index -right-) between the digested residues of each tested configuration. Horizontal bold lines represent median diversity index.

comparing Test A and Test B, probably the shorter retention time determine the absent effect on BMP results and modeled values for lag phases (Table 2, Figs. 2 and 3).

3.2. Effects of biochar addition on digestate quality

3.2.1. Chemical-physical characterization

The chemical-physical characterization of resulting digestates is available in Table 4. Biochar addition led to a higher content of TS and VS. As previously discussed, the higher amounts of solids can be due to the addition of stable graphite-like carbon, which can likely determine a higher amendment potential of digestates when applied in agricultural fields (Tambone et al., 2009). Moreover, a reduced EC and calculated VFA/TA ratio are reported for biochar-enriched digestates (Table 4). While the VFA/TA ratio is probably influenced by the alkaline nature of biochar, according to Garuti et al. (2014) the decreased salinity (i.e. decreased EC) could be due to a decrease in VFA concentration and TA, probably caused by occurred adsorption of the main salts (i.e. Na and K salts, NH_4^+ and Mg chloride) present in the digestate.

Table 4 allows an assessment of the occurred effect of biochar addition on TAN and phosphate concentration measured on water extracts derived through a leaching test on the resulting digestates. In both cases, occurring lower concentrations found in water extracts could suggest an adsorption effect onto the biochar surface and a consequent lower availability of these compounds in the produced digestate. While a higher phosphate and TAN retention capacity determines a higher fertilizing potential of a biochar-enriched organic substrate when land-applied, adsorption can likely also determine lower volatilization and slower leaching potential, thus resulting in the reduction of odorous emissions and eutrophication risk during further digestate management steps (Al Seadi et al., 2012; Malinska et al., 2014; Prasad et al., 2018). Effective phosphate and TAN adsorption potential of biochar was already investigated by previous studies, both on water and liquid fraction of digestates (Takaya et al., 2016; Kizito et al., 2017).

Unfortunately, no significant effects were reported on measured concentrations in water extracts during the conducted tests, suggesting that the used pyrolytic biochar addition did not influence ammonia and/or phosphate retention in the digestate (Table 4).

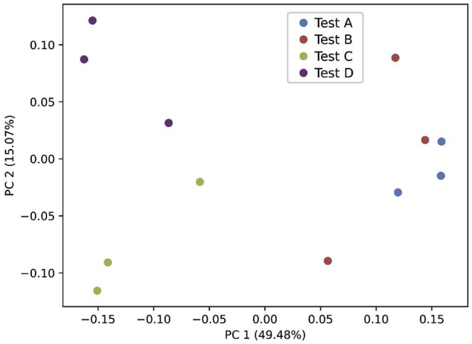

Fig. 5. Principal Coordinates Analysis of the microbial community for each tested configuration.

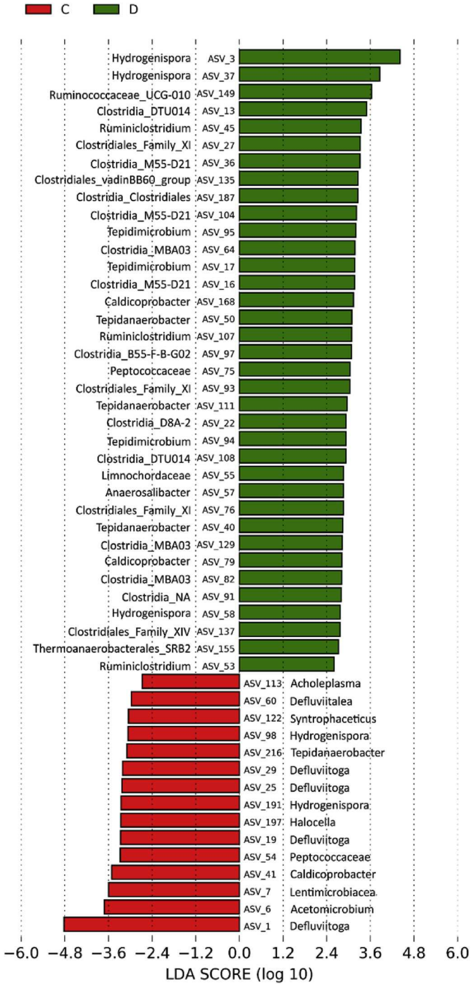

Fig. 6. - *Metagenomic biomarker analysis. Detected microbial biomarkers for digestates derived from Test C (red) versus Test D (green)*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In particular, performed adsorption tests demonstrate that used biochar could not be considered as a TAN-adsorbent material (Fig. 7), showing poor fit with applied Freundlich and Langmuir isotherms (R^2 equals to 0.62 and 0.79 respectively). Calculated TAN adsorption was recorded in the range of 3–13% of TAN equilibrium concentrations, in line with data presented by Takaya et al. (2016), who used similar test conditions. Also, previous studies reported the TAN adsorption potential of different chars (Malinska et al., 2014; Takaya et al., 2016).

As discussed for the unreported effect on methane production, the absence of a proper liquid phase due to the AD tests' dry conditions could have further negatively influenced the biochar adsorption capacity for TAN and phosphate.

The scarcity of negatively charged surface groups (i.e. oxygen and hydrogen containing functional groups) characterizing pyrolytic biochars (Benedetti et al., 2018) can be cited as an additional key factor influencing adsorption capacity. According to Gai et al. (2014), lower pyrolysis temperatures (200–400 °C) have been found suitable to produce biochar for the removal of inorganic and polar contaminants. In their study, ammonium adsorption was found to decrease when the pyrolysis temperature was increased from 400 to 700 °C. Zeng et al. (2013) observed that the involved high temperature (>600 °C) of the biochar production process caused a decrease in TAN adsorption because of the loss of biochar polar groups. Bargmann et al. (2014) and Spokas (2010) observed that char surface groups play a more important role than the surface area and porosity in biochar adsorption.

Finally, pH, contact time and initial concentration of substances to be adsorbed are important aspects determining biochar adsorption capacity (Fernando et al., 2005; Kizito et al., 2015). As previously stated, competition with other dissolved compounds found regularly in digestates could also have decreased the sorption sites available for TAN and/or phosphate adsorption (Möller and Müller, 2012; Garuti et al., 2014).

The Heavy Metals (HM) content of used biochar and digestates from tested configurations is listed in Table 4. Except for Cd, biochar shows an HM content lower than biochar-free digestates (Tests A and C): therefore, a dilution effect may have occurred in Test B and Test D digestates, equivalently characterized by HMs concentrations lower than Test A and Test C, recording the expected inverse figure for Cd content. Chemical composition of source material (i.e. woodchips) mainly determined the biochar HM content, which is known to build-up during the pyrolytic process with increasing process temperature (Jin et al., 2017; Huang et al., 2018). Aforementioned literature sources demonstrated also the role of pyrolysis in promoting changes in the chemical speciation of HM in biochar towards non-bioavailable forms, this shift being further enhanced by increasing process temperature. Therefore, according to the high temperature regime characterizing the production process of used biochar (i.e. 850 °C), the increased concentration of Cd found in biochar-enriched digestates can likely be referred as a non-toxic non-bioavailable fraction of the specific element. This assumption should be further verified by performing HM speciation assessment both in used biochar and derived biochar-enriched digestates, through leaching tests with different leaching media (e.g. DTPA, HCl, acetic acid) to detect acid soluble and reducible fractions (i.e. toxic) and oxidizable and residual portions (i.e. less toxic and stable) (Jin et al., 2017; Huang et al., 2018;

Table 4
Characterization of digestates from all tested configurations. Values followed by an asterisk are significantly different (α = 0.05).[1] European Parliament and European Council (2019).

	Test A	Test B	Test C	Test D	Biochar	Limits[1]
Chemical-physical characterization						
pH (–)	8.7 ± 0.1	8.8 ± 0.1	8.6 ± 0.1*	8.7 ± 0.1	10.5 ± 0.2	–
EC (μS cm^{-1})	3990 ± 65	3310 ± 385	4465 ± 417	3423 ± 919	–	–
TS (g $kgFM^{-1}$)	264 ± 29	291 ± 16	234 ± 21	298 ± 24	–	–
VS (g $kgFM^{-1}$)	145 ± 17	181 ± 23	133 ± 1	152 ± 29	–	–
TKN (mg N L^{-1})	2388 ± 516	2180 ± 299	2326 ± 536	2125 ± 583	–	–
TAN (mg TAN L^{-1})	2352 ± 210	1973 ± 142	2111 ± 82	1936 ± 321	–	–
Phosphates (mg PO_4^{3-} L^{-1})	720 ± 36	776 ± 83	753 ± 83	697 ± 75	–	–
VFA/TA ($mgCH_3COOH$ L^{-1})/($mgCaCO_3$ L^{-1})	0.40 ± 0.1	0.35 ± 0.05	0.52 ± 0.04	0.44 ± 0.06	–	–
Cd (mgCd $kgTS^{-1}$)	0.44	0.62	0.47	0.68	1.2	1.5
Pb (mgPb $kgTS^{-1}$)	26	23	25	17	3.1	120
Cu (mgCu $kgTS^{-1}$)	91	67	101	62	7.6	300
Hg (mgHg $kgTS^{-1}$)	0.1	0.1	0.1	<0.1	<0.1	1
Ni (mgNi $kgTS^{-1}$)	7.7	6.9	8	5	1.1	50
Zn (mgZn $kgTS^{-1}$)	226	170	233	160	82	800
Cr (mgCr $kgTS^{-1}$)	14	14	14	8	1.1	–
PAH21 (mgPAH $kgTS^{-1}$)	1.3	3.4	0.1	8.9	248	6
Biological Stability						
DRI_Max_VS (mg O_2 $kgVS^{-1}$ h^{-1})	809 ± 264	554 ± 76	n.a.	n.a.	–	–
DRI_Max_FM (mg O2 $kgTS^{-1}$ h^{-1})	2920 ± 665	1989 ± 317	n.a.	n.a.	–	–
Phytotoxicity						
EC50 (% vol/vol)	9.8	18.7	7.6	13.0	–	–

Zeng et al., 2018; Wang et al., 2019).

Besides, all digestates showed full consistency with HMs concentration limits established by the updated European Regulation on Fertilizers under the amendment category (European Parliament and European Council, 2019).

Table 4 includes data on PAH21 concentrations of biochar, which are consistent with the literature ranges for high temperature-derived commercial biochar (Kołtowski and Oleszczuk, 2015). Therefore, biochar addition led to increased PAH21 concentration on the resulting digestates (Table 4): the resulting concentrations are within the adopted Regulation limits (i.e. 6 mg/kgTS), apart from Test D digestates. However, PAH21 concentration could have been overestimated due to uneven spatial distribution of contaminants in tested samples, as a result of difficult mixing due to specific rheology (i.e. high TS content) and diffusion limitation phenomena usually characterizing digestates derived from dry AD (Bollon et al., 2013). Also, use of biochar in large scale reactors operating in continuous with digestate recirculation should take into account possible contaminant accumulation and consequent concentration build up in the resulting digestate.

Currently, post-composting of digestate from OFMSW precedes its agricultural use: in this context, the effect of the aerobic degradation on biochar-added PAHs still has to be evaluated. Recent work by Oleszczuk and Koltowksi (2018) suggested that microbial activity in the presence of nutrients and a water phase (i.e. saturated condition) can lead to significant decrease of PAHs content of biochar. In this context, biomass-driven change in hydrophilic properties of biochar surface (i.e.

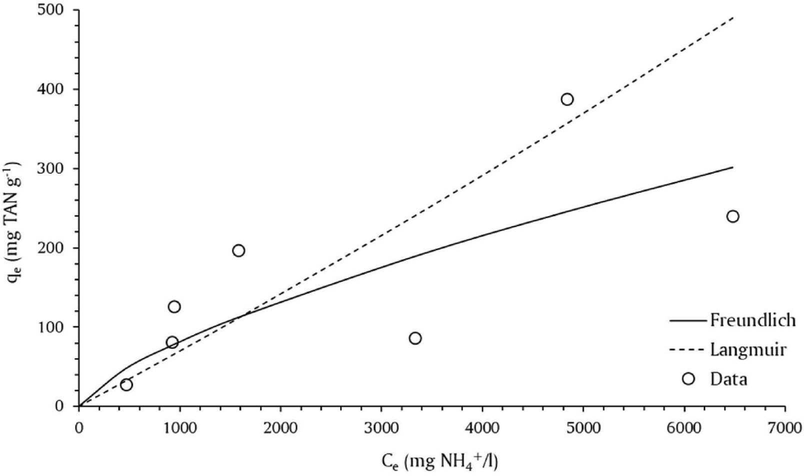

Fig. 7. Modeling results of biochar batch adsorption data performed on distilled water at various TAN concentrations.

increased O/C ratio) could lead to weaker interaction with surface-bounded PAHs and consequent increased efficiency of biomass degradative activity. However, the aforementioned findings should be verified including how composting conditions affect contaminants behavior in biochar-enriched digestate, e.g. absence of saturated water-phase, efficacy of specific aerobic biomass and designed treatment time as imposed by industrial-scale composting facilities.

3.2.2. *Biological stability*

Results from respirometric analyses show that values of DRI_{max} calculated for Test B are lower, but not significantly, than Test A. This probably suggests higher biological stability characterizing biochar-enriched digestates (Table 4). The mean values of DRI_{max} were calculated both on a total wet weight (FM) and normalized on a VS basis, to consider possible calculation biases due to the higher amount of VS of biochar-enriched digestate. However, the trend (i.e. higher stability for biochar-rich digestate) is confirmed for DRI_{max} values expressed in both VS or total wet weight. Therefore, biochar application could have contributed to a decreased activity in aerobic biomass due to the resulting lower concentration of labile organic compounds present in the same mass of tested digestate. As reported in scientific literature and discussed in the previous paragraph, biochar is not expected to influence the stock of readily biodegradable organic matter, due to its recalcitrant aromatic carbon structure (Sanchez-Monedero et al., 2018). Further studies should be performed to investigate other effects of used biochar on aerobic microflora, e.g. both by limiting or increasing the availability of inhibitory compounds due to adsorption or desorption capacity.

3.2.3. *Phytotoxicity*

Besides conformity with regulations, further analysis should be performed where the presence of organic contaminants (as in the case of Test D digestate's PAH21 concentration) could place doubts on the environmentally-sound management of biochar-enriched digestate, especially in direct land use for agricultural purposes.

For this purpose, phytotoxicity dose-response curves were determined for derived digestates from tested configurations, and Table 4 lists the specific median effect concentrations, calculated for each tested digestate. EC50 of digestates from Tests A, Test C and Test D were determined by using a logistic model (calculated S values were 2.9, 1,4 and 11 for Test A, Test C and Test D, respectively), while EC50 of digestates from TEST B were calculated by a linear-logistic model, which better fitted experimental data ($S = 4{,}2$). In fact, the dose-response curve of the Test B digestate was the only one characterized by hormetic behavior (i.e. stimulation) at lower dilutions (Da Ros et al., 2018a).

In Table 4, specific higher EC50s show a decreased phytotoxicity of biochar-containing digestates. The lower degree of phytotoxicity can be due both to a contaminant-binding capacity of biochar and to the increased carbon content able to complex with potential dissolved contaminants: both mechanisms could have reduced the bioavailability of occurring phytotoxic substances, as suggested in previous literature (Oleszczuk et al., 2012, 2013). Also, lower salinity values, Total VFA concentrations and higher biological stability (see Table 4) characterizing biochar-containing digestates could have positively influenced the germination and root elongation of cress seeds by lowering the inhibition of digestates (Pivato et al., 2016; Tigini et al., 2016). Finally, results obtained from phytoxicity testing are inversely correlated with specific PAHs concentration of investigated digestates, advocating the argument that PAHs contained in biochar are strongly bound with its solid phase, thus being characterized by low bioavailability (Kołtowski and Oleszczuk, 2015).

4. Conclusions

This study presents results from dry thermophilic AD tests in order to assess the effects of biochar addition on AD performances and final digestate quality. Analyzed biochar is currently produced from woody biomass treatment by a full-scale two-stage gasification-pyrolysis plant located in Northern Italy. Different test configurations were tested in AD semi-pilot scale reactors using OFMSW collected at a waste treatment plant.

Obtained experimental results did not show a significant increase in methane production from AD tests using biochar. A likely explanation could be due to the specific features of the biochar used (i.e. very small particle size, low adsorption potential), combined with the dry AD configuration, which could have limited interactions between biochar, input feedstocks and anaerobic microflora. However, results suggest that higher methane yield and higher microbial diversity could be obtained through increasing the retention time of biochar (e.g. through recirculation of biochar-enriched digestate), to allow more efficient bacteria acclimation and stability. The reported limited influence of used biochar should be further assessed for different dosages, while considering material handling issues and mixing constraints related to its dusty nature for subsequent application scale-up.

Biochar-enriched digestates were characterized by lower HMs concentrations but a higher PAHs content which, in one case, were not consistent with the EU Regulation on fertilizers, excluding it from possible agricultural direct reuse. Moreover, biochar determined a higher biological stability of resulting digestates: however, this was probably due to the addition of stable graphite-like carbon which determined a lower biodegradable matter content. Nevertheless, plants bioassays showed a decreased phytotoxicity of biochar-containing digestates, highlighting the possible positive effects of biochar addition related to the decreased bioavailability of phytotoxic contaminants and salinity.

Due to reported poor adsorption potential on water, biochar addition to AD did not influence the fertilizing potential of digestates, i.e. TAN or phosphate retention. The low adsorption potential of TAN and phosphates could also be related to the specific physical-chemical features of biochar, the possible competition for adsorption sites with other dissolved digestate constituents and again, to the dry conditions of the AD tests performed.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Daniela Bona: Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing. **Giovanni Beggio:** Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Tobias Weil:** Investigation, Resources, Data curation, Writing - review & editing, Visualization. **Matthias Scholz:** Investigation, Resources, Data curation, Writing - review & editing, Visualization. **Sara Bertolini:** Investigation. **Luca Grandi:** Investigation. **Marco Baratieri:** Conceptualization, Resources, Data curation. **Andrea Schievano:** Conceptualization, Supervision. **Silvia Silvestri:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing - original draft, Writing - review & editing. **Alberto Pivato:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2020.110633.

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Paper III

Beggio, G., Cerasaro, A., Bonato, T., Pivato, A. Chemically enhanced solid-liquid separation of OFMSW digestate: suspended solids removal and effects on environmental quality of separated fractions. Submitted to Journal of Water Process Engineering (07/12/2020).

Chemically enhanced solid-liquid separation of digestate: suspended solids removal and effects on environmental quality of separated fractions

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Abstract

The efficiency of chemically enhanced solid-liquid separation (CES) must be investigated when applied on digestate from organic fraction of municipal solid waste (OFMSW), to improve further membrane filterability and consequent scale-up for in situ reuse or direct discharge of the resulting liquid fractions. However, applied chemicals could decrease the environmental quality of the separated solid fraction, thus hindering its possible agricultural reuse. In this study, dosing polyaluminum chloride (PAC), epichlorohydrine-dimethylamine with ethylendiamine (DEED) and polyacrilamides (PAM) on OFMSW digestate were investigated in terms of TSS mitigation achieved in the liquid fractions and final environmental quality of the solid fractions. Results from lab-scale CES trials showed that applied CES significantly increased the removal of suspended solid (TSS) from the liquid fractions, corresponding to TSS concentrations ranging from 15,330 ± 575 mgTSS/L to 2,347 ± 281 mgTSS/L (up to 90% improved removal). Also, performed treatments led to almost complete removal of P and Heavy Metals (HMs) from the liquid fractions after centrifugation. Conditioned solid fractions showed higher Al (reaching 20 g/kgTS), organic carbon and nitrogen content due to residual PAC, DEED and PAM. However, similar concentrations of P and HMs guaranteed full consistency with regulation limits established for agricultural reuse. Further, leaching tests performed on the treated solid fractions highlight higher chlorides and soluble Al concentrations in the water extracts, but lower HMs leachability from the digestates undergone CES. Nevertheless, water extracts from treated biosolids were characterized by higher phytotoxicity, likely related with direct Al toxicity and with the increased salinity due to chemicals addition.

Keywords

OFMSW, Anaerobic Digestion, Digestate solid-liquid separation, Phytotoxicity

1. Introduction

The EU28 yearly production of digestate was estimated in almost 180 million tons, derived from the Anaerobic Digestion (AD) of both the separately collected Organic Fraction of Municipal Solid Waste (OFMSW) and the agro-industrial residues (Corden et al., 2019). In this context, digestate quality management, achieved through the application of specific treatment technologies, can determine its possible market valorization as substitute for inorganic fertilizers, while avoiding additional costs due to further treatment or disposal as a waste (Al Seadi and Lukehurst, 2012; Dahlin et al., 2015). Digestate processing technologies consist primarily on solid-liquid separation, performed to achieve i) volumes reduction, improving handling and decreasing transportation and storage costs, ii) nutrients recovery in concentrated fractions (solid and/or liquid), and iii) complete purification of the remaining liquid fraction to allow in situ reuse according to the concept of zero water discharge when applied on AD plant design (Fuchs and Drosg, 2013; Peng and Pivato, 2017).

