Bioresource Technology 313 (2020) 123557

Contents lists available at ScienceDirect

Bioresource Technology

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

Uncovering new indicators to predict stability, maturity and biodiversity of compost on an industrial scale

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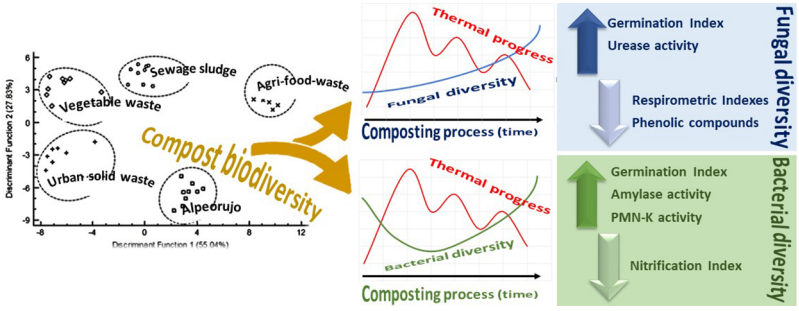
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Industrial compost
Biodiversity
Stability
Maturation
Respirometric index

ABSTRACT

Currently, the metagenomic study of the composting process has gained great importance since it has allowed the identification of the existence of microorganisms that, until now, had not been isolated during the process by traditional techniques. However, it is still complex to determine which bioindicators could reveal the degree of maturity and stability of a particular compost. Thereby, the main objective of this work was to demonstrate the possible correlation between traditional parameters of maturity and stability of compost, with other indicators of biodiversity in products highly heterogeneous from composting processes on an industrial scale.

The results demonstrated the enormous influence of the raw materials in characterizing the products obtained. Even so, important relationships were established between the Chao1 and Shannon indexes, and certain parameters related to the maturity, stability and toxicity of the samples, such as nitrification index, humification rate, phenolic content, germination index or oxygen consumption.

1. Introduction

Composting is an aerobic biotransformation process of organic matter, which occurs under controlled conditions of humidity, temperature and aeration. In recent years, due to environmental problems related to the generation and accumulation of anthropogenic wastes, composting has become an effective, ecological and sustainable alternative, aimed at the re-valorization of organic waste of enormous agronomic value (Tittarelli et al., 2002; Onwosi et al., 2017).

Some authors have reported that the proper transformation of organic matter and the quality of the final compost depends on the qualitative and quantitative composition of microorganisms associated

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https://doi.org/10.1016/j.biortech.2020.123557
Received 17 March 2020; Received in revised form 15 May 2020; Accepted 17 May 2020
Available online 19 May 2020
0960-8524/

with composted materials (Pepe et al., 2013). However, deep knowledge of the microorganisms that form a compost pile is not an easy task because of culture medium limitations (Chandna et al., 2013).

Traditionally, physicochemical and enzymatic characterization of composting process have served as a basis for assessing the stability and the degree of compost maturation. The evolution of several parameters, such as the C/N ratio, humic/fulvic ratio (Iglesias-Jiménez and Pérez-García, 1992), ammonium/nitrate ratio (Bernal et al., 1998), germination index (Emino and Warman 2004), oxygen conpsumption (AT_4 or DRI_{24}) (Barrena-Gómez et al., 2006), as well as dehydrogenase, glucosidase, phosphatase, esterases, protease or urease activities (Tiquia, 2002), have been traditionally taken into account when establishing the degree of stability and maturity of a particular compost.

Metagenomic study of the composting process has gained great importance since it has allowed us to know the existence of microorganisms that until now had not been isolated in this process through traditional techniques. In the last decades, several works have correlated the succession of microorganisms, organic matter degradation, and functions of microorganisms during composting processes using genomic sequencing, and phylogenetic analyses of microbial communities (Langarica-Fuentes et al., 2014, 2015; Wang et al., 2018). However, no previous research has attempted to determine which biodiversity microbial indicators reveal both a successful composting and an optimal-stabilized and mature compost.

Given the problem raised above, the main objectives of this work were (i) to characterize the products derived from 15 composting processes on an industrial scale from different anthropogenic organic wastes from a physical–chemical and enzymatic point of view; (ii) to demonstrate the relationship between physicochemical and enzymatic parameters, and microbial bioindicators (Shannon and Chao1); and (iii) to determine the influence of raw materials on the biodiversity degree of compost on an industrial scale and the potential relation between stability, maturity and biodiversity.