Complete purification of the liquid fraction of digestates can be effectively achieved through the application of the whole range of membrane technologies, from microfiltration to reverse osmosis (Vaneeckhaute et al., 2019; Waeger et al., 2010). However, when applied on digestate, membrane processes should include mandatory pretreatment aimed at decreasing the specific high concentration of total suspended solids (TSS), which is the main cause of membrane fouling, decreased separation efficiency and short membrane service life (Guo et al., 2012; Meixner et al., 2015; Shi et al., 2019). Chemically-enhanced solid-liquid separation (CES), consisting on the dosage of a combination of chemical products (mostly polyelectrolytes and organic polymers) followed by solid-liquid separation step (e.g. centrifugation, screw press, etc.), has been implemented to solve this issue and proved to outperform in terms of low cost and simple operations (Even-Ezra et al., 2011; Hjorth et al., 2010). Chemical conditioning improve the efficiency of the following solid-liquid separation by charge neutralization of suspended particles (i.e.

coagulation) and further aggregation of destabilized particles (i.e., patch flocculation and polymers bridging) (Metcalf and Eddy, 2013). Polyaluminum chloride (PAC), polyacrylamide (PAM) and epichlorohydrine-dimethylamine with Ethylendiamine (DEED) are among the most efficient coagulant and flocculant products used in wastewater treatment (Bu et al., 2016). Also, some authors demonstrated the efficacy of PAC and cationic PAM on digested residues from AD of slurries and agro-industrial substrates (Camilleri-Rumbau et al., 2019; Luo et al., 2018; Meixner et al., 2015). Further, Borowski et al., (2018) assessed the effects of several CES products on digestates from a pilot-scale codigestion of lab-reconstructed food waste, slaughterhouse waste and municipal sewage sludge. Nevertheless, to the authors best knowledge, no previous study investigated the efficiency of chemical conditioning when applied on digestates derived from a full-scale biogas plant treating separately collected OFMSW.

The potential use as organic fertilizer of the separated solid fraction of digestate has been largely supported by the scientific literature (Lukehurst et al., 2010; Tambone et al., 2017, 2010, 2009). Consequently, they were recently included within the updated regulation on CE-marked fertilizers as potential components to manufacture “Solid organic fertilizers” (European Parliament and European Council, 2019). This regulation establishes requirements in terms of process specifications and chemical-physical and biological parameters, which can be considered “End-of-waste” criteria for those substrates derived from the AD of OFMSW. Consequently, the nonconformity with these limits can determine their further disposal as wastes. In this context, CES application could rise doubts regarding the compliance of treated biosolids with legal requirements established for agricultural reuse. Also, changes in chemical-physical properties and environmental behavior (e.g., Heavy Metals (HM) leachability) due to the addition of selected chemical products must be assessed to support scientifically sound decisions on the agricultural reuse or disposal of digestate solid fractions undergone CES. To the best of authors knowledge, the integrated approach between leaching test and phytotoxicity testing was efficiently used to understand the overall environmental quality of treated digested residues (Bona et al., 2020; Pivato et al., 2016).

To address these gaps, several lab-scale CES trials involving PAC, DEED and PAM in various dosages were performed on OFMSW digestate derived from a full scale AD plant located in Northern Italy. The choice of conditioning products was based on the experience of the manufacturing company in the wastewater sector and the consequent possibility of market expansion in the field of biogas plants treating separately collected OFMSW. Results allowed to assess i) CES efficiency in terms of suspended solids mitigation in the separated liquid fraction and ii) the influence of used chemicals on the overall environmental quality of treated solid fraction with a view to possible agricultural reuse.

2. Materials and Methods

2.1. OFMSW digestate and conditioning products

About 50 L of digestate was sampled once at the output of 4 anaerobic digesters, operated in parallel in wet thermophilic conditions (~ 10%TS, 55 °C) with an hydraulic retention time of 21 days and treating yearly about 120,000 tons OFMSW, separately collected from several municipalities located in Veneto region in North-East Italy. Sampled digestate was stored in a 50 L PE container at 4 °C for no more than 3 weeks. Physicochemical features of the sampled digestate are listed in Table 1.

Table 1 – Physicochemical characterization of sampled unseparated OFMSW digestate used for lab-scale CES experiments. All data are expressed on a dry basis (TS), except pH, EC and TS (FM = fresh matter).

Parameter	Unit	Value
pH	-	8.2
EC	mS cm^{-1}	46.9
TS	g kg^{-1} FM	103
VS	g kg^{-1} TS	523
TKN	g kg^{-1} TS	83.3
TAN	g kg^{-1} TS	69.3
P	g kg^{-1} TS	10.7
Cd	mg kg^{-1} TS	0.43
Pb	mg kg^{-1} TS	15.37
Cu	mg kg^{-1} TS	57.55
Hg	mg kg^{-1} TS	0.07
Ni	mg kg^{-1} TS	5.84
Zn	mg kg^{-1} TS	168.00
Cr	mg kg^{-1} TS	10.75

Conditioning tests involved the use of 3 coagulant products: polyaluminum chloride (PAC), Epichlorohydrine-dimethylamine with Ethylendiamine (DEED), a mixture of 30% (w/w) PAC with 70% (w/w) DE (PACDE). Further, 2 flocculants, a cationic and an anionic polyacrilamide characterized by high molecular weight (CPAM, APAM), were tested. The investigated products are commercially available to the wastewater treatment plant sector (i.e., currently not sold for digestate treatment). Manufacturing process and the exact products composition cannot be reported since they are patented information.

2.2. Experimental setup

The experimental activity was conducted stepwise. A preliminary phase was first performed to assess the effective range of dosages of the chosen chemical products on the investigated digestate. First, the dosages were applied according to the existing know-how of the manufacturer, which is based on the application of CES on urban and industrial wastewater treatment, and not on digestate processing. During this phase, the efficacy of the tested products was assessed by expert judgement through visual examination of rate of coagulation and flock settling velocity. Therefore, 16 effective treatments were tested in the final definitive phase, resulting from the mixed dosage of the 3 coagulants (PAC, PACDE, DEED) and 2 flocculants (CPAM and APAM). Further, one control treatment involved no chemicals addition. The tested treatments (products and dosages) applied on the definitive phase are resumed in Table 2.

During both preliminary and definitive phases, conditioning tests were performed by use of Jar Test apparatus equipped with impellers. For each performed treatment, a volume of 1 liter of whole (i.e., unseparated) OFMSW digestate was poured into a 2 L glass container, upon shaking and mixing it within the 50 L PE container, to avoid eventually occurred sedimentation during storage.

Whole digestate was initially stirred for 10 seconds at 300 rpm to allow homogenization. While being continuously stirred, chemicals were spiked according to the chosen dosages: PAC and PACDE were added directly to the digestate, while DEED and polyacrylamides (APAM and CPAM) were first dissolved in distilled water to 10% and 0.2% (vol/vol) concentrations, respectively. Afterwards, conditioned digestate was stirred for other 60 seconds at 300 rpm, followed by slow stirring for 600 seconds at 50 rpm. Only for the definitive phase, the entire test sample was finally centrifuged at 4000 rpm for 10 min to obtain, for each performed treatment, separated liquid and solid fractions.

Table 2 – Treatments and relative dosages of conditioning products investigated in the definitive phase of the lab-scale CES experiments. Dosages are expressed in mass (g) of compound per unit volume (L) of whole unseparated digestate.

Treatment	PAC g L^{-1}	DEED g L^{-1}	PAC+DE g L^{-1}	APAM mg L^{-1}	CPAM mg L^{-1}
Control	-	-	-	-	-
PAC1	1.8	-	-	0.2	-
PAC2	1.8	-	-	-	0.2
PAC3	3.6	-	-	0.2	-
PAC4	3.6	-	-	-	0.2
DEED1	-	0.1	-	0.2	-
DEED2	-	0.1	-	-	0.2
DEED3	-	0.2	-	0.2	-
DEED4	-	0.2	-	-	0.2
DEED5	-	0.6	-	0.32	-
DEED6	-	0.6	-	-	0.32
PACDE1	-	-	0.54 + 7	0.2	-
PACDE2	-	-	0.54 + 7	-	0.2
PACDE3	-	-	1.00 + 14	0.2	-
PACDE4	-	-	1.00 + 14	-	0.2
PACDE5	-	-	3.2 + 42	0.32	-
PACDE6	-	-	3.2 + 42	-	0.32

2.3. Analyses

2.3.1. Physicochemical characterization

Total Suspended Solids (TSS) were analyzed on the derived 17 liquid fractions according to standard methods (IRSA-CNR, 2003). TSS removal (%) was calculated for each treatment according to the TSS value measured in the liquid fraction of the control. The liquid fractions derived from the control and the 3 lab-scale CES tests characterized by the higher obtained TSS removal were characterized for pH, Total Solids (TS), Conductivity (EC), Total Kjeldahl Nitrogen

(TKN), Total Ammonia Nitrogen (TAN), total Phosphorus (P), chlorides (Cl^-), total Aluminum (Al), and total HMs (Cd, Pb, Cu, Hg, Ni, Zn, Cr) (IRSA-CNR, 2003). Equivalently, the resulting 4 solid fractions obtained from the same treatments were tested for fertilizing properties TS, Volatile Solids (VS), Total Organic Carbon (TOC), TKN, TAN (IRSA-CNR, 1984, 1985, 1986) and HMs (Cd, Pb, Cu, Hg, Ni, Zn, Cr) (USEPA, 1994; 1996a, 2014). Water extracts of the same solid fractions, derived according to a Liquid-to-Solid ratio of 10:1 (EN, 2002), were also evaluated for pH, EC, TKN, TAN, P, Cl^-, Al, HMs (Cd, Pb, Cu, Hg, Ni, Zn, Cr) (IRSA-CNR, 2003) and phytotoxicity.

2.3.2. Phytotoxicity testing

Seed Germination Bioassays were performed on prepared eluates according to APAT (2004), ISO (2005) and USEPA, (1996a, 1996b). Each water extract was tested in five different test dilutions with distilled water (0% (Control), 5%, 15%, 50% and 100% vol/vol) and four replicates for each dilution. For each replicate, 5 mL of prepared test dilution were poured in a Petri dish and covered by filter paper. Then, 10 dicotyledon cress seeds were placed on top of the filter paper and incubated in dark conditions for 72 h at 25 °C. At the end of incubation period, the shooting seedlings from each replicate were removed from the Petri dish and their length was measured to derive dilution specific Germination Index (GI). Median effect concentrations (EC50) were determined through logistic and linear-logistic regression: the best fitting model was used according to the standard deviation of the distances between fitted and measured data (Bona et al., 2020).

3. Results and Discussion

3.1. Influence of CES on the quality of the liquid fraction of OFMSW digestate

Several CES experiments were conducted to assess TSS removal efficiency of PAC, DEED and PAM products when applied on OFMSW digestate. During testing, treatments PAC1, PAC2, PACDE1, PACDE2, PACDE3 and PACDE4, led to visible coagulation of solid particles with fast occurrence of a net distinction between solid fraction and clearer supernatant. Conversely, all DEED treatments were characterized by the absence of visible coagulation/flocculation and no clear separation of solid and liquid fractions. Instead, the trials PAC3, PAC4, PACDE5 and PACDE6 showed the formation of a thick foam, both during and at the end of the conditioning phase, which did not allow to observe the possible occurrence of coagulation and phase separation. Here, foam formation was noted also in Meixner et al., (2015) as due to the volatilization of carbonates alkalinity to carbon dioxide caused by the hydrolysis of $FeCl_3$. However, foam disappeared in the interphase between conditioning test and the following centrifugation step.

Obtained TSS removal values (%) are depicted in Figure 1. It could not be scientifically sound to compare the obtained data with published literature, since available papers do not assess CES efficiency specifically on OFMSW digestate, while on different digested substrates, characterized by different substrates origin and thus not comparable chemical-physical features, mainly in terms of TS content and type (suspended or dissolved). However, the sole or combined use of aluminum-based coagulants and CPAM was reported as efficient in decreasing TSS concentration of the liquid parts of swine digested slurries (Camilleri-Rumbau et al., 2019; Luo et al., 2018) and of residues from anaerobic codigestion of sewage sludge, food waste and slaughterhouse waste (Borowski et al., 2018).

On the contrary, to the authors knowledge, no previous studies investigated the flocculation potential of DEED on digested residues.

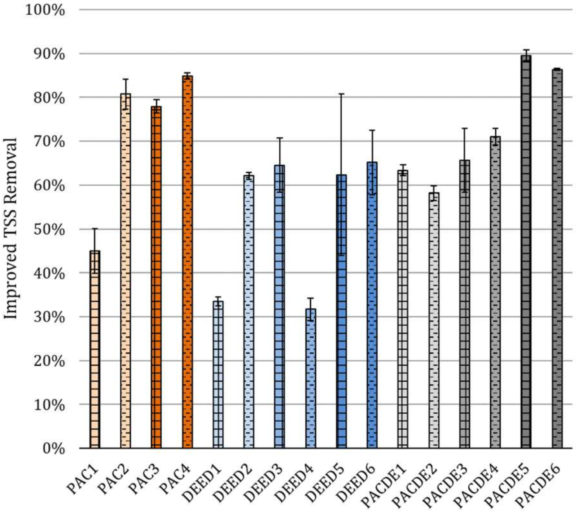

Figure 1. TSS removal improvement (%) calculated according to the ratio between the TSS values measured in the liquid fractions after each performed CES treatment and the control.

Despite the type of involved chemical, each treatment led to a significative increase of TSS removal if compared to the control TSS (i.e., 22.447 ± 1.150), ranging from DEED4 with 32 ± 1% to PACDE5 with 90 ± 1%, corresponding to the TSS concentrations of 15,330 ± 575 and 2,347 ± 281 mg/L, respectively (Figure 1). Measured means and standard deviations of TSS concentration derived from each treatment are included in the Supplementary Material (Table S1).

Reported TSS concentrations in the liquid fractions derived from CES test with only PAC and PAM dosages are characterized by lower values if compared to all the liquors undergone DEED and PAM application, except for PAC1 (Supplementary Material, Table S1). Further, anionic polyacrylamide APAM determined significative higher TSS removal than the same dosage of cationic polyacrylamide CPAM when coupled with lower dosages of PAC (45% and 81% for PAC1 and PAC2, respectively) and DEED (34% and 62% for DEED1 and DEED2, respectively). This latter trend is inverted with medium applied dosages of DEED (65% and 32% for DEED3 and DEED4, respectively). In all the other cases, no significative difference was reported concerning the different efficiency between CPAM and APAM (Fig. 1). These results suggest either that tested DEED and PAM dosages cannot be considered optimal and/or that TSS mitigation was mainly determined by PAC-led coagulation. At the same time, patch flocculation and polymers bridging, which are more related to DEED and PAM actions, could have played a minor role. As suggested by Hjorth et al., (2010), this latter aspect could be due to the high conductivity of the treated substrate (i.e., 46.9 mS/cm, Table 1), which could have determined flocculants inefficiencies due to the coiling up of the tested polymers. However, PACDE5 and PACDE6 allowed to obtain the highest TSS removal, showing that a higher dosage of DE and PAM polymers could have enhanced the charge neutralization abilities of Al-based coagulant by adding adsorption (i.e. patch flocculation) and bridging potential (Bu et al., 2016; Hjorth et al., 2010).

Results of chemical-physical characterization of liquid fractions of the control and the 3 CESs characterized by the higher obtained TSS removal are shown in Table 3.

The application of CES led to the variation of the chemical physical characteristics of the liquid fractions of treated digestate (Table 3). All investigated CES treatments showed lower pH values, as a result of the possible protons release during hydrolysis of PAC molecules (Davis and Edwards, 2014). Further, lower values of TS could be noted for treated liquid fractions, indicating that the CES step efficiently facilitated the particulate matter removal during centrifugation (Camilleri-Rumbau et al., 2019).

Table 3. Chemical-physical characterization of liquid fractions obtained after centrifugation of control and the 3 CES treatments characterized by lower TSS concentration. Applied treatments are described in Table 2.

Parameter	Units	Applied Treatments			
		Control	PAC4	PACDE5	PACDE6
pH	-	8,2	7,3	7,5	7,4
TS	g L^{-1}	47	42	42	43
EC	$mScm^{-1}$	46.4	52.6	58.1	56.3
TAN	g L^{-1}	4.3	3.5	3.9	3.9
P	m g L^{-1}	132	37	3	3
Cl^-	g L^{-1}	9.1	17.5	18.7	16.2
Cd	µg L^{-1}	< 10	< 10	< 10	< 10
Pb	µg L^{-1}	201	< 10	< 10	< 10
Cu	µg L^{-1}	1,027	178	106	151
Hg	µg L^{-1}	< 0.5	< 0.5	< 0.5	< 0.5
Ni	µg L^{-1}	238	218	171	176
Zn	µg L^{-1}	5,433	670	460	433
Cr	µg L^{-1}	314	54	< 10	38

Also, similarly increased values of EC can be due to the higher Cl^- concentrations characterizing the treated liquors, which are determined by the same PAC dosing. About nutrients, liquid fractions undergone CES are characterized by slightly lower ammonia concentration and strong reduction in P content. In fact, TAN is known to be less prone to flocculate due to the high solubility and limited capacity of polymers adsorption. Conversely, P concentration in liquid phase was reported significantly lower in PAC4 (72%), PACDE5 and PACDE6 treatments (98%). As suggested by Luo et al. (2018), P removal could have been related more with the absorption of orthophosphates by PAC than precipitation with the suspended particles. Furthermore, a lower dissolved P concentration was reported as a key factor in decreasing inorganic fouling of membranes caused by precipitation of orthophosphates salts (Even-Ezra et al., 2011; Guo et al., 2012).

Further, the HMs content of treated liquors showed a significant decrease for each assessed CES trials (Table 3). Pb content was found under the LOQ from the analyzed liquors and on average 86%, 90% and 85% of Cu, Zn and Cr concentrations were respectively removed during the performed experiments. Also, Ni concentrations were subjected to a decrease after CES performance, but to a lesser extent (i.e., approximately 21% on average between the applied treatments). The same HMs decreasing behavior in liquid fractions was reported after PAC and PAM application of digested chicken slurry and explained by the binding of HMs molecules by the formation of flocs during CES, which likely migrated to the separated solid fractions (Luo et al., 2018).

3.2. Influence of CES on the quality of the solid fraction of OFMSW digestate

3.2.1. Chemical physical characterization

The characteristics of solid fractions separated after CES performance were investigated to evaluate the reached overall environmental quality for its possible land-spreading in agricultural fields. The results of the analyses conducted on the solid fractions of control and the 3 best performing CES treatments are shown in Table 4, together with the requirements established for the category PFC 1(A)(I) of "solid organic fertilizers" in the European Regulation of CE-marked organic fertilizers (European Parliament and European Council, 2019).

All analyzed fractions resulted compliant with minimum fertilizing potential requirements established by the EU regulation. Besides, applied CES treatments led to significant changes in terms of VS, TOC and nitrogen content of treated solid fractions, while no big changes in TS can be reported. In particular, higher VS values characterize solid fractions derived from trials PACDE5 and PACDE6 (22% and 18% higher than control), while a slighter increase in VS can be reported for PAC4.

Table 4. Chemical-physical characterization of solid fractions separated after centrifugation of control and the 3 CES treatments characterized by lower TSS concentration in the liquid fractions. All data are expressed on a dry basis (TS), except TS (FM). [a] *Requirements for the category "Solid organic fertilizer", i.e. PFC 1(A)(I) established in European Parliament and European Council, (2019). Applied treatments are described in Table 2.*

Parameter	Units	Applied Treatments				Limits[a]
		Control	PAC4	PACDE5	PACDE6	
TS	g kg^{-1} FM	280	290	270	280	-
VS	g kg-^{1}TS	500	510	610	590	-
TOC	g kg-^{1} TS	263	285	324	332	≥150
TKN	g kg-^{1} TS	26.5	33.7	37.6	44.1	≥10
TAN	g kg-^{1} TS	19.4	21.8	18.6	18.5	-
P	g kg-^{1} TS	11.0	11.5	11.1	10.8	≥10
Al	mg kg-^{1} TS	6,547	20,376	12,552	11,526	-
Cd	mg kg-^{1} TS	0.72	0.65	0.62	0.63	1.5
Pb	mg kg-^{1} TS	43	31	29	29	120
Cu	mg kg-^{1} TS	109	106	99	98	300
Hg	mg kg-^{1} TS	0.27	0.22	0.24	0,20	1
Ni	mg kg-^{1} TS	9	9	8	8	50
Zn	mg kg-^{1} TS	303	295	302	285	800
Cr	mg kg-^{1} TS	23	20	18	18	

This trend is also reflected in the TOC values, which results 7%, 22% and 25% higher in the biosolids undergone PAC4, PACDE5 and PACDE6 treatments, respectively. Also, reported TKN values highlight a similar figure, showing increased nitrogen concentrations of 11% for PAC4 and 46% and 66% for PACDE5 and PACDE6. Conversely, similar TAN concentrations between the different treatments likely suggest the non-soluble polymeric origin of increased carbon and nitrogen content of substrates, originated by DEED and PAM dosages. This can also be attributed to the presence of several non-reported nitro-contaminants in the composition of conditioning products, as reported in Letterman and Pero (1990). However, the possible contribution of the flocculated organic matter from the treated liquid fraction to the increase of the final solid TKN contents should also be better clarified.

On contrary, no significative variation in HMs and P content can be reported for the solid fractions if compared with the control treatment (Table 4). In fact, from a mass balance point of view, most of the HMs and P content could have been already present in the part of the solid fraction separable through centrifugation alone, i.e., without the chemicals aid. At the same time, the foreseen mass extra-contributes derived from the flocculated particles in the liquid fractions could have played a minor role in determining the final elements concentrations.

The treated solid fractions show full compliance with the regulation limits imposed for land-spreading, consistently with the general trend reported for several digestate typologies (Kupper et al., 2014; Nkoa, 2014; Tambone et al., 2017; Vaneeckhaute et al., 2019). Instead, aluminum content increased remarkably in the treated solid fractions as a result of PAC dosages, ranging from about 2-times the control content in PACDE5 and PACDE6 to more than 3-times the control Al concentration in PAC4 treatment (Table 4). Currently, no limits on Al concentration are set in the regulations for organic fertilizers. However, residual bioavailable Al could show phytotoxic effects, such as decreased plant uptake of essential elements as P, K, Ca, Mg, Mo and B, inhibition of root elongation and biomass production and disruption of plant cellular membrane functions, mainly if related with digestate application on acidic soils (Luo et al., 2018; Vaneeckhaute et al., 2019, Shetty et al., 2021).

3.2.2. Fertilizing potential, leachability of HMs and phytotoxicity

Water extracts derived from the solid fractions of control and the 3 best performing CES trials were characterized to assess possible changes induced by used chemicals on fertilizing characteristics, HMs leachability and phytotoxicity. Table 5 shows the results from the performed analyses on the water extracts together with the outcomes of the plant bioassay in terms of EC50 (% vol/vol).

Applied CES trials influenced the fertilizing potential of the treated substrates, in terms of nutrient leachability from the tested solid fractions. If compared to the untreated digestate, almost 50% higher TKN concentrations can be noted in the water extracts derived from separated solids undergone PACDE5 and PACDE6, together with a slighter increase in the PAC4 eluate.

Table 5. Chemical-physical characterization and phytotoxicity assessment of water extracts (L/S ratio = 10) of the solid fractions of the control and the 3 best performing CES treatments. Applied treatments are described in Table 2.

Parameter	Units	Applied Treatments			
		Control	PAC4	PACDE5	PACDE6
Chemical-physical characterization					
pH	-	8.7	8.1	8.2	8.3
EC	mS cm^{-1}	11.4	16.9	15.9	16.5
TKN	mg L^{-1}	933	1,043	1,361	1,350
TAN	mg L^{-1}	913	904	1,067	1,027
P	mg L^{-1}	45.8	16.3	2.8	3.1
Cl^-	g L^{-1}	1,589	4,283	4,740	4,659
Al	µg L^{-1}	1,060	2,291	1,884	1,780
Cd	µg L^{-1}	< 10	< 10	< 10	< 10
Pb	µg L^{-1}	105	11	< 10	< 10
Cu	µg L^{-1}	163	36	70	29
Hg	µg L^{-1}	< 0.5	< 0.5	< 0.5	< 0.5
Ni	µg L^{-1}	131	111	74	75
Zn	µg L^{-1}	1,829	280	189	141
Cr	µg L^{-1}	23	19	7	7
Phytotoxicity					
EC50	%(vol/vol)	23	14	20	19

Together, calculated TAN/TKN ratios decrease significantly in the eluates of test portions treated with PAM and DE (i.e., 87% in PAC4, 78% in PACDE5 and 76% in PACDE6 eluates, against 98% calculated in the control). Here, higher TKN could have been determined by the presence of hydrolysis byproducts of aminic groups or other contaminants present in the dosed products, derived from DE polymers already present in the solid fractions (Table 4) (Letterman and Pero, 1990). For this reason, as suggested in the previous paragraph, the increased nitrogen concentration found in the treated water extracts does likely not indicate increased fertilizing or amendment potential. Instead, the potential long-term ecotoxicity and soil-water-plant transfer mechanisms of DEED and PAM by-products must be properly investigated before intended land reuse (Bamba et al., 2017; Bhat and Gogate, 2021; Letterman and Pero, 1990). Further, considerably lower P concentrations can be found in treated eluates (Table 5). This could be due to the low solubility constant of Al-PO_4 complexes occurring in the solid fractions after CES, as suggested in Luo et al. (2018).

The analyzed water extracts showed a decreased concentration of Pb, Cu, Ni, Zn and Cr (Table 5), indicating a general lower water solubility of these elements and reduced environmental risk due to a decreased HMs leaching potential. This was also reported in several solid fractions of sewage sludge coagulated/flocculated with different chemicals as a result of metallic cations fixation (Liang et al., 2020; Xiong et al., 2018). However, the lower solubility of Cu and Zn, which can be considered as plant micronutrients, together with the lower P leachability could suggest a decreased nutrient efficiency of chemically treated solid fractions.

Consequently, remarkably increased EC values recorded in the treated eluates (almost 50% higher than in the control in each treated eluates) were most likely determined by the higher chlorides and soluble aluminum concentrations in the analyzed water extracts. In particular, PAC4 treatment increased leachable Cl by almost three times while leading to double contents of Al ionic forms if compared to untreated digestate. In particular, Al content in treated eluates reached a concentration of 85 µM (PAC4), which is reported to inhibit root growth in most agriculturally important plants (Samac and Tesfaye, 2003; Shetty et al., 2020; Silva et al., 2001). Nevertheless, it must be pointed out that the cited sources refer to Al^{3+} solutions derived from acidic soils (with pH values slightly higher than 4), which is not the case for this study, where the pH of the eluates resulted slightly alkaline. This aspect could have led to a different speciation of soluble Al towards less phytotoxic ionic forms (e.g., $Al(OH)^{2+}$ or $Al(OH)_2^{+}$) (Shetty et al., 2020).

Lower values of EC50 (% vol/vol) were in fact calculated based on the outcomes from the performed plant bioassays (Table 5). Further, dose-response curves of treated eluates were not characterized by the typical biostimulation behavior characterizing digestates at the lower dilutions (Pivato et al., 2016). Besides possible aluminum direct phytoxicity, also high level of EC and salinity characterizing digested residues, could be the main cause of acute phytotoxicity (i.e., inhibition of seed germination and root elongation) (Bona et al., 2020; Pivato et al., 2016; Teglia et al., 2011; Tigini et al., 2016). In this context, because of the lower leachable (i.e., bioavailable) concentration of HMs, higher phytotoxicity of treated solid fractions seems to be more correlated with increased salinity of tested eluates, indirectly determined by the soluble Al and Cl^- concentrations, resulting from the application of tested products.

4. Conclusions

The effects of CES were investigated at lab-scale when applied on OFMSW digestate derived from a full scale AD plant, in terms of i) improved TSS reduction in the liquid fractions, which can improve the efficiency of further membrane treatment and ii) reached overall environmental quality of solid fraction to assess possible impacts due to its agricultural reuse.

All applied CES treatments demonstrated to be effective when applied on OFMSW digestate, in terms of improved TSS reduction, which can result in significantly improved membrane filterability. In particular, high dosages of PAC and DEED led to the highest TSS removal rate in the liquid fractions separated after centrifugation. In addition, investigated CES treatments led to remarkable P and HMs removal from the liquid fractions, which likely migrated to the solid fractions.

However, no significative changes in P and HMs content were noted in the conditioned solid fractions, suggesting a minor role of the mass extra-contributes flocculated from the treated liquors. Also, treated biosolids showed increased organic carbon and nitrogen content, likely related to the residual amines polymers. The eventual polymeric origin of the higher nitrogen content was likely supported in the water extracts derived from the leaching tests performed on the treated solid fractions. This suggests the need to further investigate possible long term ecotoxicity to the soil compartment related with land-spreading of biosolids undergone CES. However, treated eluates highlights lower P and HMs leachability, likely decreasing the nutrient efficiency of treated fractions. Nevertheless, plant bioassays demonstrated higher phytotoxicity of the conditioned biosolids if compared with untreated digestate, which can be explained both by the direct action of reported soluble Al concentrations in the treated eluates and the higher salinity values recorded in the relative water extracts, indirectly related with increased chlorides and aluminum concentrations resulting from PAC dosing.

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Paper IV

Beggio, G., Bonato, T., Schievano, A., Garbo, F., Ciavatta C., Pivato, A. Agricultural application of digestates derived from agricultural and municipal organic waste: a health risk-assessment for heavy metals. Submitted to Human and Ecological Risk Assessment: An International Journal (30/01/2021).

Agricultural application of digestates derived from agricultural and municipal organic wastes: a health risk-assessment for heavy metals

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Abstract

Digestate is considered as a potential component of CE-marked organic fertilizers. Also, digestate application to soil must not lead to significant risks to ecosystems and human receptors, as prescribed by the regulation protecting agricultural soil quality.

Outcomes are discussed of a human-health risk assessment performed on an agricultural site in North-East Italy undergoing yearly application of digestate derived from agricultural and municipal organic wastes. The assessment estimated the risk related to human intake of Heavy Metals (HM) present in crops grown on the investigated agricultural site. The soil-plant transfer model and toxicological parameters were used as recommended by the US Environmental Protection Agency. Eight different scenarios were investigated, according to different digestate type, soil background concentrations and application techniques.

Non-risky situations related to HMs intake resulted in all scenarios involving digestate application,. The totality of calculated non-carcinogenic Hazard Indexes (HI) and carcinogenic total risk ($RTOT_C$) resulted below 0.02 and 10^{-9}, respectively. Conversely, non-carcinogenic risks were associated with the considered background concentrations, with HIs up to 1.7 for child receptors, while carcinogenic risk was calculated below the concern threshold (i.e., $RTOT_C < 10^{-5}$). This is likely due to the conservative assumption which considered the HMs background concentrations as 100% available for the soil-plant transfer.

Keywords

Digestate, Heavy Metals, Human Risk Assessment, Soil-plant transfer, Background concentrations

1. Introduction

The use of mineral fertilizers is currently being discouraged because of decreasing worldwide natural pools of mineral resources, particularly for phosphorus, and the related negative environmental effects caused by the simultaneous use of fossil components for their production. Here, the decreased use of mineral products in agriculture can be compensated by the supplementary use of organic and organic-based fertilizers. Among them, digestate has already been acknowledged for its specific fertilizing properties and soil amendment potential together with its key role in supporting the bioeconomy. For these reasons, it has been included as a component to manufacture CE-marked organic fertilizer, whether compliance is ensured with quality requirements established in terms of chemical/biological parameters (e.g., Heavy Metals (HMs), PAHs, presence of pathogens, etc.) and with a positive list of allowed input feedstocks to the digester, including agro-industrial residues, manures, energy crops and separately collected Organic Fraction of Municipal Solid Waste (OFMSW) (European Parliament and European Council, 2019). While several sources are present in the scientific literature discussing the presence and potential impacts of HMs in digestate (Knoop et al., 2018; Kupper et al., 2014; Stürmer et al., 2020), information about the presence of organic pollutants are still scarce and should be better investigated (Beggio et al., 2019).

Besides products requirements, digestate application must be compliant with the law framework protecting the agricultural soil quality. Some countries, like Italy, have established legal thresholds on several soil chemical parameters to identify possibly occurred contamination on agricultural soil due to unsustainable fertilization practices (Ministero dell'Ambiente, 2019). The set of considered chemical concentrations in soil includes HMs, PAHs, pesticides, PCDDs/PCDFs and asbestos. These concentration limits, if exceeded, trigger the need to perform a site-specific human risk assessment related with the indirect exposure due to the ingestion of crops grown on the assessed agricultural site. The results of a risky situation can bring severe and legal consequences, such as criminal conviction for contamination and mandatory performance of remediation measures, often accompanied by reasonable open questions on who is the responsible of the pollution and of the cost of remediation. For these reasons, the implemented modeling approach should be based on realistic assumptions (i.e., with a fair degree of precaution) and scientific-sound reasoning.

The Italian law framework establishes a tiered procedure to perform the human risk assessment for agricultural sites, consisting in 3 different steps, which can be performed in sequence or alternatively according to the available data (Ministero dell'Ambiente, 2019). The first step consists on the comparison between concentrations of chemicals measured in the considered plant edibles (i.e., fruits, root and non-root plants) and the limits established in the relative food regulation. Whether these data or limits are missing, a second step, called "EU approach", can be performed, by comparing the value of the contaminants intake rate through crops ingestion with a toxicological parameter expressed in terms of contaminant-specific or Tolerable Daily Intake (TDI) or Tolerable Weekly Intake (TWI) (EFSA, 2020). If these latter are not available (as in the case of the list of chemicals required by the aforementioned regulation), the third tier allows to estimate risk according to the well-known approach of USEPA, based on the comparison between the contaminant intake by crops ingestion and the specific toxicological parameters assessing possible non-carcinogenic and carcinogenic effects, i.e., the Oral Reference Dose (oRfD) and the Oral Slope Factors (oSF), respectively (USEPA, 2020, 2019, 1996).

The direct measure of chemical concentrations in food is useful to minimize the uncertainties related with risk estimations, but these data are often not available. In this case, soil concentrations of contaminants can be used to predict chemicals content in plants through the application of soil-plant transfer models. Assuming equilibrium conditions, these models provide simple contaminant-specific linear relationships (Eq. 1) between the concentrations in soil C_s and plants C_{veg} through the definition of the so-called "transfer factor" coefficient F_T

$$F_T = \frac{C_{veg}}{C_s} \qquad \text{(Eq. 1)}$$

Currently, two different types of transfer models are usually applied, according to the organic or inorganic nature of the contaminant. For organic species, two formulations are available providing the F_T as a function of the lipophilicity of the compound. Due to their dependence on octanol-water partition coefficient K_{ow}, these approaches cannot be applied to inorganic contaminants (e.g., HMs). The first one, introduced by Travis and Arms (1988), can be applied only for above-ground parts of the plants ("non-root" vegetables). Besides, it presents other limitations, due to the fact that i) the results cannot be adjusted according to different values of soil organic carbon content and ii) no root uptake is considered, thus doubts could arise from the possible contributions to the plant concentration due to atmospheric deposition or foliar uptake of gaseous chemicals. To solve these issues, a second approach by Briggs et al (1982) is suggested by the USEPA with some modifications, applied to consider influence of different values of organic carbon content in the soil (Spence and Walden, 2001). Only for inorganic compounds, the model developed by Baes III et al., (1984) allows to estimate the transfer factor for both "root" and "non-root" plants based on an exponential model of the dissociation constant K_d. Also, the European Chemicals Bureau proposed an approach based on a conceptualized uptake from pore water and air into a consistent, one-compartment model, considering a soil-plant-air system. (European Commission, 2003a). Nevertheless, this model is considered too complex, and it has a numerical solution (European Commission, 2003a; Spence and Walden, 2001). Here, the issue related with the bioavailability of soil background content of HMs to the soil-plant transfer should be better investigated to refine the reliability of the outcomes from the human risk assessment procedures (Díez et al., 2009; Zhong and Jiang, 2017).

In this paper, the results derived from a human risk assessment are presented to i) check the compliance of digestate land spreading with the Italian regulation on contaminated agricultural soils and ii) discuss how resulting risk estimations can be influenced by the applied assumptions. According to the aforementioned regulation and data availability, the authors focused on the indirect exposure to HMs in crops grown on an agricultural site fertilized with digestate. Digestate characterization data were taken from the outcomes of a statistical analysis previously performed by the authors (Beggio et al., 2019). Different digestate application methods were investigated. Further, the considered agricultural site was assumed characterized by the soil background concentrations derived for the Brenta river alluvial area (Veneto Region, North-East Italy) by the regional environmental agency (ARPAV, 2019). In particular, the USEPA approach was selected to conduct the assessment and the Baes III et al., (1984) was implemented to simulate the soil-to-plant transfer.

2. Materials and Methods

2.1. Conceptual model

According to the risk-assessment procedure established in the Italian regulation on agricultural contaminated sites (Ministero dell'Ambiente, 2019), this study identified a conceptual model (Figure 1) considering the indirect exposure of two recipients (i.e., average Italian adult and child) to the bioavailable HMs possibly found in i) a portion of agricultural soil undergone to the application of different types of digestate and ii) a portion of agricultural soil generally occurring in the Brenta river alluvial area (Veneto Region, North-East Italy). Here, the oral intake of crops cultivated in the investigated soil portion was considered as the indirect exposure pathway. The transfer of contaminants from the soil to the edible part of the plant was modeled according to the sole soil-water partition coefficient, defined as “the ratio of elemental concentration in soil to that in water in a soil-water system at equilibrium” (Baes III et al., 1984). Two types of edible plants were considered: non-root (e.g., leaves and stems) and root(e.g. tubers and seeds). Consumption of pasta and bakery products were not taken into account, since they usually derive from transformation of crops likely not cultivated in the area under investigation.

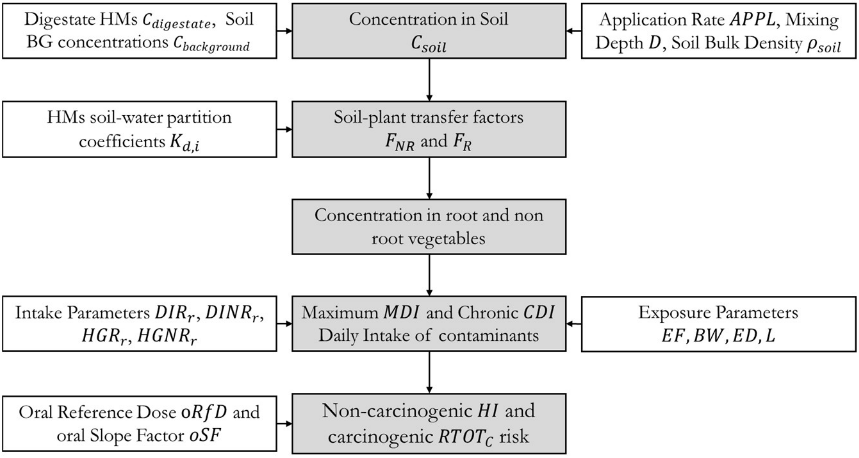

Figure 1. Conceptual model of the performed human risk assessment procedure.

2.2. Hazard identification

2.2.1. Digestates characterization and soil background concentrations

The human health risk assessment was performed considering the HMs content of seven HM (Cr, Pb, Cd, Ni, Cu, Hg, Zn) including two oxidation states of Cr (Cr III and Cr VI) of the soils after digestate land-spreading on agricultural fields. Three different types of digestates were defined in terms of HM concentrations (see Table 1). The first one, named AGRO, represents an average digestate originating from the anaerobic digestion (AD) of agro-industrial feedstocks (e.g., wastewaters from agro-food activities, olive mills wastewater, animal manures, etc.). The second one, named OFMSW, stands for an average digestate derived from the AD of separately collected organic fraction of municipal solid waste. Data from the first two categories were collected from the mean concentration values calculated by a previous work, based on real digestate characterization data (Beggio et al., 2019). Currently, agricultural usage is allowed only for AGRO digestate, while OFMSW digestate land spreading is still forbidden by the specific Italian regulation (Ministero delle Politiche Agricole, 2016). However, this restriction is no more present within the updated European Directive regulating the marketing of fertilizers (European Parliament and European Council, 2019). Therefore, the third category, named LIM, represents those digestates characterized by HMs contents equivalent to the concentration limits established for the category "organic fertilizer" in the above mentioned EU Regulation for CE-marked fertilizer (European Parliament and European Council, 2019).

Further, the risk assessment procedure was conducted on a soil type (BG) considered as characterized by the background concentrations of investigated HMs, estimated for the Brenta river alluvial area (Veneto Region, North-East Italy) (ARPAV, 2019). Finally, a second soil substrate (ASV) was included for the analysis, being representative of the Agricultural soil Screening Values, which, if exceeded, trigger the need to perform a Site Specific Risk Assessment, as established by the Italian regulation on contaminated sites (Ministero dell'Ambiente, 2019).

It must be highlighted that, for each investigated digestate and soil type, the total value of the considered HM concentration was conservatively assumed to be 100% available to plant uptake, as no other detailed indications are present in the regulation and consulted technical guideline. Table 1 resumes the characterization data used for the risk assessment of the selected substrates.

*Table 1. Assumed HM concentrations of the digestate types (AGRO, OFMSW and LIM) and soil types (BG and ASV) used for the performed human health risk assessment. * Considered as Cr III. a Beggio et al., (2019); b European Parliament and European Council, (2019); c ARPAV, (2019); d Ministero dell'Ambiente, (2019). All data are expressed on a dry basis (TS). All HM are reported as total content. Total contents are assumed to be 100% available to plant uptake.*

Parameter	Unit		$C_{digestate}$		$C_{background}$	
		AGRO[a]	OFMSW[a]	LIM[b]	BG[c]	ASV[d]
Cr-tot	mg kg^{-1} TS	8.7	12.7	-	63	150
Cr VI	mg kg^{-1} TS	-	-	2	-	2
Pb	mg kg^{-1} TS	4.7	18.6	120	56	100
Cd	mg kg^{-1} TS	0.4	0.6	1,5	0,9	5
Ni	mg kg^{-1} TS	8.2	11.0	50	38	120
Cu	mg kg^{-1} TS	62.2	53.2	300	110	200
Hg	mg kg^{-1} TS	0.05	0.08	1	0.5	1
Zn	mg kg^{-1} TS	280	233	800	143	300

2.2.2. Contaminants characterization data

Information on contaminant-specific partitioning behavior and related human toxicity are needed to characterize the type of soil-plant transfer and assess the human exposure via oral intake. In this context, the values for the soil-water partition coefficients $K_{d,i}$ (L kg^{-1}) are presented in Table 2 for each considered HM and based on ISS and INAIL (2015) and USEPA (2020). Together, Table 2 includes the specific oral Reference Dose values 〖oRfD〗 _i (mg kg-1 d-1) and the oral Slope Factors $oRfD_i$ (mg kg^{-1} d^{-1}) for the non-carcinogenic and the carcinogenic effects, respectively, derived according to OEHHA (2011) and USEPA (2019). According to the used sources, only Cr VI and Pb are considered carcinogenic for the oral exposure, while the other are currently not referenced with a value specific for the oral intake (OEHHA, 2011).

Table 2. Soil-water partition coefficients and toxicity factors of the HMs considered in the risk assessment procedure. [a] *USEPA, (2020).* [b] *USEPA, (2019).* [c] *OEHHA, (2011).*

Parameter	K_d [a] (L kg^{-1})	$oRfD$ [b] (mg kg^{-1} d^{-1})	oSF [c] (mg kg^{-1} d^{-1})
Cr III	1.8E+06	1.5	-
Cr VI	1.9E+01	-	4.2E-01
Pb	9.0E+02	-	8.5E-03
Cd	7.5E+01	5.0E-04	-
Ni	6.5E+01	2.0E-02	-
Cu	3.5E+01	4.0E-02	-
Hg	5.2E+01	3.0E-04	-
Zn	6.2E+01	3.0E-01	-

2.3. Exposure assessment

2.3.1. Soil-plant transfer model

The used transfer model allowed to determine the amount of considered HMs adsorbed from soil to the plant system. For each considered digestate type (Table 1), the contaminant-specific concentration in soil resulting just after digestate application ($C_{soil,i}$) was determined according to the equation mentioned in European Commission (2003), as follows:

$$C_{soil,i} = (C_{digestate,i} * APPL)/(D/\rho_{soil}) \qquad \text{(Eq. 2)}$$

where:

$C_{digestate,i}$ (mg kg^{-1}TS) is the HM-specific concentration of the chosen digestate types (see Table 1);

$APPL$ (tTS ha^{-1}) is the yearly application rate of digestate, assumed occurring once in a year;

D (m) is the depth of the mixing horizon;

ρ_{soil} (g cm^{-3}) is the bulk density of the soil mixing horizon after digestate application.

In Eq. (2) complete and uniform soil-digestate mixing was assumed after the application. Further, no contributes from crops interception were taken into account, assuming that digestate spreading was performed on bare soil (i.e., no grassland) just before the sowing, consistently with good agricultural practices.

For each digestate type, a value of 0.4 kgTS m^{-2} y^{-1} was attributed to the application rate APPL. This value was assumed according to the maximum N application rate on non-vulnerable areas of 340 kg N ha^{-1} y^{-1} (Ministero delle Politiche Agricole, 2016) and the average between the mean N concentrations reported for AGRO and OFMSW, i.e., 65 gN kg^{-1}TS and 110 gN kg^{-1}TS, respectively (Beggio et al., 2019). Further, according to Pivato et al. (2016), two different soil-mixing procedures were considered, a “good practice” and a “bad practice”, characterized by a soil-digestate mixing layer D of respectively 0.2 and 0.04 m and a resulting soil bulk density ρ_{soil} of 1.25 kg dm^{-3} and 1.00 kg dm^{-3}, respectively. While the “good practice” is assumed to be representative of digestate sub-surface placement minimizing the possibility of volatile losses of ammonia as well as managing odor emissions (e.g., through injection trails), the “bad practice” configuration refers to the so-called “splash-plate” spreading, i.e., inadequate agricultural practices consisting in uneven over-surface distribution of digestate and uncontrolled N application rate (Figure 2). By assuming these two configurations, digestate can be considered “diluted” in soil with a resulting concentration of 0.2 and 1% (kgTS kg^{-1}TS), respectively, for the “good practice” and the “bad practice”.

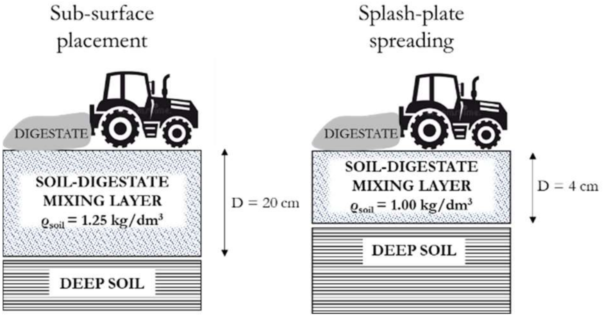

Figure 2. Graphical schematization of the mixing practices considered for the performed risk assessment procedure.

In Eq. (2) no background concentration were considered in the determination of the soil concentration, to assess the sole risk posed by digestate application. Conversely, the resulting contaminant-specific concentrations in soil ($C_{soil,i}$) for the two considered soil types (BG and ASV) were considered equal to the relative concentrations $C_{BG,i}$) reported in Table 1, as:

$$C_{soil,i} = C_{background,i} \tag{Eq. 3}$$

Due to their inorganic nature, no degradation in soil was assumed for the considered HMs concentration. Therefore, the calculated values for each $C_{soil,i}$ can be considered as Predicted Environmental Concentrations, usable as inputs for the risk assessment procedure.

The soil-plant transfer of the investigated contaminants was modeled through the use of the so-called soil-to-plant concentration factor. This factor is defined as a dimensionless parameter defined as the ratio of the concentration of a single compound in a plant, or a portion of it, to that in the soil. The soil-to-plant factors were calculated both for "non-root" (F_{NR}) and "root" crops (F_R) based on the contaminant-specific soil-water partition coefficient $K_{d,i}$ (see Table 2) according to the formulation proposed by Baes III et al. (1984):

$$F_{NR,i} = e^{[2.67-1.12\ln(K_{d,i})]} \quad \text{(Eq. 4)}$$

$$F_{R,i} = e^{[2.87-1.52\ln(K_{d,i})]} \quad \text{(Eq. 5)}$$

where:

$K_{d,i}$ is the i-th contaminant specific soil-water partition coefficient (see used values in Table 2).

2.3.2. Human exposure

The human exposure to the resulting concentration of the investigated contaminants in soil was determined based on the maximum possible intake of that specific contaminant, estimated according to the exposure parameters shown in Table 3 and the contaminant-specific soil-plant transfer factors (Eq. 4 and Eq. 5).

The used exposure parameters were derived from previous elaboration on food diet of different recipients in Italy (Leclercq et al., 2009). This study made use of parameters derived for both adults and children, whose food diet was assumed composed 100% by crops (both root and non-root) cultivated on the soil investigated.

For each considered i-th non-carcinogenic compound (i.e., Cd, Cr, Cu, Hg, Ni, Zn) and for each r-th recipient (i.e., adult and child), the Ingestion Exposure $ENC_{i,r}$ (kgTS kg^{-1} d^{-1}) and the Maximum Daily Intake $MDI_{i,r}$ (mg kg^{-1} d^{-1}) of root and non-root was calculated according to the following formula:

$$ENC_{i,r} = \frac{(FR_i*DIR_r*HGR_r+FNR_i*DINR_r*HGNR_r)*EF}{BW_r*365} \quad \text{(Eq.6a)}$$

$$MDI_{i,r} = ENC_{i,r} * C_{soil,i} \qquad \text{(Eq.6b)}$$

Where:

FR_i is the i-th contaminant specific soil-to-plant transfer factor for "root" crops (Eq. 4);

FNR_i is the i-th contaminant specific soil-to-plant transfer factor for "non-root" crops (Eq. 5);

DIR_r (kgTS d^{-1}) is the r-th recipient-specific root crops vegetable daily intake;

$DINR_r$ (kgTS d^{-1}) is the r-th recipient-specific non-root crops daily intake (kgTS d^{-1});

HGR_r (%) is the r-th recipient-specific homegrown fraction of "root" crops;

$HGNR_r$ (%) is the r-th recipient-specific homegrown fraction of "non-root" crops;

EF (d y^{-1}) is the exposure frequency, equal for each recipient;

BW_r (kg) is the r-th recipient-specific body weight.

$C_{soil,i}$ (mg kg^{-1}) is the resulting soil concentration of non-carcinogenic compounds after digestate application (see previous paragraph 2.3.1)

Intake for the considered i-th carcinogenic element (i.e., CrVI and Pb), the Lifelong Ingestion Exposure EC_i (kgTS kg^{-1} d^{-1}) and the Chronic Daily Intake CDI_i (mg kg^{-1} d^{-1}) of both root and non-root crops was used for both considered recipients, and determined as follows:

$$EC_i = \frac{(ENC_{i,child} * ED_{child}) + (ENC_{i,adult} * ED_{adult})}{L} \qquad \text{(Eq.7a)}$$

$$CDI_i = EC_i * C_{soil,i} \qquad \text{(Eq.7b)}$$

where:

ED_{child} and ED_{adult} are the exposure durations for the considered recipients;

L (d) is the lifetime;

$C_{soil,i}$ (mg kg^{-1}) is the resulting soil concentration of carcinogenic compounds after digestate application (see previous paragraph 2.3.1)

The values used in the Equations (6a) and (7a) are listed in Table 3.

Table 3. Exposure parameters used for the human exposure assessment. [a] *Leclercq et al., (2009).*

Parameter	Unit	Recipient	
		Adult[a]	Child[a]
BW	kg	70	15
EF	d y^{-1}	350	350
ED	y	24	6
L	d	25,550	25,550
DIR	kg TS d^{-1}	0.0065	0.0020
$DINR$	kg TS d^{-1}	0.0386	0.0165
HGR	%	100%	100%
$HGNR$	%	100%	100%

2.4. Risks calculation for the considered scenarios

The risk assessment related with the intake of the i-th non-carcinogenic compound was estimated by the so-called Hazard Quotients $HQ_{r,i}$ (USEPA, 1996), calculated for each r-th recipient based on the contaminant-specific i-th Maximum Daily Intake $MDI_{i,r}$ (mg kg^{-1} d^{-1}) (Eq. 6b) and the related value of oral Reference Dose $oRfD_i$ (mg kg^{-1} d^{-1}) (Table 2), as:

$$HQ_{r,i} = \frac{MDI_{i,r}}{oRfD_i} \qquad \text{(Eq. 8)}$$

The risk related with human exposure due to the intake of the i-th carcinogenic substance $R_{C,i}$ was instead calculated for each recipient by multiplying the Chronic Daily Intake CDI_i (mg kg^{-1} d^{-1}) (Eq. 7b) with the related value of the oral Slope Factor oSF_i (mg kg^{-1} d^{-1}) (Table 2), thus:

$$R_{C,i} = CDI_i * oSF_i \qquad \text{(Eq.9)}$$

An estimation of the total risk associated with all considered non-carcinogenic substances to the r-th recipient was provided by the so-called Hazard Index HI_r (USEPA, 1996), determined as follows:

$$HI_r = \sum HQ_{r,i} \qquad \text{(Eq. 10)}$$

Also, the total risk derived from specific exposure to carcinogenic substances $RTOT_C$ was determined as the sum of each risk value $R_{C,i}$, thus:

$$RTOT_C = \sum R_{C,i} \qquad \text{(Eq. 11)}$$

In this context, if calculated value for HI_r results higher than the unity, there is enough evidence to admit possible manifestation of non-carcinogenic impacts on the specific recipient. For carcinogenic substances, the limit for the occurrence of carcinogenic effects is usually set for $RTOT_C$ between 10^{-6} and 10^{-4} (USEPA, 1996). A threshold of 10^{-5} was used in this study to interpret the calculated $RTOT_C$.

The recipient-specific Hazard Index HI_r and the total carcinogenic risk $RTOT_C$ were calculated for 8 different scenarios, identified according to the different source of HMs in soil (digestate or background concentration) and digestate application methods. The considered scenarios are resumed in Table 4.

Table 4. Scenarios considered for the risk assessment procedure. [a] these scenarios were used as a base case to perform the sensitivity and uncertainty analysis (see Paragraph 2.5).

Scenario	Source (Table 1)	Digestate application (Figure 2)
S1	AGRO	Splash-plate spreading
S2	AGRO	Sub-surface placement
S3[a]	OFMSW	Splash-plate spreading
S4	OFMSW	Sub-surface placement
S5	LIM	Splash-plate spreading
S6	LIM	Sub-surface placement
S7[a]	BG	-
S8	ASV	-

2.5. Sensitivity and uncertainty analysis

A sensitivity analysis was performed to assess the parameters most influencing the calculated risk values. Further, an uncertainty analysis was conducted to estimate the influence on the calculated risk values of the uncertainty related with model's inputs variables. Both analyses were implemented on scenarios 3 and 7 (see Table 4) by making use of the Microsoft Excel plug-in Oracle Crystal Ball™.

The most influencing parameters were identified through the Tornado chart, which graphically depicts the variability of the calculated risk values (both non-carcinogenic and carcinogenic) according to the imposed variation of the used parameters. In particular, the parameters were varied by ± 10% to build the Tornado chart.

A Monte Carlo sampling was performed with 10,000 iterations to conduct the uncertainty analysis. Probability distributions and statistical dependency of the involved input variables must be assumed. Here, all selected input variables are assumed to be independent between each other. HMs concentrations of both digestates and soil types considered in the analysis were assumed as normally distributed, as suggested in Beggio et al. (2019). Probability distributions for the exposure parameters were assumed according to Spence and Walden (2001). The distributions for the remaining parameters were conservatively assumed as uniform.

3. Results and Discussion

The performed risk assessment procedure allowed to estimate both the non-carcinogenic and carcinogenic risk related to the indirect exposure of adult and child recipients to HMs in soil after the application of different types of digestate and according to two soil spreading and mixing techniques. Further, two soil types were equivalently assessed for risk: one proxying the background values characterizing the site where digestate application is assumed to occur and the other representative of the HMs concentrations of an agricultural site declared as contaminated according to the involved regulation (Ministero dell'Ambiente, 2019).

Only the oral intake of vegetables cultivated in the investigated soil portion was considered as the indirect exposure pathway, as prescribed by the Italian regulation (Ministero dell'Ambiente, 2019). In this context, the oral intake, particularly of site-grown vegetables, already proved to be the major way of exposure of the considered receptors to the contaminants present in soil after different biosolids application (INERIS, 2008; Martí et al., 2016). Further, the risk related to the oral intake is currently considered a key pathway for the dissemination of the risk assessment outcomes in terms of "risk perception" (CTTC, 2016).

The performed risk assessment procedure was based on exposure values consistent with the Italian scenario (Leclercq et al., 2009). Among the most up to date references, the so-called USEPA "Exposure Factors Handbook" (EFH) reports root and non-root crops intake values estimated according to the most recent market basket survey of the US population (USEPA, 2018). These values are currently suggested also to perform human health risk assessment procedures by the RISC4 software (Spence and Walden, 2001). Accordingly, the intake rate reported in the EFH are higher than the values reported for the Italian scenario. However, only a fraction of the reported intake is considered to be grown on the analyzed site (i.e. HGR_r and $HGNR_r$ of 10% for Spence and Walden (2001) vs. 100% assumed here), thus leading to values almost similar to the ones used in this study. Furthermore, the exposure durations EF assumed here for the considered recipients (i.e., 24 and 6 years for adults and children, respectively) are more conservative than what is suggested in Spence and Walden (2001), i.e., 9 and 6 for adult and child recipients, respectively.

The results calculated for non-carcinogenic risk are reported in Table 5 for both adult and child recipients, while carcinogenic risk values are listed in Table 6.

Table 5. Calculated non carcinogenic $HQ_{r,i}$ and HI_r associated with indirect exposure of adult and child recipients to concentrations of some resulting HMs concentrations in soil according to the different assumed scenarios.

	Scenario							
	S1	S2	S3	S4	S5	S6	S7	S8
Adult								
Cr III	4.39E-11	7.03E-12	6.40E-11	1.02E-11	0.00E+00	0.00E+00	3.17E-08	7.54E-08
Cr VI	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E-03	3.20E-04	0.00E+00	2.00E-01
Cd	5.03E-04	8.05E-05	7.29E-04	1.17E-04	1.89E-03	3.02E-04	1.17E-01	6.29E-01
Ni	3.03E-04	4.85E-05	4.08E-04	6.52E-05	1.85E-03	2.96E-04	1.40E-01	4.44E-01
Cu	2.32E-03	3.72E-04	1.99E-03	3.18E-04	1.12E-02	1.79E-03	4.11E-01	7.47E-01
Hg	1.59E-04	2.54E-05	2.54E-04	4.06E-05	3.18E-03	5.08E-04	1.62E-01	3.18E-01
Zn	7.28E-04	1.16E-04	6.06E-04	9.69E-05	2.08E-03	3.33E-04	3.72E-02	7.80E-02
HI_{adult}	4..02E-03	6..43E-04	3..98E-03	6..38E-04	2.22E-02	3.55E-03	8.68E-01	2.42E+00
Child								
Cr III	8.76E-11	1.40E-11	1.28E-10	2.04E-11	0.00E+00	0.00E+00	6.31E-08	1.50E-07
Cr VI	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.92E-03	6.28E-04	0.00E+00	3.92E-01
Cd	9.93E-04	1.59E-04	1.44E-03	2.30E-04	3.72E-03	5.96E-04	2.31E-01	1.24E+00
Ni	5.98E-04	9.57E-05	8.05E-04	1.29E-04	3.65E-03	5.84E-04	2.77E-01	8.76E-01
Cu	4.57E-03	7.32E-04	3.91E-03	6.26E-04	2.21E-02	3.53E-03	8.09E-01	1.47E+00
Hg	3.13E-04	5.01E-05	5.01E-04	8.02E-05	6.26E-03	1.00E-03	3.19E-01	6.26E-01
Zn	1.44E-03	2.30E-04	1.20E-03	1.91E-04	4.11E-03	6.57E-04	7.34E-02	1.54E-01
HI_{child}	7.92E-03	1.27E-03	7.85E-03	1.26E-03	4.37E-02	7.00E-03	1.71E+00	4.76E+00

Table 6. Calculated carcinogenic risk values $R_{C,i}$ *and* $RTOT_C$ *associated with indirect exposure of the considered recipients to some HM concentrations in soils according to the different assumed scenarios.*

	Scenario							
	S1	S2	S3	S4	S5	S6	S7	S8
Cr VI	0	0	0	0	3,53E-09	5,65E-10	0	3,53E-07
Pb	2,12E-12	3,39E-13	8,45E-12	1,35E-12	5,45E-11	8,72E-12	2,54E-09	4,54E-09
$RTOT_C$	2,12E-12	3,39E-13	8,45E-12	1,35E-12	3,58E-09	5,73E-10	2,54E-09	3,57E-07

Scenarios involving the application of the considered digestate types (i.e., S1 to S6) are characterized by significantly lower HI_rs values than the threshold of concern, here assumed as the unity, for both adult and child recipients (Table 5). In particular, the $HQ_{r,i}$s related with Cu and Zn exposure in S1 to S4 scenarios count for almost 70% on average of the specific HI_rs, thus explaining the fact that AGRO digestates, being characterized by higher concentrations of these elements, determined relatively higher HI_rs than OFMSW digestates. Conversely, the highest $HQ_{r,i}$s in S5 and S6 scenarios were determined by the Cu and Hg exposure, accounting for 50% and 14% of the HI values, respectively. However, the highest HI_r value was calculated in S5 for both recipients (0.02 and 0.04 for adult and child, respectively), i.e., in the considered "worst case scenario", consisting in the application of a digestate characterized by max HMs content for agricultural reuse and spread through bad/inefficient practices (e.g., splash-plate soil distribution). Further, scenarios characterized by "good practice" application (i.e., S2, S4 and S6) led to almost 85% lower risk values, if compared with "bad practice" scenarios (even if these latter resulted in non-risk situations whatsoever). Besides the reported odors minimization and ammonia decreased leaching potential (Orzi et al., 2018; Riva et al., 2016), this last consideration should draw the public attention (i.e., law-makers and controllers) also on the performed agricultural practice behind digestate utilization, other than focusing on the sole achievable digestate quality.

Similarly, carcinogenic risk values $RTOT_C$ calculated for the scenarios S1-S6 were all reported remarkably below the assumed limit triggering the possibility of occurrence of carcinogenic effects (i.e., 10-5), ranging from 3.4E-13 (S2) to 3.6E-09 (S5) (Table 6). In particular, the reported $RTOT_C$ were mainly determined by Pb content of digestate. In fact, the scenarios S1 and S4 did not consider the $R_{C,i}$ related with CrVI, which is instead taken into account in S5 and S6, according to the prescriptions of European Parliament and European Council (2019). Nevertheless, this latter can be considered a conservative assumption, since CrVI would immediately reduce to CrIII after soil application, as instead assumed in the risk procedure made by INERIS (2008) and reported elsewhere (Stürmer et al., 2020).

These findings highlight both the non-significant risk related to digestate spreading on agricultural field and the overprotective nature of the established limits on avoiding possible situation of concern (European Parliament and European Council, 2019). Anyway, the reported results are consistent with a previous study, which also declared as negligible the risk related with human exposure through vegetable intake to HMs in soil after application of quality-assured digestate from OFMSW (Longhurst et al., 2019). These considerations are explained in the inherent scientific literature by a proved general compliance of quality-assured digestate to the established legal requirements and according to the fact that also the application of mineral fertilizers and the atmospheric deposition should be considered as additional HMs loadings to agricultural soils (Beggio et al., 2019; Kupper et al., 2014; Stürmer et al., 2020).

Conversely, resulting non-carcinogenic risk estimations show a possible situation of concern for the adult receptors related with the exposure scenario S8 (i.e., HI_{adult} of 2.2) and a border condition for S7 (i.e., HI_{adult} of 0.9) (Table 6). Further, both S7 and S8 could led to possible occurrence of non-carcinogenic effects to child receptors (i.e., HI_{child} higher than the unity) (Table 6). The calculated values of HI_{child} for S7 and S8 are depicted in Figure 3 as the sum of the contaminant-specific contributions (i.e., HQs).

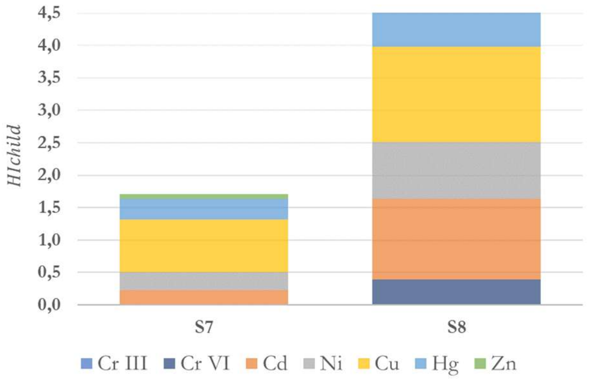

Figure 3. Total and substance-specific non-carcinogenic risk of child receptors calculated for S7 and S8 scenarios.

Here, while the risk for S7 is mainly due to the soil content of, in order, Cu>Hg>Ni>Cd>Zn>CrIII, the calculated risk value of S8 is influenced by children indirect exposure to Cu>Cd>Ni>Hg>Cr VI>Zn>CrIII. Instead, no carcinogenic risk was reported for these two exposure scenarios (Table 8). In summary, the oral exposure to the HMs present in the soil as background concentration (S7) and proxying a contaminated agricultural site (S8) determined higher non carcinogenic risk situations if compared to the sole HMs contribution due to digestate application. These results were consistent with the findings of a previous human risk assessment procedure evaluating the exposure to HMs after sewage sludge application (INERIS, 2008). These results were consistent with the findings of a previous human risk assessment procedure evaluating the exposure to HMs after sewage sludge application (INERIS, 2008).

Accordingly, the outcomes related to S8 suggest the consistency between the values imposed by the Italian regulation on contaminated agricultural sites and their specific aim, i.e., to identify a situation of concern and to trigger, if actually exceeded, the need to perform site-specific risk assessment and possible site remediation (Ministero dell'Ambiente, 2019).

On the contrary, the risky situations estimated for S7 seem to conflict with the current definition of the background concentrations. In fact, background values are here intended as the HMs concentrations found in the superficial soil horizon (layer) of an agricultural site, resulting from the past deposition and accumulation of HMs due to both pedogenetic and anthropogenic inputs, not affecting soil functions or posing significant environmental impacts (ARPAV, 2019). Consistently, no situation of concern should be expected when assessing the risk related with an average background soil type. Here, it must be recalled that this risk assessment procedure was performed assuming that each reported HM concentration is considered "totally bioavailable", i.e., totally adsorbable by plant roots (according to the applied soil-plant transfer model), thus potentially uptaken by the recipient through vegetable ingestion. The same assumption was made by other human risk assessment procedure for agricultural land spreading of sewage sludge (INERIS, 2008). However, the bioavailable fraction of each HM is likely lower than its measurable total content. Also, it is determined by the relative equilibrium conditions characterizing the specific soil under investigation. Therefore, this assumption, and similarly the outcomes from the risk assessment procedure concerning the scenario S7, can be considered too conservative. Overestimated risk due to the use of total concentration of trace elements for regulatory purposes was also already reported in the scientific literature (Díez et al., 2009; Zhong and Jiang, 2017). A possible solution could be to measure the real bioavailable fractions for each considered HM through the application of leaching tests on a soil portion making use of different leaching media characterized by increasing solubilization potential, e.g., distilled water, acetic acid and DTPA solutions and aqua regia following the technical standard ISO (2011). According to a preliminary study conducted by the Italian regional environmental control agency ARPAV (2019) on the soil types investigated in this study, the bioavailable fractions of Cu and Ni are suggested around 10% of the total content, while no bioavailable fraction for Hg was recorded. Here, the performance of the risk assessment procedure upon these last preliminary findings would have determined a non-risky situation for non-carcinogenic HMs.

The sensitivity analysis performed on S3 and S7 scenarios provided the Tornado charts depicted in Figure 3. Results revealed that the variables $APPL$, D and ρ_{soil} are crucial in influencing both the non-carcinogenic (Figure 4a) and carcinogenic (Figure 4b) risk associated with digestate spreading (i.e., scenario S3), together explaining almost 45% and 25% of the total variability of HI_{adult} and $RTOT_{C}$, respectively. Also, the estimated non-carcinogenic risk for the scenario S7 is likely afflicted mainly by the variability of the involved exposure factors (i.e., about 72% of the HI_{adult} variability explained by BW, EF, $DINR$ and HGR) and on a lesser extent by Cu characterization data (Figure 4c). Conversely, the variability affecting characterization data of the carcinogenic Pb (i.e., $K_{d,Pb}$ and 〚oSF_{Pb}) and its assumed soil background concentration (i.e., $C_{background,Pb}$) could be the major responsible for the carcinogenic risk estimation.

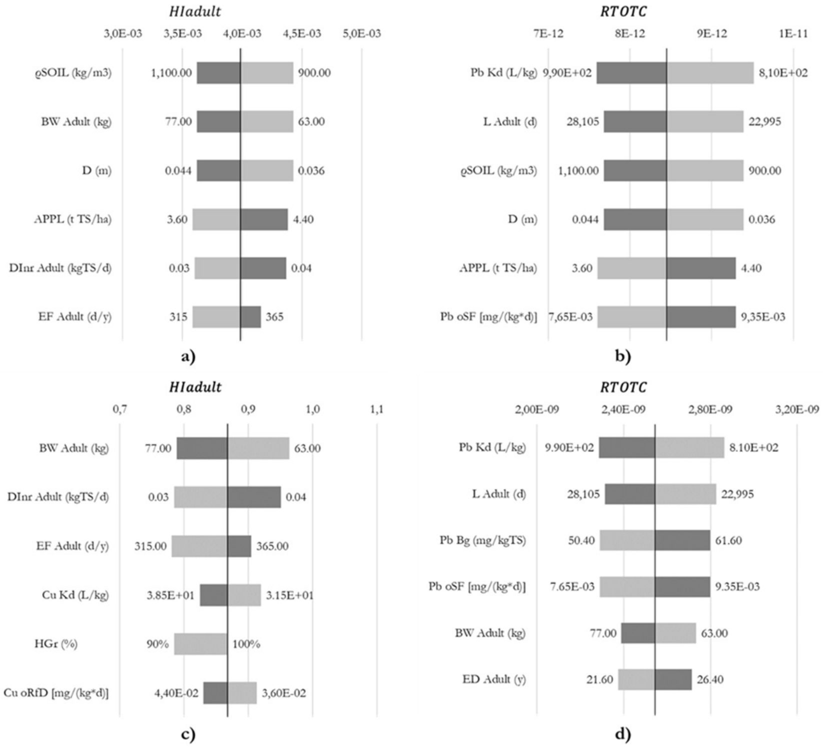

Figure 4. Tornado charts determined for HI_{adult} and $RTOT_C$ in S3 (Figure 4a and Figure 4b) and S7 (Figure 4c and Figure 4d). The dark-grey bar indicates a risk value calculated by assuming the variable's lower bound (i.e., case base -10%), while the light-grey bar indicates a risk value produced by the variable set as upper bound (i.e., case base + 10%). The black vertical bar represents the risk values for the case base (see Table 6 and Table 7).

Monte Carlo simulations performed on S3 and S7 scenarios allowed to estimate the range related to each risk values according to the probability distributions of values assumed for the model's inputs. To do so, each risk value within the calculated is assigned with a probability to achieve it. In particular, Figure 5 displays the Probability Density Function calculated for non-carcinogenic risk related with adult exposure to soil background concentrations in the site under investigation (S7), since the base case resulted in a boarder conditions (i.e., HI_r almost 0.9). Here, the base case, was not characterized by the highest probability to occur. Also, it must be highlighted that, according to the depicted results, there is an almost 30% possibility that the risk related to the human exposure to non-carcinogenic substances could be higher than the unity, thus identifying possible situation of concern.

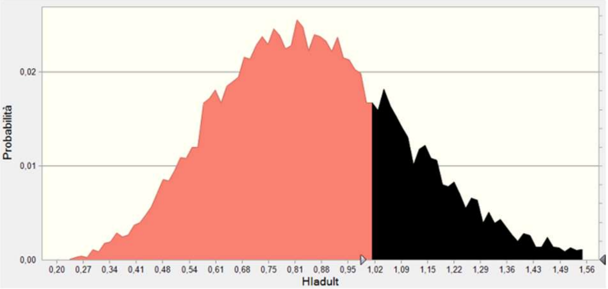

Figure 5. PDF for HI_{adult} calculated for scenario S7 through the application of a Monte Carlo analysis (n = 10,000). The black area represents the cumulative probability to obtain a value for HI_{adult} higher than the unity, according to the assumed probability distributions of values assigned to the model input.

4. Conclusions

This paper discusses the results derived from a human-health risk assessment procedure, considering indirect exposure to HMs in vegetables grown on an agricultural site undergone fertilization through application of agricultural and OFMSW digestates.

Independently from digestate type and mode of application, both carcinogenic and non-carcinogenic risk values were calculated remarkably under the assumed thresholds identifying possible situations of concerns for the considered human recipients (i.e., adults and children). However, the resulting total risk was mainly determined by the digestate Cu and Ni contents, which led to higher risk related with agro-industrial digestates, these latter being characterized by higher concentrations of these elements if compared with OFMSW digestates. Further, scenarios investigating digestate application through “good practice” led to almost 85% lower risk values than “bad practice” scenarios. Conversely, the oral exposure to the HMs present in the analyzed soil as background concentrations and proxying a contaminated agricultural site could lead to significant non-carcinogenic risk situations if compared to the sole HMs contribution due to digestate application. In particular, the reported non-carcinogenic risk was mainly due to Cu, Hg, Ni and Cd content of soil. These results are likely explained according to the applied assumption on considering the total background concentration of the considered HMs as available for plant uptake. This assumption can be considered overprotective and led to unrealistic risk estimation. Determining the bioavailable fractions of considered contaminants, e.g., through the application of leaching tests exploiting leaching media characterized by different solubilization potential, could increase the reliability of the outcomes of human risk assessment procedures.

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PART III

Additional Papers

Additional Paper I

Pivato, A., Beggio, G., Raga, R., Soldera, V., 2019. Forensic assessment of HP14 classification of waste: evaluation of two standards for preparing water extracts from solid waste to be tested in aquatic bioassays. Environ. Forensics 20, 275–285. https://doi.org/10.1080/15275922.2019.1630517

ENVIRONMENTAL FORENSICS
https://doi.org/10.1080/15275922.2019.1630517

Forensic assessment of HP14 classification of waste: evaluation of two standards for preparing water extracts from solid waste to be tested in aquatic bioassays

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ABSTRACT

Misclassification of waste hazardousness could lead waste operators to be charged of illegal trafficking. Among different aspects characterizing waste hazardousness, Ecotoxicity is acknowledged as the most frequent Hazard Property classifying wastes as hazardous. In this context, a conventional, scientifically based and agreed-upon procedure for ecotoxicity assessment can help forensic activities to verify the conditions of possibly occurred illegal trafficking of waste.

Currently, European Regulation declares a waste ecotoxic according to a calculation method based on its chemical composition. Whether outcomes from calculation method would result in an unrealistic assessment, results from ecotoxicity testing can be used for waste hazardousness classification. However, each Member State is allowed to establish a specific experimental procedure, thus resulting in a fragmented legal framework regulating ecotoxicological testing. This study gives a description of the two main approaches followed by European Member States in regulating ecotoxicity assessment of wastes. This work reports how both investigated approaches address three main methodological issues: which sample preparation standard must be carried out, which test battery should be performed and which concentration limits will trigger hazard classification.

Further, a comparison is presented between two different standardized protocols (GHS Annex X and EN 14735) for leaching tests of solid wastes, performed on Automotive Shredder Residues (ASR) samples to obtain water extracts to be further tested in aquatic bioassays. EN 14735 resulted more conventional for routine waste classification efforts, both in terms of achieved sample representativeness and time needed to derive test portions. Then, results from chemical physical analyses on derived water extracts suggested that the lower Liquid-to-Solid ratio required by EN 14735 could have played a key role in determining the resulting higher contaminant concentrations. For this reason, it could also be considered as the most conservative approach for further testing on aquatic organisms. However, this assumption must be further validated by further research on different waste streams, speciation mechanisms of dissolved contaminants, the role of pH adjustment of the test portion and the influence of specific dilution media.

KEYWORDS
hazard classification of waste; ecotoxicity of waste; illegal trafficking of waste; leaching test; ecotoxicity testing

Introduction

The lack of legal clarity and international harmonization of waste classification criteria is a major driver of unlawful waste management practices (Baird et al., 2014). Management of non-hazardous residues can offer savings up to 300 per cent, compared to operations related to hazardous wastes (UNEP, 2015). Further, the scarce number of disposal sites authorized for hazardous waste management can force companies to transboundary movement of waste to countries where different classification system is in force. Consequently, either unintentionally or intentionally circumventing waste classification can avoid compliance costs, but at the same time can negatively influence market competition and bring environmental impacts. For these reasons, management of misclassified waste streams can lead involved businesses to be charged of illegal waste trafficking (UNEP, 1989).

In Europe, the European "List of Waste" (LoW) allows to classify every produced waste stream either as absolute hazardous, absolute non-hazardous or "mirror", i.e., waste type that can be classified for

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hazardousness only after further compliance assessment (European Commission, 2000). In this context, a lack of clarity in classification criteria can provide an opportunity to falsely declare hazardous waste as non-hazardous to gain illegal economic benefits (Baird et al., 2014). To understand the scale of the topic, in 2016, the total production of "mirror-entry" wastes, which need classification efforts, is assumed to have reached 2 billion tons, representing about 80% of total waste production (on a weight base) within the EU28 group (European Commission, 2018).

According to the Waste Framework Directive (WFD) (European Parliament, European Council, 2008a), a "mirror entry" must be classified as hazardous if specific assessment could attribute to the tested waste at least one of the 15 Hazard Properties (HP), as described in the Annex III of the same Regulation. Both LoW and Annex III of WFD undercame amending process aiming for harmonization with Regulation on Classification, Labelling and Packaging of chemical products (CLP) (European Parliament, European Council, 2008b).

Among the 15 HPs, HP14 "Ecotoxic" is acknowledged as being the most frequent Hazard Property classifying entries of LoW as hazardous (Hennebert et al., 2014). As established by Regulation 997/2017/EC, HP14 can be assigned according to the application of a calculation method based on chemical speciation and composition (i.e., mass fractions) of specific waste constituents, already classified by the CLP with one or more Hazard Statement Codes related to ecotoxicity, i.e., H400, H410, H411, H412, H413, H420 (European Council, 2017). However, due to the heterogeneous and multi-component nature of waste matrix, real elemental speciation (and consequently, bioavailability) can only be hypothesized or assumed under conservative "worst case assessment", thus influencing the reliability and goodness of results given by the established quantification method (Stiernström et al., 2016; Hennebert, 2018).

To overcome this limitation, European Council (2017) stated that results gathered through ecotoxicological testing must prevail on the outcomes of the calculation method. Further, single Member States (MS) are allowed to establish testing procedures, which must be consistent to test methods pursuant to CLP or "other internationally recognized test methods and guidelines" (European Council, 2008, 2017).

An experimental methodology intended to classify waste for ecotoxicity purposes can be defined by three main features:

1. Which sample preparation procedure should be carried out before testing;
2. Which battery of bioassays should be performed;
3. Which limits concentrations (e.g., in terms of EC50) should trigger hazard classification.

The suitability and representativeness of different batteries of bioassays together with consistency and reliability of proposed concentration limits were already well studied and discussed both in scientific literature and technical guidelines (Pandard et al., 2006; Pandard and Römbke, 2013; Hennebert et al., 2014; ECHA, 2015; Hennebert, 2018; Römbke, 2018). Instead, to the authors knowledge, sample preparation is investigated by fewer scientific publications, which focused on the influence of different operative conditions involved in water extracts preparation from solid waste (i.e., leaching test) on the results of ecotoxicity surveys: applied Liquid-to-Solid ratio, eventual pH adjustment of the test portion, use of specific dilution media and complexation reactions with occurring DOC concentration (Postma et al., 2009; Van der Sloot and Van Zomeren, 2009; Stiernström et al., 2016).

In this context, this study wants to support actors involved in forensic activities related to wrong hazardousness classification of wastes and possible consequent illegal trafficking, also in relation to extranational transport, with the ultimate aim to suggest a conventional and scientifically sound procedure for sample preparation prior to ecotoxicity assessment of waste. First, a description is presented briefly describing two different approaches characterizing MS proposals of methodologies for ecotoxicity testing of wastes. Second, results are presented on the application of two standard protocols for water extracts preparation prior to testing, in order to give indications about the most conservative and suitable approach for waste classification purposes. The resulting water extracts were derived from leaching tests performed on samples of Automotive Shredder Residues (ASR), also known as Car Fluff, classified in the LoW as "mirror entry".

Current approaches for experimental HP14 classification of wastes

Currently, there is no common proposal able to summarize, at the European level, the heterogeneity of approaches in ecotoxicity testing, thus leading to possible harmonization issues between MS. However, among actions undertaken in single Member States, two main orientations can be clearly distinguished. Table 1 summarizes the key features between the 2

Table 1. Comparison between current European approaches for testing methodologies for HP14 testing of waste.

		Approach A	Approach B
Sample Preparation		GHS Annex X (OECD, 2001)	EN 14735 (ISO, 2005a)
Test Battery	*Aquatic organisms*	Fish (OECD, 1992)	Crustacean -Daphnia (ISO, 2012b)
		Crustacean - Daphnia (OECD, 2004)	Freshwater Algae (ISO, 2012a)
			Bacteria (ISO, 1998)
		Freshwater Algae (OECD, 2011)	
	Terrestrial Organisms	–	Bacteria (ISO, 2018)
			Plant (ISO, 2013)
			Earthworms (ISO, 2008)
Classification Criteria (waste is classified hazardous if)	*Aquatic organisms*	EC50 $\leq$ 100 mg/l[1]	EC50 (ISO, 2012b) $\leq$ 15 %[2]
		NOEC $\leq$ 1 mg/l	EC50 (ISO, 2012a) $\leq$ 10 %[2]
			EC50 (ISO, 1998) $\leq$ 10 %[2]
	Terrestrial Organisms	–	EC50 (ISO, 2018) $\leq$ 5 %[3]
			EC50 (ISO, 2013) $\leq$ 15 %[3]
			EC50 (ISO, 2008) $\leq$ 5 %[3]

[1] This condition must be assessed for each performed test included in the required battery

[2] Volumetric fraction (% vol/vol). See (Hennebert, 2018)

[3] Weight fraction (% w/w). See (Hennebert, 2018)

approaches, denoted with Approach A and B. As explained in the Introduction section, each proposal addresses the three main aspects defining a testing strategy for ecotoxicity classification purposes, i.e., i) standard protocol for sample preparation ii) test battery and iii) classification criteria.

Approach A, supported by Italian Environmental Agency, imposes that HP14 classification by testing must be fully compliant with the requirements established in CLP, in terms of test battery, limit concentrations and sample preparation procedure, being this Regulation the sole source of requirements institutionally established (ISPRA, 2018). What stands out is that this approach does not require to perform any terrestrial bioassays, thus not needing to perform tests on solid waste samples (ECHA, 2015), being the CLP deficient of criteria assessing terrestrial toxicity. In fact, the hazard statement codes related to ecotoxicity (HSC400, HSC 410, HSC 411, HSC 412, and 4 HSC13) depend on aquatic organisms exclusively. Only liquid waste or water extracts derived by leaching test performed on solid waste can be tested by aquatic bioassays, which are acute toxicity of fish (OECD, 1992), acute immobilization of daphniae (OECD, 2004) and algae/cyanobacteria growth inhibition test (OECD, 2011). Tested liquid wastes and water extracts must be prepared according to OECD document No. 23 on ecotoxicity testing difficult substances and mixtures (OECD, 2000), or to a more standardized protocol laid down to assess, through chemical analysis the transformation and dissolution of metals or metallic compounds in substances (OECD, 2001).

Finally, a waste is declared hazardous (i.e., "Ecotoxic") if at least one between the median effect concentration, (EC50), and the No Observed Effect Concentration (NOEC), gathered from each required bioassay, is found lower than 100 mg/l and 1 mg/l respectively (ECHA, 2015; European Parliament, European Council, 2008b).

Criticism could arise by the application of Approach A: being based on testing protocols purposely designed to test products, which are generally constituted by single substances or mixture of well-known compounds, it hardly addresses the needs for classifying heterogenous multicomponent waste matrixes, thus missing to assess important exposure pathways (i.e., through terrestrial bioassays), requiring tests not suitable for frequent classification efforts (i.e., fish test) and establishing sample preparation procedures not specifically designed for ecotoxicological testing. This latter aspect is further discussed in following paragraphs.

Method B is based on works coming from the scientific community devoted to providing conventional lab practices specifically developed to test ecotoxicity of wastes. Adopted test battery is suggested by the work of Pandard et al. (2006): it encompasses tests on 3 trophic levels of both terrestrial and aquatic organisms. The proposed battery includes bioassays testing bacteria (ISO, 1998), plant (ISO, 2013) and earthworms (ISO, 2008) for solid and liquid wastes, and bacteria (ISO, 1998), crustacean (ISO, 2012a) and algae (ISO, 2012b) for liquid waste or water extracts of solid waste samples. Preparation of solid samples, liquid samples and water extracts must be performed according to ISO (2005), officially designed for waste sample preparation for ecotoxicity testing. Concentration limits, i.e., EC50, are established specifically for each of the 6 bioassays to perform in terms of dilution of samples with a dilution media, as provided in Hennebert, (2018). This approach is currently adopted in France, Germany and Austria, the latter with the exception of requiring just aquatic biotests, supporting the opinion that terrestrial bioassays currently result inconsistent with HP14 definition, as established by the European Regulator (CEN/TC 444/WG 1, 2018).

Materials and Methods

Samples of ASR were used to perform the different leaching test procedures as established by Approach A and Approach B. ASR is defined as the light fraction derived from aeraulic separation of prior crushed End Life Vehicles (ELV), the whole process aimed at metal recovery. This study assumes ASR as example of a mirror entry waste in the LoW and comprehensive data on production processes, disposal and characterization are available in scientific literature (Cossu and Lai, 2015; Morselli et al., 2015). Currently, ASR can be classified with nonhazardous and hazardous waste codes, respectively 19.10.04 and 19.10.03* (European Commission, 2000). Finally, ASR samples used for the purposes of this study resulted hazardous (HP14) according to the calculation method, based on previously performed chemical characterization.

ASR Sampling

ASR was sampled from two different private plants treating ELV for metal recovery, the first located in central Italy (sample A) and the second located in northern Italy (sample B). For this reason, sampling plans, which provided sample A and sample B, were designed according to the principles laid down in ISO (2005b). Sampling procedure was carried out in two phases. Primary samples of about 250–300 kg were collected for sample A and B respectively. Final samples were obtained through the so-called 'quartering' procedure as established by UNI (2013). In both cases, a final sample of about 30 kg FM was collected and delivered to the laboratory for the analysis.

Experimental design

Figure 1 illustrates the experimental design of this work. Water extracts were obtained from sample A and sample B through the application of the 2 different protocols, which are GHS Annex X (OECD, 2001) and EN ISO 14735 (ISO, 2005a), as required by Approach A and Approach B, respectively. Methodological prescriptions of performed leaching tests are listed in Table 2 and described in detail in the following paragraphs. Each leaching test was performed in triplicate. Each derived water extract was assessed by chemical-physical characterization.

Leaching test A: GHS - ANNEX 10 guidance on transformation/dissolution of metals and metal compounds in aqueous media

As required by the standard, ASR particles were reduced to a size of 1 mm using a saw mill, taking care to remove non-crushable materials present (e.g., bigger nuts and bolts) to avoid possible blockages of used devices. It seems important to report that size reduction of particles up to 1 mm brought difficulties from the operational point of view, thus resulting exceedingly time consuming. Cutting device had to be operated intermittently with small amounts of waste in order to avoid sample heating, which could have modified intrinsic features, and frequent blockages.

For each sample, leaching test according to OECD, (2001) was conducted by adding 100 mg TS of sampled ASR on 1 liter of leaching media. Reconstituted Standard Freshwater (ISO, 2012b) was used as leaching media. The leaching was conducted at a temperature between 20 ÷ 23 °C in 2 L HDPE bottles, roughly mixed in end-over tumbler (at about 10 rpm) for 7 days. pH was kept in the range 6÷8.5. At the end of the leaching test, the mixture was allowed to settle for 5 min and the resulting supernatants were filtered through a 0.2 μm membrane filter.

Leaching test B: EN ISO 14735 preparation of waste samples for ecotoxicity tests

EN 14735:2005 standard relies on the requirements established in EN 12457-2:2004 for water extract preparation: this protocol describes a standardized procedure for leaching test of granular waste and waste sludge, to assess consistency with Landfill Acceptance Criteria. Solid particles of ASR were size reduced to 4mm with the same crushing device used for Leaching test A. No particular difficulties linked to this size reduction step were worth to be noted. About 95 g TS of ASR samples were added to 2 l HDPE bottle with 1L of deionized water as leaching medium. The leaching was conducted at a temperature between 15 ÷ 25 °C, with no pH adjustment. Containers were gently shaken in end-over tumbler (at about 10 rpm) for 24 ± 0.5 h. At the end of the leaching test, the mixture was allowed to settle for 15 ± 5 min to permit the liquid-solid separation and the supernatants were filtered through a 0.45 μm membrane filter with a vacuum pump.

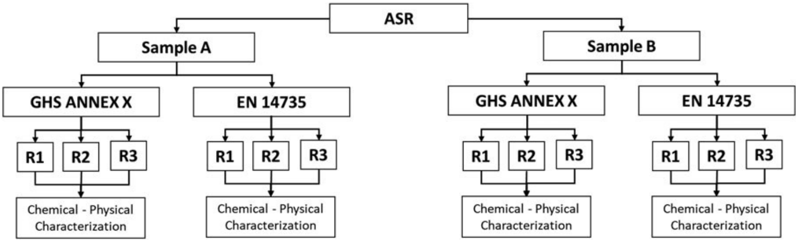

Figure 1. Graphical scheme of the implemented experimental design.

Table 2. Experimental features of leaching tests investigated for deriving water extracts to be further tested in aquatic bioassays.

	EN 14735	GHS Annex X
Particle Size	≤ 4 mm	≤1 mm
Loading Rate	100,000 mg/L	100 mg/L
L/S ratio	10	10,000
Leaching medium	Distilled Water	Reconstituted Standard Freshwater (ISO, 2012b)
Test duration	24 h	7 d
Solid/Liquid Separation	0.45 μm filtration	0.2 μm filtration
Test Temperature	15–25 °C	20–23 °C
Reference	ISO, (2005a)	OECD, (2001)

Analyses

EC, pH, DOC, TDS, Chlorides (as Cl–), Fluorides (as Fl–), Sb, As, Ba, Cd, Cr-tot, Hg, Mo, Ni, Pb, Cu, Se and Zn were quantified in derived water extracts with standardized wastewater techniques (IRSA-CNR, 2003). Results of chemical characterization of water extracts were compared with the Italian landfill acceptance criteria for non-hazardous waste (Ministero dell'Ambiente, 2010), in order to assess consistency with this Regulation. Landfill acceptance criteria are set for resulting chemical concentrations found in water extracts derived from leaching tests on waste performed according to ISO (2004), completely consistent with ISO (2005a).

Results and discussion

General observations on performed laboratory activities: sample representativeness and time consumption

Ecotoxicity of granular waste is related to chemical species present in high concentration in a very small number of particles constituting the heterogeneous multicomponent waste matrix. A representative waste sample should reflect adequately the waste generating technological process and the properties of interest (e.g., ecotoxic metals content) in the waste matrix (ISO, 2005b). According to Gy's theory (Gy, 1979), included in standardized sampling methodologies, required degree of sample representativeness can be guaranteed by a specific mass and particle size of sampled waste material (ISO, 2006). The aforementioned theory makes use of key parameters that must be assumed, increasing the uncertainty of results obtained by further analysis and classification efforts (Stelling and Sjerps, 2005). Furthermore, in waste-related forensic activities it is important to select a reliable standardized procedure for waste sample preparation, which should be enough conservative to avoid further uncertainty, but also enough operative to ensure that ecotoxicological testing could become a conventional experimental analysis (Hennebert, 2018).

Given the background, the difference between the two performed standardized procedures in terms of laboratory efforts for providing a fair degree of sample representativeness is evident. Assuming a constant volume of leaching medium for both studied protocols, EN 14735 requires higher amount of waste tested with higher particle size, thus ensuring higher degree of sample representativeness. Further, GHS Annex X, due to the small mass of waste sample required, involves greater efforts for particle size reduction to guarantee the same degree of sample representativeness, which leaded to operational difficulties, also reported in a previous study (Stiernström et al., 2016). Finally, due to the 7 days leaching time required, GHS Annex X is much more time consuming than

EN 14735: this issue can lead to operational drawbacks for actors involved in waste management, which often need to perform frequent material classification.

Chemical-physical characterization of ASR water extracts

Table 3 shows the results of the chemical characterization for sample A and B together with Italian acceptance limits for non-hazardous waste disposal in landfill (Ministero dell'Ambiente, 2010). It is recalled that compliance with landfill acceptance criteria must not replace waste hazardousness classification through ecotoxicological testing (Hennebert, 2018). Beside compliance, results from chemical analysis allows to make suggestions on which of the two protocols used could be considered the most conservative, in terms of higher concentration of toxic compounds resulting in the aqueous extracts to be tested in further aquatic bioassays.

Results are expressed as average and standard deviation of the resulting replicates. Chemical concentrations found under the quantification limits were assumed equivalent to the quantification limits, under conservative assumption.

The totality of prepared water extracts shows full consistency with Italian landfill acceptance criteria for nonhazardous waste. Therefore, from a regulatory point of view, samples of ASR meet the criteria for non-hazardous waste disposal in landfill.

All water extracts are characterized by similar pH values, which result both slightly more and consistently less alkaline than pH values found in literature, respectively in Fiore et al. (2015) and Cossu and Lai (2013). Based on these results, no pH adjustment should be performed before testing water extracts derived with GHS Annex X protocol. Further, neutral pH is consistent with needed pH range of survival of both proposed test batteries. EC values, which are known to be a key factor influencing ecotoxicity, are comparable. However, EC from sample B results higher for eluates obtained through the T/D protocol: this trend is inverted in water extracts from sample A, suggesting that resulting EC is not strongly influenced by different set up of leaching tests. EN 14735 water extracts of the two samples are characterized by

Table 3. Chemical characterization of obtained water extracts.

	Sample A GHS ANNEX X	Sample A EN 14735	Sample B GHS ANNEX X	Sample B EN 14735	LAC[1]
EC	556.667	779.333	554.000	489.000	
µS/cm	±1.528	±29.670	±1.732	±12.288	
TDS	516.667	729.167	519.333	626.000	10000
mg/l	±6.429	±12.829	±9.452	±38.158	
pH	7.580	7.600	7.273	7.573	
(−)	±0.164	±0.066	±0.100	±0.181	
Sb	0.030	0.018	n.a.	n.a.	0.07
mg/l	±0.000	±0.002	±0.000	±0.186	
As	0.020	0.020	0.020	0.022	0.2
mg/l	±0.000	±0.000	±0.000	±0.003	
Ba	0.071	0.406	0.052	0.232	10
mg/l	±0.016	±0.060	±0.008	±0.035	
Cd	0.001	0.002	0.001	0.001	0.1
mg/l	±0.000	±0.000	±0.000	±0.001	
Cr	0.005	0.005	0.005	0.013	1
mg/l	±0.000	±0.001	±0.000	±0.007	
Hg	0.001	0.001	0.001	0.001	0.02
mg/l	±0.000	±0.000	±0.000	±0.000	
Mo	0.022	0.035	0.021	0.038	1
mg/l	±0.003	±0.005	±0.001	±0.013	
Ni	0.005	0.122	0.005	0.057	1
mg/l	±0.000	±0.000	±0.000	±0.003	
Pb	0.005	0.059	0.005	0.243	1
mg/l	±0.000	±0.023	±0.000	±0.186	
Cu	0.010	0.298	0.010	0.339	5
mg/l	±0.001	±0.067	±0.000	±0.140	
Se	0.005	0.005	0.005	0.005	0.05
mg/l	±0.000	±0.000	±0.000	±0.000	
Zn	0.069	3.604	0.074	2.752	5
mg/l	±0.032	±0.981	±0.041	±1.861	
DOC	5.937	124.667	5.480	160.667	100-[2]
mg/l	±0.164	±16.073	±0.147	±17.039	
Chlorides (as Cl−)	169.130	63.846	138.637	31.911	2500
mg/l	±59.552	±6.876	22.573	±3.563	
Fluorides (as Fl−)	0.100	0.467	0.100	1.267	15
mg/l	±0.000	±0.058	0.000	±0.351	

[1] LAC = Italian Landfill Acceptance Criteria (Ministero dell'Ambiente, 2010).

[2] Limits for DOC concentrations for the involved sample are considered compliant in derogation from LAC.

higher TDS. Similarly, DOC concentrations of EN 14735 eluates are found to be much higher (i.e., 2 order of magnitude) than GHS Annex X water extracts, but lower than DOC concentrations of water extracts derived equivalently in a previous study (Fiore et al., 2015). A likely explanation is that higher DOC values are a result of higher mass of material involved during leaching test. This trend could be confirmed to a less extent also for TDS concentration. Higher values of DOC could likely determine an higher portion of dissolved contaminants associated with dissolved organic matter, thus potentially decreasing ecotoxicity of the test portion, which is related to free ionic form, i.e., not associated (Moser and Römbke, 2009; Newman, 2014).

Inorganic content of water extracts (i.e., fluorides and metals) derived through EN 14735 is comparable in terms of order of magnitude with analyzed car fluff eluates derived with the same procedure in previous studies (Cossu and Lai, 2013; Fiore et al., 2015). Differences with literature data are likely caused by the high degree of variability characterizing ASR samples originating from independent metal recovery plants performing different mechanical treatments (Cossu et al., 2014).

What stands out in Table 3 is the higher concentrations of these compounds in water extracts derived according to EN 14735 with respect to eluate obtained from GHS Annex X. While this aspect is not clearly distinguished according to some elemental concentrations (i.e., Cd, Hg, and Se), the described trend is more visible for Ba, Mo, Ni, Pb, Cu, and Zn, these differences better illustrated in Figure 2.

By comparing the requirements established in the two analyzed leaching procedures, three experimental features of leaching tests could be thought to influence resulting total concentration of contaminants in water extracts and consequent bioavailable fractions. Smaller particle size (i.e., increased particle surface in contact with leaching media) and longer contact time could lead more favorably to equilibrium conditions, thus determining higher solubilization potential (Dijkstra et al., 2006). Then, assuming an equal distribution coefficient between equilibrium concentration in solid and liquid fractions, L/S ratio of orders of magnitude higher will likely result in lower aqueous concentrations (Grathwohl and Susset, 2009). Accordingly, results in Table 3 suggest L/S ratio as the experimental factor most influencing dissolution of inorganic contaminants. The only exception is the inverted trend characterizing Chlorides content (see Table 3): this can be easily justified by the presence of chlorides in the leaching medium used for leaching test according to GHS Annex X, i.e., Standard Freshwater reconstituted according to (ISO, 2012b).

Leading to higher elemental concentrations in eluates, higher degree of sample representativeness and lower analytical efforts, EN 14735 might be considered as the most efficient and precautionary approach in deriving waste extracts to be further tested in aquatic bioassays. However, the aforementioned approach still misses to completely clean the field from other analytical issues influencing the assessment of the reliability of what is measured through bioassays. First, EN 14735 sets the actors involved in waste characterization free to perform or not pH adjustment of derived water extracts, where resulting pH values could be found out of established test-ranges (i.e., not in this study). In these cases, the results of bioassays performed on not pH-adjusted water extracts could be predicted beforehand. On the other hand, pH adjustment could rise doubts on the reliability of ecotoxicity results, which can be considered certainly not representative of the waste material at the production stage. In this context, further testing and subjective expert judgments are still needed.

Further, this interpretation assumes that higher total dissolved concentration will lead to higher ecotoxic response. However, higher aqueous concentration is not always positively correlated with higher bioavailability of contaminants (i.e., present as "free" ionic or unbound form), the speciation being influenced by possibly occurring association with DOC and colloids or by changes due to dilution of the test portion with established control medium for the preparation of the different tested concentrations (Postma et al., 2009). Therefore, the reported higher values of DOC characterizing water extracts obtained according to the EN 14735 (Table 3) could likely determine a higher portion of dissolved contaminants associated with dissolved organic matter, thus potentially decreasing ecotoxicity of the test portion and ultimately resulting in contrast with the precautionary assumption (Postma et al., 2009; Van der Sloot and Van Zomeren, 2009; Newman, 2014).

The aforementioned issues should be further investigated by performing ecotoxicological testing integrated with geochemical and release modeling of wastes (e.g., LEACHXS-ORCHESTRA), this latter tool being acknowledged to be powerful tools able to determine speciation at equilibrium for test portions and dilutions of derived water extracts (Van der Sloot and Van Zomeren, 2009). Finally, the discussed findings, observed for both studied approaches, must be

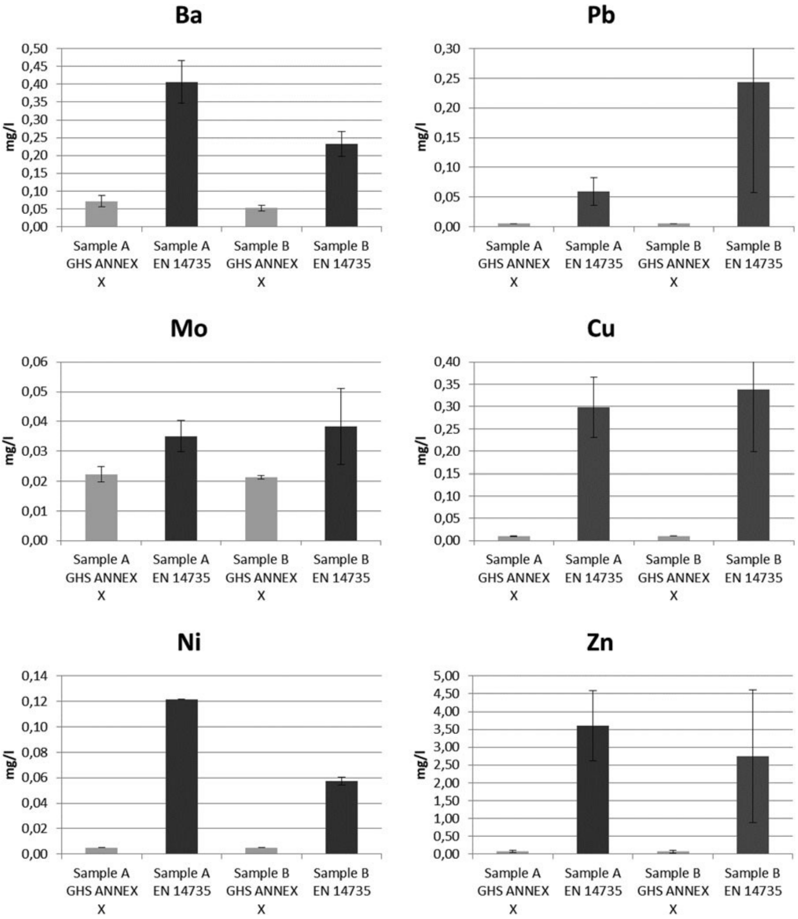

Figure 2. Graphical comparison of Ba, Pb, Mo, Cu, Ni and Zn concentrations of obtained water extracts.

further verified and validated to increase the possibility of being accepted by involved actors, by applying the comparison also on other wastes classified as "mirror" entries in the European LoW.

Conclusions

In a forensic context, a clear, scientifically sound and harmonized classification system will at the same time help to verify the conditions of occurred illegal trafficking of waste and to avoid unsustainable compliance costs.

First, this study described the current European legal framework regulating experimental assessment for HP14 classification of wastes classified as "mirror entries" in European LoW. Among national proposals, two main approaches can be distinguished, based on different methodological frameworks regarding involved battery of bioassays, chosen classification criteria and performed procedures for preparation of water extracts from waste to be tested through aquatic bioassays. In this context, the differences characterizing water extracts obtained through the application of different procedures, as could be the case of the two investigated approaches, can lead to discrepancies in results from further bioassays, thus leading to different waste classification and consequent legal implications in extranational transport.

Then, results from an experimental activity were presented to assess which approach requires a standard for sample preparation (GHS Annex X or EN 14735), which can be considered most suitable to derive water extracts to be further tested in required battery of aquatic bioassays. Two ASR samples were used as example wastes for leaching tests. ASR samples were previously classified as HP14 by use of the calculation method established by the Regulation 997/2017/EC.

According to the greater efforts in particle size reduction to obtain representative test samples and to longer contact time required by the leaching procedures, GHS Annex X presents issues likely causing difficulties during frequent waste classification activities, thus making EN 14735 the most operative and conventional between the two protocols.

Despite higher particle size and contact time required, EN 14735 allowed to obtain an eluate characterized by higher concentrations of DOC and Heavy Metals, probably because of the lower L/S ratio required. For this reason, it could be considered also the most conservative approach. However, being bioavailable fraction of contaminants not always correlated with total concentration, further research should be performed to validate this assumption, taking into account the real speciation of dissolved compounds, as influenced by DOC presence and specific dilution media.

For these reasons, while adopting EN 14735 can ease the process of making bioassays conventional, this approach is currently still far to allow a correct and univocal interpretation of ecotoxicological tests, which could be adopted by the international regulations on classification and extranational transportation of waste.

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Additional Paper II

Pivato, A., Beggio, G., Hennebert, P., Bonato, T., Favarin, M., Raga, R., 2020. Proposal of a Testing Program for the Hp14 (Ecotoxic) Classification of Automotive Shredder Residues (Asr) By a Battery of Ecotoxicological Bioassays. Detritus 13, 12–22. https://doi.org/10.31025/2611-4135/2020.14032

detritus | Multidisciplinary Journal for Waste Resources & Residues

CISA

PROPOSAL OF A TESTING PROGRAM FOR THE HP14 (ECOTOXIC) CLASSIFICATION OF AUTOMOTIVE SHREDDER RESIDUES (ASR) BY A BATTERY OF ECOTOXICOLOGICAL BIOASSAYS

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Article Info:
Received:
24 October 2019
Revised:
7 July 2020
Accepted:
31 July 2020
Available online:
30 November 2020

Keywords:
Ecotoxicity
HP 14
Test battery
Leaching Test
Automotive Shredder Residues

ABSTRACT

This manuscript describes a full procedure to be used in performing HP 14 classification of Automotive Shredder Residue (ASR). Sampling instructions and conditions for the transport and storage of ASR samples are included. Additionally, the steps to be followed in preparation of test portions (both solid samples and water extracts) prior to chemical characterization and subsequent ecotoxicological testing are defined. The established test battery includes all bioassays proposed in Pandard and Römbke (2013). Only aquatic bioassays are proposed as mandatory in this paper, which leaves the possibility of performing tests on terrestrial organisms based on the results of chemical characterization of the solid samples. Finally, the proposed set of concentration limits triggering HP 14 classification is fully consistent with thresholds described by Hennebert (2018a).

1. INTRODUCTION

Ecotoxicity is acknowledged as the most frequent property classifying wastes as hazardous (Hennebert et al, 2014). In Europe, wastes are classified for ecotoxicity (HP 14) according to the Regulation 2017/997/EC (European Parliament and European Council, 2017). The latter introduces a calculation method for the HP 14 classification, based solely on chemical concentrations of the hazardous substances occurring in the waste to be classified (i.e., classified with a Hazard Statement Code (HSC) H400, H410, H411, H412 and/or H413) according to the Regulation on Classification, Labeling and Packaging of Products and Substances 2008/1278/EC (European Parliament and European Council, 2008a). However, it does not include any specific guidance for performing the HP 14 classification through ecotoxicological testing. At the same time, it states that each Member State can adopt specific analytical protocols on conducting bioassays and the derived outcomes supersede results from chemical composition analyses.

The lack of a clearly established testing procedure can lead to erroneous waste classifications of those waste streams typically classified as mirror entries by the European Waste Catalogue (EWC), which must be declared as hazardous based on mandatory compliance assessment (European Commission, 2000).

For this reason, the objective of this paper is to propose a testing procedure allowing for a complete HP 14 classification of a classic example of a mirror entry, (i.e., Automotive Shredder Residues (ASR)), which is classified in the EWC with the couple of codes 19 10 03* (hazardous) and 19 10 04 (non hazardous). Therefore, ASR is here intended as the so-called "light fluff", which is the lighter fraction separated through air classification from the shredded hulk (Cossu et al., 2014). ASR is a highly heterogeneous waste stream, both in terms of granulometry and materials composition: it includes plastic, foam, textiles and metallic (magnetic, non-magnetic and PVC covered cables) particles, characterized by broad size distribution (Cossu and Lai, 2015). Significant environmental issues related to ASR management could arise due to the reported presence of trace elements, heavy metals and possible organic contaminants (i.e. PAHs, PCBs and mineral oil) (Cossu et al, 2014).

The choice of addressing HP 14 classification for this

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Detritus / Volume 13 - 2020 / pages 12-22
https://doi.org/10.31025/2611-4135/2020.14032

specific kind of waste was based on opportunity. However, the rationale behind this proposal can be applied to other mirror entries by taking care of fine tuning this procedure according to observable specific features. These specific features mainly relate to sampling and analytical procedures (e.g., particles size distribution and size reduction, extreme values of pH in the water extracts, and the need to investigate the presence of specific contaminant concentration).

This paper also includes detailed instructions about sampling, transport and storage of ASR laboratory samples. Furthermore, it describes stepwise procedures for liquid and solid test portion preparation, which will undergo further chemical analysis and ecotoxicological characterization. Also, the rationale behind the various requirements is explained. Elements of the whole procedure were not proposed from scratch but reference both to specific international and European technical standards as well as European ongoing regulations.

Regulation 2017/997/EC considers as the appropriate biotest procedures the ones which are consistent with the relevant methods established in the Regulation 2008/440/EC, pursuant to the Regulations 2006/1907/EC and 2008/1272/EC on products and substances (naming REACH and CLP, respectively), or "other internationally recognized test methods and guidelines" (European Parliament and European Council, 2006, 2008a, 2008b, 2017). In particular, the proposed ecotoxicological test battery includes all bioassays proposed in Pandard and Römbke, (2013). Therefore, the compliance with Regulation 2017/997/EC is assumed according to the fact that the chosen approach relies on technical standards referring to internationally acknowledged EN ISO series.

The CLP Regulation classifies products and substances (both liquid and solid) only for ecotoxicity in aquatic environment, thus requiring only chemical characterization on the water extracts or biotests performed on aquatic organisms. Although, chemical investigations on solid samples and bioassays carried out on terrestrial organisms could assess the presence of contaminants able to bind to soil particles and related exposure pathways, this behavior cannot be investigated solely by analyses on liquid samples (e.g. non-soluble or non-leachable substances). Given this background as well as considering the principle of the technical and economic feasibility as sanctioned by the Directive 2008/98/EC (European Parliament and European Council, 2008,b), the authors propose to include a chemical characterization of solid waste, which can trigger the need to assess related toxicity patterns through specific terrestrial ecotoxicity testing. In this context, ecotoxicological tests and chemical characterization are assumed to act not independently but synergistically for waste classification.

Lastly, the classification criteria of the results from bioassays were derived based on the concentration limits listed in Hennebert, (2018), which were proposed consistently with the EWC. The authors acknowledge that the EWC is not fully based on scientific evidence, as it is partly the result of political compromises and lobbying. Nevertheless, it is currently the only source which can be used as a reference for classification purposes.

2. METHODOLOGY

2.1 General Procedure

The general objective of the proposed testing program is to assess the classification of ASR as hazardous by HP 14. The consequent level of testing is the compliance testing level. The different phases of the proposed experimental protocol are outlined in the flowchart depicted in Figure 1. The stepwise procedure starts from the sampling phase, which is aimed at producing two laboratory samples that would be assessed by fractional composition analysis and chemical- ecotoxicological characterization, respectively. The obtained samples are then transported and stored in the laboratory where analyses will be performed. One laboratory sample undergoes Fractional Composition Analysis without any further preparation steps. Then, the solid test portion is prepared from the other laboratory sample to undergo i) chemical characterization and ii) leaching testing aimed at obtaining the relative water extracts. Chemical characterization and aquatic bioassays are then performed on the resulting aqueous test samples (i.e., water extracts). Terrestrial bioassays completed on the solid test samples are only executed if the results of the chemical characterization are not compliant with the proposed concentration limits for solid samples (outlined in Table 3). Finally, the ASR sample is classified for HP 14 based on the results of the comparison between the proposed concentration limits and the obtained results from the performed aquatic (Table 4) and terrestrial (Table 5) bioassays.

The rationale behind the requirements described for each phase of the proposed procedure is illustrated in the following sections.

2.2 Sampling, Transport and Storage

The proposed protocol defines a Sampling Plan consistent with the standards outlined in EN 14899:2015, UNI EN 10802:2013, and EN 15002:2015. Furthermore, instructions for Transport and Storage are consistent with EN 14735:2005.

If possible, it is recommended that sampling is performed by picking up the material dynamically from the conveyor belt placed downstream of the occurring aeraulic classifier. This approach is recommended to minimize spatial segregation due to the possible differential gravity settling occurring in the waste heap configuration. If dynamic sampling cannot be accomplished, sampling actions should be performed statically, either by stopping the conveyor belt or by carrying out sampling from temporary storage (heap configuration). Subsampling should be performed according to the "quartering and coning" procedure as explained in EN 15002:2015. Also, the so-called "long pile procedure" can be equivalently performed for the reduction of sample mass, according to EN ISO 14780:2017.

The calculation method used, and the parameters values assumed to determine the minimum sample mass of both primary sample and laboratory sample is described in Paragraph SM.1 of the Supplementary Material. Different values should be justified according to the results of further material investigations (e.g. bulk density) or granulometric analyses (e.g. definition of alternative D95) per-

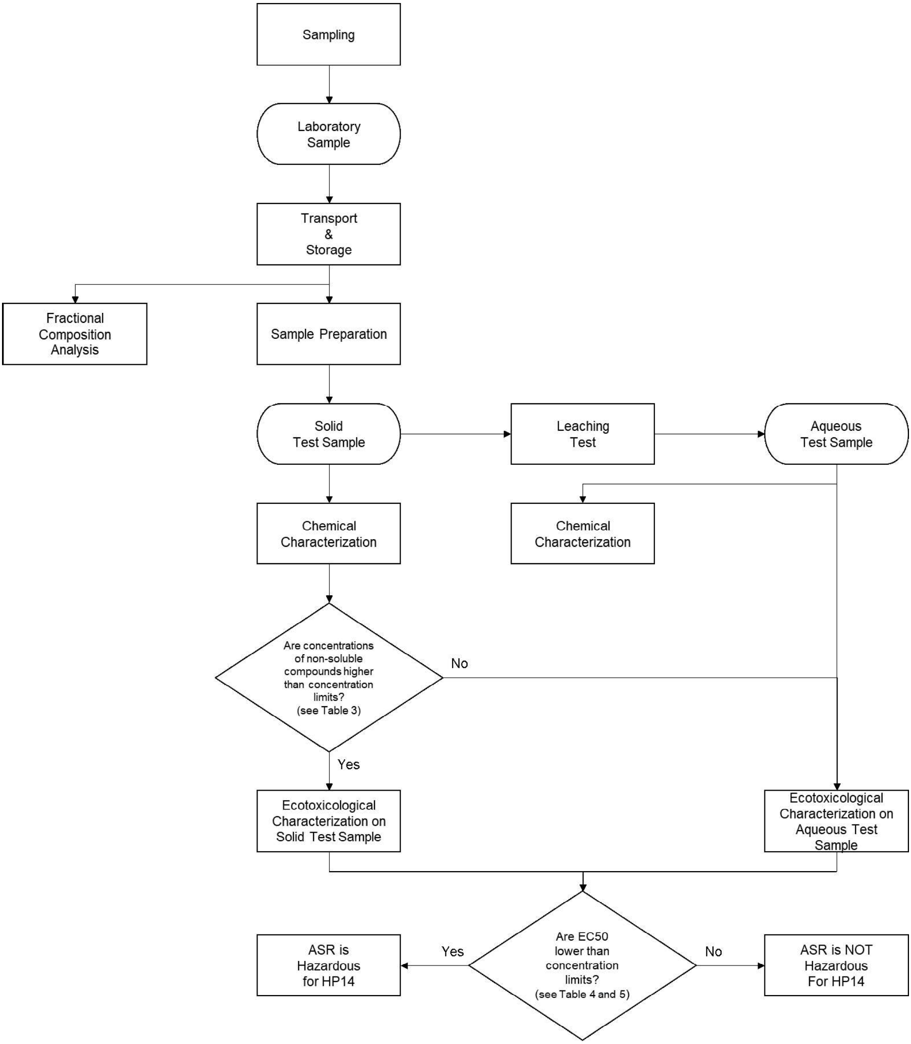

FIGURE 1: Stepwise procedure for HP14 classification of Automotive Shredder Residues (ASR).

formed on untreated sample (i.e., not size reduced). Here, ASR can be characterized by the presence of fines (0-20 mm) up to 70% (w/w) of the total sample, which can significantly lead to overestimation of the size of the laboratory sample (Cossu et al, 2014).

2.3 Fractional Composition Analysis

Fractional composition analysis is included in the procedure in order to determine the different material fractions making up the laboratory sample. Results of the fractional composition analysis are used to determine qualitatively the composition of the resulting laboratory sample and will not be used for any compliance assessments. This assumption allows us to perform such analysis on test portions which cannot be considered representative because of the suggested sample size.

2.4 Test Samples Preparation – Leaching Test

Instructions for the preparation of solid test samples are consistent with the principles reported in EN 15002:2015 for chemical analysis and EN 14735:2005 for terrestrial bioassays. The leaching test procedure is proposed in line with EN 12457-2 to derive water extracts for chemical analysis and aquatic bioassays.

While there is an agreement on the leaching procedure to derive water extracts for chemical characterization (i.e. EN 12457-2, here adopted), two main approaches are acknowledged for the preparation of waste eluates for ecotoxicological testing, both related to specific standards:

1. EN 14735 - Characterization of waste – Preparation of waste samples for ecotoxicity tests (EN 14735, 2005, currently under revision). Here, ASR can be considered as "granular waste". Consequently, specific water extracts must be derived following the "leaching test" procedure as laid out in EN 12457-2:2004;
2. OECD Document, nr. 23 and OECD Document, nr. 29 - Guidance on transformation/dissolution of metals and metal compounds in aqueous media. These standards are recalled by CLP Regulation (OECD, 2001, 2019).

Among the different requirements established by the cited standards (e.g. test duration, particle size), the factor most influencing the chemical profile of the obtained water extracts is the prescribed loading rate, 100 g/L for EN 12457-2 and 100 mg/L for OECD n.29. For the same amount of waste material, the consequent L/S ratios (10 L/kg vs. 10.000 L/kg for EN 12457-2 and OECD n.29, respectively) mimic long term release conditions which differ of several orders of magnitude in terms of years of occurred percolation or contact with pore water. Consequently, EN 12457-2 led to water extracts characterized by higher inorganic chemical concentrations (e.g. Heavy Metals) compared to OECD Documents nr. 23 and 29, when applied on the same ASR samples (Pivato et al., 2019). In this situation, a water extract should likely be considered more relevant to aquatic organism when derived applying the lower L/S ratio as practically feasible (Van der Sloot et al., 1997, Van der Sloot and Dijkstra, 2004). Besides, the requirements laid down in EN 12457-2 results in a more operative process to prepare a representative test portion (i.e. less efforts for both size reduction and subsampling) (Pivato et al., 2019). Finally, chemical and ecotoxicological characterization conducted on eluates identically derived allow an integrated assessment (Van der Sloot and Dijkstra, 2004). For these reasons, the experimental protocol being proposed, adopts the EN 12457-2 for liquid sample preparation prior to chemical characterization and ecotoxicity testing.

2.5 Chemical Characterization

Chemical characterization of both solid sample and waste eluates should be conducted in triplicate to assess the representativeness of the derived solid test portions after the subsampling process. In fact, the analyzed solid test portions can be considered representative if the coefficients of variation, calculated for each measured parameter as standard deviation divided for the mean of concentration, is low enough low to represent the lowest possible degree of variability, i.e. due to the performed analytical protocol. In this context, a CV of 0.1 can be taken as a reference value (CEN, 2006, Hennebert, 2019).

Results of the bioassays prevail on the assessment based on liquid concentration data for the sake of HP 14 classification (European Parliament and European Council, 2017). In this context, chemical characterization of ASR aqueous samples could allow to evaluate the leachable fraction of inorganic compounds (e.g. HMs), which can be considered responsible for the effects on the aquatic organisms. In fact, liquid concentration data can be used to calculate element-specific Toxic Units, by dividing measured values by the respective EC50 values as found in scientific literature or ecotoxicological repositories, to help interpret the outcomes from bioassays. The list of parameters which should be assessed on ASR eluates could be taken by what is prescribed by European Council, (2003).

Conversely, chemical characterization of solid test samples is needed to investigate the presence of non-soluble compounds likely occurring in ASR, i.e. Mineral Oil, PAHs and PCBs, whose effect in terms of toxicity cannot be assessed through aquatic bioassays (Cossu et al, 2014). It should be highlighted that ASR (as any other mirror entry) could be classified as hazardous for HP 7 and HP 11 in case concentrations of substances, classified with carcinogenic HSCs, are reported over specific thresholds established by specific regulations (European Commission, 2014). Therefore, the presence of one of the listed substances below the lowest thresholds specifically established by the regulations should trigger the need to perform the battery of terrestrial bioassays. In particular, the list of parameters which should be investigated in ASR solid test samples should include Mineral Oil (as Total Petroleum Hydrocarbons) i.e. sum of C<12 and C>12, PCBs and the list of PAHs required by the regulation (European Commission, 2014). This is reflected in the proposed protocol.

2.6 Ecotoxicological Characterization

The choice of the test battery has been determined following method proposed by Pandard and Römbke, 2013. Currently, there is consensus within the scientific community about this proposition, specifically when performed for fully comprehensive ecotoxicological characterization of waste (Moser and Römbke, 2009, Pandard and Römbke, 2013, Römbke, 2018). It should be assumed as a minimum set of assays, since it can be proved that the results from each ecotoxicological test will not be correlated with the other required tests, thus highlighting difference in the mechanisms of toxicity.

According to the principle of technical and economic feasibility (European Parliament and European Council, 2008b), each suggested bioassay should be performed on a unique test portion, constituted by the combination of three equal aliquots of the available test samples undergone chemical characterization (both solid and liquid).

The need to perform the set of terrestrial bioassays is triggered only if the derived concentration level of a defined list of substances in the solid test samples is not compliant with specific concentration limits proposed in national and international regulations.

2.7 Criteria for HP 14 Classification of ASR

The choice of concentration limits triggering the HP14 classification of the tested samples were established according to the proposition of Hennebert (2018a and 2018b). At the time of developing this approach, the proposed set of concentration limits were not applied in the national or international regulations. However, what is proposed in Hennebert, (2018) is currently the only available set of limits which can consistently be compared with the results of the proposed test battery, when performed according to the cited international guidelines (from EN ISO series, e.g. EN ISO 11348-3, EN ISO 8692 and EN ISO 6341).

3. PROPOSED PROCEDURE FOR HP 14 CLASSIFICATION OF ASR

3.1 ASR Sampling

- The technical goals of the sampling plan should be:
    1. the production of a laboratory sample representative of ASR regularly produced in a specific treatment plant considering the potential variability of input materials and produced waste in terms of both composition and granulometry;
    2. the production of a laboratory sample characterized by a sample size suitable, in terms of mass, to perform the planned analysis.
- To achieve the aforementioned technical goals, sampling of ASR is performed according to a probabilistic random sampling approach.
- The laboratory sample is obtained through a stepwise procedure:
    1. Primary sample (i.e. composite sample) production, and
    2. Quartering procedure.
- Two sampling techniques can be performed to obtain the primary sample:
    1. Sampling from temporary storage (see Paragraph 3.1.1);
    2. Sampling from a conveyor-belt (see Paragraph 3.1.2).
- The proposed methodology ensures that the laboratory samples are representative in terms of both quality and composition of each part of a monthly produced amount of ASR.
- A primary sample must be characterized by a total fresh mass of about 1.300 ± 50 kg.
- At the end of the quartering procedure, the laboratory sample which will undergo chemical and ecotoxicological characterization should be characterized by a total fresh mass of 30 ± 5 kg. The obtained laboratory sample should be characterized by a D95 ≤ 2 cm. Therefore, on-site size reduction (e.g. through portable shredding equipment) could be needed before performing the Quartering procedure.
- At the end of the quartering procedure, the laboratory sample which will undergo fractional composition analysis should be characterized by a total fresh mass of 60 ± 5 kg and should not undergo any size reduction for any reason.
- Different mass values for both primary and laboratory samples should be justified according to the results of further material investigations (e.g. bulk density) or granulometric analyses (e.g. definition of alternative D95) performed on untreated sample (i.e., not size reduction).
- Paragraph SM.2 of the Supplementary Material contains the minimum set of information that the sampling operator should complete and send to the laboratory with the collected sample.

3.1.1 Sampling from temporary storage

- A primary sample from a temporary storage heap must be performed monthly.
- The primary sample is a composite sample obtained by mixing together at least 20 increments, withdrawn from the ASR storage heap. Each increment must be characterized by a minimum increment weight of about 65 kg (i.e. 1.300 kg divided by 20 increments). It is preferred that increments are withdrawn with a mechanical bucket. Otherwise, increments should be collected using a shovel characterized by an opening size of at least 30 cm.
- Increments must be taken from the temporary ASR storage at different heap heights (see Figure 2 for examples of sampling locations based on heap subdivision). At least 3, 7, and 10 samples must be taken from the top, medium, and bottom layers of the heap, respectively. Additional increments could be obtained but care must be taken to maintain a ratio of 2:4:6 between increments withdrawn from the top, medium, and bottom layers, respectively. Each layer must be characterized by approximately the same height.
- The primary sample is then obtained after homogenizing (e.g., through mixing with shovels) the increments collected.

3.1.2 Sampling from conveyor-belt

- A primary sample from the conveyor belt must be collected monthly.
- The primary sample is a composite sample obtained by mixing together at least 20 increments, withdrawn from the conveyor belt during normal daily operations. Each increment must be characterized by a minimum size of about 65 kg (i.e. 1.300 kg divided by 20 increments).
- Each increment is sampled directly from the conveyor belt (during normal operation) preferably by use of cross stream sampler. Otherwise, a container can be used with enough of a capacity (i.e., characterized by an opening size of at least 30 cm), handled by a lifter, ensuring that a constant velocity is maintained through all the cross section of the conveyor belt.

3.1.3 Quartering procedure

- The production of the laboratory sample is achieved according to the so-called quartering procedure, as follows (Figure 3):
    I. Distribution of the primary sample of ASR on a cemented pavement and formation of a circular cake;

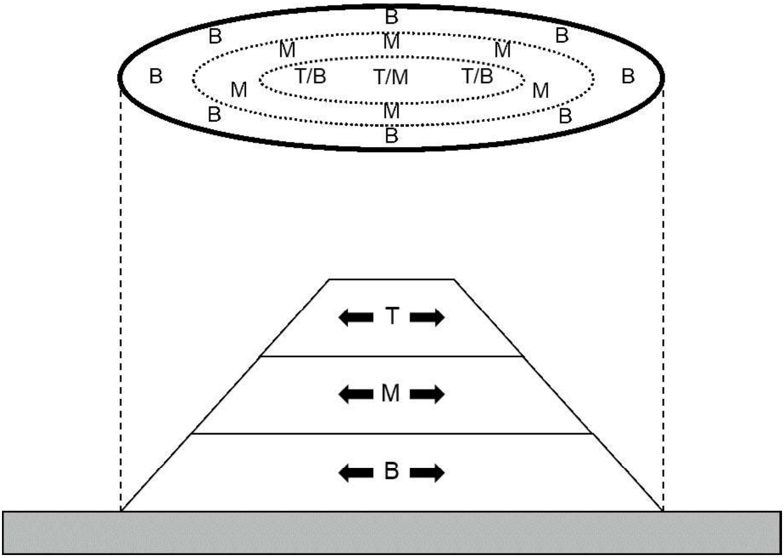

FIGURE 2: Schematization of ASR storage heap subdivision for increments withdrawal (T = Top layer, M = medium layer, B = Bottom layer).

II. Superficial subdivision with two perpendicular diametrical lines and complete removal of the material belonging to two opposite quadrants with a shovel and broom;
III. Mixing of the ASR from the remaining quadrants and formation of a new circular cake;
IV. Superficial subdivision with two orthogonal diameters, displaced by 45° with respect to the two previous tracers and complete removal of the material belonging to two opposite quadrants with a blade and broom;
V. Mixing of the remaining parts (i.e., about a quarter of the original one) and formation of a cake, ensuring to maintain approximately the same layer thickness while reducing the overall diameter;
VI. On the new circular cake, the same steps carried out previously on the initial cake are iterated enough times so that with the last quartering results in a sample characterized by a weight of 30 ± 5 kg, which is defined as the “laboratory sample”.

- Instead of the quartering procedure as here described, the “long pile” procedure can be performed equally for the reduction of mass of the primary sample, according to EN 14780:2017.

3.2 Transport and storage

3.2.1 Transport

- The laboratory samples should be sent to the laboratory and stored in a resealable container that also ensures that the samples are kept in dark conditions.
- The container material shall be appropriate and not be a cause any type of contamination.
- Transport times of the laboratory samples should be as short as possible. Transport times shall be included in the overall storage time. A transport time of less than 12 h under refrigerated conditions (4 ± 2) °C shall be maintained in order to preserve the original properties of the laboratory samples and to avoid the migration of volatile fractions. If it is demonstrated that volatile compounds are not present in the sample, different transportation conditions can be permitted.

3.2.2 Storage

- Storage should be carried out in the same containers used for transport. Possible changes may be considered, and storage conditions shall be designed accordingly in order to limit the effects of such changes on the results of any tests. Any applicable changes must be reported in the final test report;
- Storage time starts from the collection of laboratory samples and ends with the start of definitive tests and should be as short as possible;
- A storage time of less than two months and at low temperature conditions (4 ± 2) °C shall be established in order to appropriately maintain the properties of the waste samples.

3.3 Fractional composition analysis

- Fractional composition analysis is performed according to the following stepwise procedure:

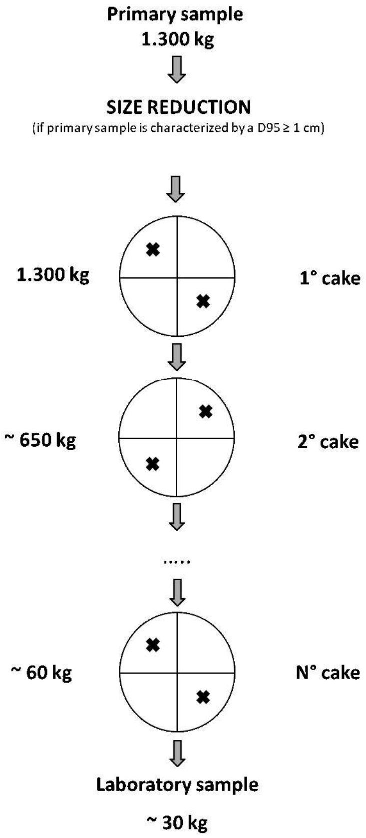

FIGURE 3: Quartering procedure for the preparation of the final laboratory sample (derived from EN 15002:2013).

I. Three test portions of about 20 kg are obtained through the quartering and coning procedure from the subsample derived from the previous step. No size reduction processes are performed on the 3 derived test portions;
II. Each obtained test portion is sieved through a 20 mm sieve;
III. The over-sieve fraction is divided into different categories (e.g., plastic foam and rubber, metals, cellulosic materials (wood and paper), textiles, electric cables, composites, and undersieve < 20 mm);
IV. Each fraction is weighed to determine the specific weight percentages;

- The analysis is performed in triplicate, (i.e., 3 test portions (in total 3*20 kg = 60 kg));
- An example of the report table to complete while performing the analysis and a exemplificatory figure for the graphical representation of the results are included in Paragraph SM.3 in the Supplementary Material.

3.4 Preparation of test samples for chemical and ecotoxicological analysis

- Chemical analyses are performed on the following samples:
  - ASR solid test samples (see Paragraph 3.5.1), and
  - ASR aqueous test samples which are derived from the leaching procedure (see Paragraph 3.5.2).
- Ecotoxicological Analyses are performed on a defined mixture of a dilution media (as required by each selected bioassay) and:
  - ASR solid test samples for terrestrial tests (see Paragraph 3.6.1), or
  - ASR aqueous test samples for aquatic tests, derived from a leaching procedure performed on the solid test portion of the ASR subsamples (see Paragraph 3.6.2).

3.4.1 Solid test sample preparation

- The preparation of the solid test sample is a stepwise process: each phase must be performed according to the following approach:
  I. A subsample of the laboratory sample, characterized by a weight of about 15 kg, must be obtained through a quartering and coning procedure.
  II. Non-crushable material occurring in the subsample characterized by a particle size > 4 mm (e.g. metallic parts such as nuts, bolts, scrap) that can possibly damage crushing equipment, must be selected and removed from the subsample before size reduction occurs. The particles withdrawn must be reported and classified according to the material composition and weight. The removed fraction should not exceed 10% (mass) of the total aliquot of the sample considered, otherwise the selected subsample must be discarded.
  III. At least 95% (on a weight base) of the ASR test sample must be characterized by a grain size of less than 4mm. If oversized (i.e. grain size > 4mm) materials exceed 5% (on a weight base), the oversized materials must be reduced in size with an appropriate crushing apparatus (e.g. shredder). Materials with a high particle size (e.g., plastic foam, long electric cables, bigger pieces of textiles) can be cut with other kinds of manual or mechanical devices prior to crushing, in order to avoid blockages of the crushing equipment.
  IV. Place the size-reduced subsamples within a container of adequate capacity and mix carefully with an appropriate tool (e.g. shovel, scoop, or trowel).
  V. The size of the prepared test samples must be determined according to the need of the following analytical determinations. Therefore, the needed test samples are:
    - Test sample (about 300 g TS) for aqueous test sample preparation through a leaching test (see Paragraph 7.2),
    - Test sample (about 300 g TS) for chemical cha-

racterization of ASR, and
  - Test sample (the remaining amount of ASR test sample) for ecotoxicological characterization on terrestrial organisms.

3.4.2 Aqueous test sample preparation – Leaching test

Leaching test is performed in triplicate and according to the following stepwise procedure:

I. Place a solid ASR test portion characterized by a total dry mass of 90 (± 5) g TS (i.e., prepared according to Paragraph 9.1) in a glass container with a nominal volume of 2 L.
II Add distilled water to reach 900 ml of water including the moisture of the sample to ensure a liquid-solid ratio (L/S) of 10 L/kgTS ± 2%.
III. Agitate the glass container containing the ASR solid test portion and the distilled water using an end-over-end tumbler at 5-10 rpm at room temperature (15°C to 25°C) for 24 ± 0,5 h.
IV. Allow suspended solids to settle for 15-30 min and centrifuge the eluate for 30 min at 2500 g, whether or not incomplete separation of solid and liquid phases occurs.
V. Filter the obtained eluate through a 0,45 μm membrane filter using a vacuum or pressure filtration device. Rinsing the filter is not permitted after filtration.
VI. Measure and report conductivity in μS/cm (EN 10523:2012) and pH (ISO 10523:2008) of the resulting water extract immediately.
VII. Do not adjust the pH in any case during the leaching test procedure;
VIII. Take a subsample (e.g. ~1/3 L) from each of the water extract replicates to reconstitute the test sample to be used in ecotoxicological bioassays on aquatic organisms.
IX. The required volume (in ml) of the prepared test samples must be determined according to the

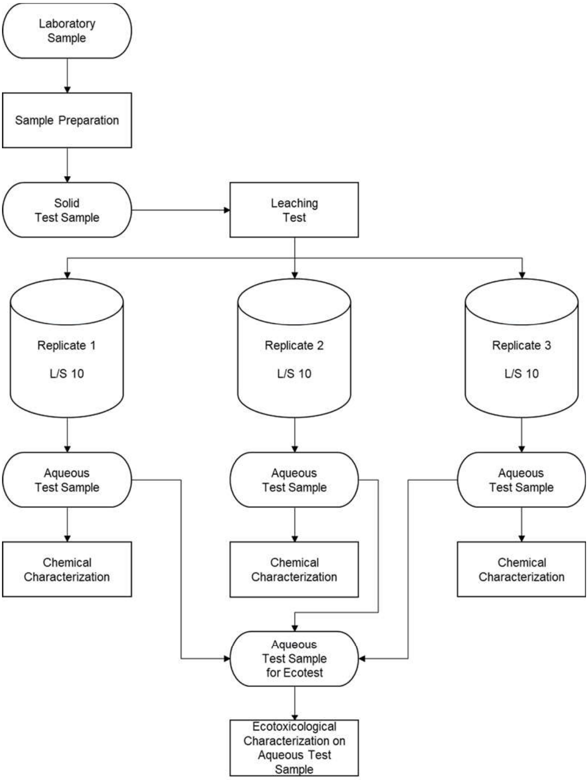

FIGURE 4: Detailed graphical procedure for deriving aqueous test samples through leaching tests for chemical and ecotoxicological analysis.

requirements of the following analytical determinations. Therefore, the necessary amount of test samples are:
  - Test sample (about 100 ml for each water extract replicate) for chemical characterization;
  - Test sample (about 400 ml) for ecotoxicological characterization of aqueous test sample reconstituted as described in step VIII.
- The proposed procedure is graphically described in Figure 4.
- Further details of the leaching procedure may be found in EN 12457-2 and EN 14735.

3.5 Chemical characterization

- Chemical Analysis are performed on:
  - ASR solid test samples, and
  - ASR aqueous test samples, (i.e., derived from a leaching procedure (see Paragraph 3.4.2)).

3.5.1 Chemical characterization of ASR solid test sample

Table 1 lists the chemical concentrations that must be measured in the prepared ASR solid test samples

Chemical analysis listed in Table 1 must be performed on 3 ASR test samples, where each one is characterized by an amount of 90 (± 5) g TS.

3.5.2 Chemical characterization of ASR aqueous test sample

- The aqueous test samples, derived from each replicate leaching test (see Paragraph 3.4.2), must undergo chemical characterization for the parameters established in Table 2;

3.6 Ecotoxicological characterization

- Compare concentration values obtained from solid test samples (see Paragraph 3.5.1) with the concentration ranges listed in Table 3. Whether at least one concentration values of non-soluble compounds is within the listed ranges, it will trigger the need to implement terrestrial bioassays. Otherwise, only aquatic test must be implemented.
- Results of ecotoxicological tests are expressed in terms of EC50 (%vol/vol and %w/w for aquatic and terrestrial tests respectively), which is the tested dilution of the test samples (with respect to the specific dilution media) generating 50% of the considered effect in the specific bioassay.

TABLE 1: List of parameters to be measured in solid test samples.

Analytes	Unit of measure	Standards
∑ TPH (C<12 + C>12)	% Organics	EN 14346:2007
∑ TPH (C<12 + C>12)	mg/kg$_{TS}$	EN 14039:2005
Benzo(a)antracene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
Benzo(b)fluorantene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
Benzo(j)fluorantene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
Benzo(k)fluorantene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
Pyrene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
Crisene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
Dibenzo(a,h)antracene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
Naftalene	mg/kg$_{TS}$	EN 16181:2018 or EN 15527:2008
∑(PCB/PCT)	mg/kg$_{TS}$	EN 12766-1 and EN 12766-2

3.6.1 Bioassays on terrestrial organisms

- The test battery presented in Table 4 must be implemented on the ASR solid test samples prepared following the procedures described in Paragraph 3.4.1.
- The ranking of the sensitivity of the test battery listed in Table 4 is: Arthrobacter > Eisenia > Brassica.
- Each test is carried out following the instructions laid out in the specific standard listed in Table 4.

TABLE 2: List of parameters to be measured in aqueous test samples.

Analytes	Unit of measure	Standards
DOC	mg/L	EN 1484:1997
TDS	mg/L	EN 14346: 2007
Chlorides (as Cl^-)	mg/L	EN ISO 10304-1:2009
Fluorides (as Fl^-)	mg/L	EN ISO 10304-1:2009
Sulphates (as SO_4^-)	mg/L	EN ISO 10304-1:2009
Metals and metalloids		
Antimony	mg/L	EN ISO 11885:2009
Arsenic	mg/L	EN ISO 11969:1996
Barium	mg/L	EN ISO 11885:2009
Cadmium	mg/L	EN ISO 11885:2009
Chromium Total	mg/L	EN ISO 11885:2009
Copper	mg/L	EN ISO 11885:2009
Lead	mg/L	EN ISO 11885:2009
Mercury	mg/L	EN ISO 12486:2012
Molybdenum	mg/L	EN ISO 11885:2009
Nickel	mg/L	EN ISO 11885:2009
Selenium	mg/L	EN ISO 11885:2009
Zinc	mg/L	EN ISO 11885:2009

TABLE 3: Concentration ranges on ASR solid samples that trigger the need to perform terrestrial bioassay. a – derived from European Council, 2004 and further amendments. b – derived from European Parliament and European Council, 2019.

Substance	Unit of measure	Concentration range[a]
∑ TPH (C<12 + C>12)	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Benzo(a)antracene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Benzo(b)fluorantene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Benzo(j)fluorantene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Benzo(k)fluorantene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Pyrene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Crisene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Dibenzo(a,h)antracene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
Naftalene	mg/kg$_{TS}$	$0.001 \leq x < 1{,}000$
∑(PCB/PCT)	mg/kg$_{TS}$	$0.0001 \leq x < 50$[b]

3.6.2 Bioassays on aquatic organisms

- Test battery presented in Table 5 must be implemented on the ASR aqueous test samples prepared following procedures described in Paragraph 3.4.2.
- Ecotoxicological tests should be carried out within 72 h from the preparation of the aqueous test sample, which shall be stored in glass bottles with a minimal headspace at (4 ± 2) °C.
- No pH adjustments of the test sample shall be carried out. pH of the mixture should be measured immediately at the beginning and at the end of the test.
- The ranking of the sensitivity of the test battery listed in table 5 is: Pseudokirchneriella > Daphnia magna > Vibrio Fischeri.
- Each test is carried out following the specific standard listed in Table 5.

3.7 Classification criteria

- ASR is classified as HP 14 if at least one of the performed bioassays resulted in an EC50 not compliant (i.e. strictly lower) than the concentration limits listed in Tables 4 and 5 for terrestrial and aquatic tests, respectively.

4. CONCLUSIONS

This paper proposes an experimental procedure for the HP 14 classification of ASR through ecotoxicological testing. In addition, this manuscript includes the founding principles behind the proposition.

TABLE 4: Recommended ecotoxicological test battery on terrestrial organisms. Rounded limits are suggested according to Hennebert (2018b) based on thresholds proposed in Hennebert (2018a) for HP14 classification of waste. Concentration limits are reported as dilution % expressing dry mass of solid test sample on dry mass of specific dilution media, as required from the specific test.

Organism	Concentration limits for HP 14 classification	Standards
Soil bacteria (Arthrobacter globiformis)	EC50 < 5% (w/w)	ISO 18187:2016
Plants (Brassica rapa)	EC50 < 15% (w/w)	EN ISO 11269-2:2012
Soil invertebrates (Eisena fetida)	EC50 < 5% (w/w)	ISO 17512-1:2008

TABLE 5: Recommended ecotoxicological test battery on aquatic organisms. Rounded limits are suggested according to Hennebert (2018b) based on thresholds proposed in Hennebert (2018a) for HP14 classification of waste. Concentration limits are reported as dilution % expressing volume of aqueous test sample on volume of specific dilution media, as required from the specific test.

Organism	Concentration limits for HP 14 classification	Standards
Aquatic bacteria (Vibrio Fischeri)	EC50 < 15% (vol/vol)	EN ISO 11348-3
Algae (Pseudokirchneriella subcapita)	EC50 < 10% (vol/vol)	EN ISO 8692
Crustaceans (Daphnia magna)	EC50 < 10% (vol/vol)	EN ISO 6341

The document includes technical detailed instructions for each step of the procedure, from the production of the laboratory sample to ultimate classification based on the comparison of the results of a battery of bioassays with a proposed set of concentration limits. Furthermore, the chemical and physical characterization is required for both solid test samples and aqueous test samples. These samples are obtained through the performance of a leaching test. The proposed ecotoxicological tests include 3 aquatic and 3 terrestrial tests. However, the need to perform terrestrial tests is triggered when concentrations of several non-soluble and non-leachable contaminants in the solid test samples are recorded within proposed concentration ranges.

The compliance with European Regulation for HP 14 classification is ensured through the reference to international technical standards derived from the EN ISO series for each step of the proposed procedure.

Through this paper, the authors aimed to share with the scientific community the principles of this procedure and their technical application on a specific waste stream, while providing a basis for the development of sound scientific procedures for HP 14 classification by testing for other types of wastes, classified as mirror entries in the EWC.

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