2. Material and methods

2.1. Sampling strategy

Samples were taken from 15 companies dedicated to the composting of different raw materials. The main criteria to select those companies was the starting materials used. So, 15 full-scale composting facilities operating in the Southeast of Spain were contacted. The entire sampling process took place in one day in facilities working in continuous, i.e. Vegetable Waste (VW), Urban Solid Waste (USW) and Sewage Sludge (SS). In the case of Agri-food Waste (AW) and Olive Mill Waste or "Alpeorujo" (ALP) facilities, due to the seasonality of the raw material, sampling was performed when the specific composting phases were reached

A total amount of 45 samples (5 input wastes, three facilities per waste and one composting phase for each) were collected from November 2016 to November 2017. During each stage, a sample of nine different locations (300 g of each site) was taken, so that all possible areas were covered. These samples were mixed to obtain a representative and homogeneous sample (approximately 3 kg). For analytics, each sample was divided into three parts. In the case of USW samples, all inappropriate materials (plastic, glass, metal, etc.) were removed manually. After this, samples were crushed in a food processor (Moulinex Cousine Companion HF800A13) and then immediately stored in vacuum bags and frozen at −20 °C. They were defrost at room temperature for 24 h before analysis.

All analyses were performed in triplicate.

2.2. Monitoring parameters

To test the quality of the compost samples, four basic physical–-chemical parameters were analyzed based on Spanish current legislation (BOE 999/2017). Moisture (M) was determined by drying at 105 °C for 24 h. OM content was evaluated by determining the weight loss by ignition at 550 °C. Total nitrogen (TN) and total carbon (TC) were determined in solid dry samples by combustion at 950 °C using a LecoTruSpec C-N elemental analyzer (Leco Co., St. Joseph, MI, USA), and then C/N Ratio was calculated. Temperatures reached in the thermophilic phase was measure on-site by a long-handled (50 cm) PT100 probe. The thermophilic temperature data was provided by the 15 collaborating companies, when thermal peaks were reached in the bioxidative phase of each composting process.

2.3. Maturity parameters

Humic fractions were obtained by the modified method of Cavani et al. (2003). 2 g of sample were incubated 48 h at 65 °C and 120 rpm with 100 mL of 0.1 M NaOH and 0.1 M $Na_4P_2O_7 \times 10\ H_2O$ in a thermostatic water bath. The extracts were centrifuged 10 min at 10,000 rpm, and then the supernatant filtered using a 0.8 mm cellulose acetate filter (standard MF-Millipore membrane, EMD Millipore Corporation, Billerica, MA, USA, 2013). This filtered solution constituted the total extractable carbon. Fractioning methodology of Ciavatta et al. (1990) was applied to separate humic (C_{HA}) and fulvic (C_{FA}) acids through polyvinylpyrrolidone. Total organic carbon from all fractions was analyzed by combustion in a TOC analyzer (TOC-VCSN, Shimadzu, Japan). The Humification Rate was calculated:

$$HR = ((C_{HA} + C_{FA}) / TC)\ x\ 100$$

where: C_{HA}: Organic carbon from humic acids (%), C_{FA}: Organic carbon from fulvic acids (%), TC: Total Carbon (%)

Samples were diluted 1:5 in distilled water to evaluate nitrate content ($N\text{-}NO_3^-$. Suspensions were incubated for 10 min at 20 rpm. After the incubation period, the samples were filtered under vacuum. The resulting extracts were used for nitrate measurement using the system Nitracheck 404. On the other hand, to evaluate ammonium content ($N\text{-}NH_4^+$), fresh samples were diluted 1:10 in distilled water. Suspensions were incubated and filtered under the same conditions above cited. 0.1 M $MgSO_4$ was added to the supernatant in a 1:10 ratio. The ammonium concentration was measured using a reference electrode (Reference no. 5044, HACH, Loveland, Colorado, USA) and an ammonium ion-selective electrode (Reference no. HI4101, HACH, Loveland, Colorado, USA). Nitrification Index was expressed as the relation between ammonium / nitrate content (Bernal et al., 1998).

2.4. Stability parameters

Phenolic acids were extracted in sodium pyrophosphate ($Na_4P_2O_7$) following the method described by Morita (1980) and quantified as previously described by Marambe and Ando (1990). It was measured at a wavelength of 725 nm in a multiwell spectrophotometer (Eon-Biotek).

According to Zucconi et al. (1981), phytotoxicity tests were performed using watercress seeds (*Lepidum sativum* L.). Compost was mixed with water to reach a moisture content equivalent to 65%. After 30 min, a 10% aqueous extract was obtained and filtered through a 0.45 mm pore size membrane. Four mL of the filtrate were added to square Petri dishes (12 × 12 cm) with 25 seeds of *L. sativum* located on a sheet of filter paper as a support. Four replicates were used for each sample providing a total of 100 seeds. The same procedure was performed using distilled water instead of compost extracts (control seeds). Plates were placed in a growth chamber at 25 °C for 48 h in the dark. After this period, germination percentage and root lengthening were measured, and finally, Germination Index (GI) calculated based on the following formula:

$$GI = ((G\% \times L) / (G_c\% \times L_c)) \times 100$$

where: G%: Germination percentage from seeds exposed to compost extracts, L: mean of root lengthening from seeds exposed to compost

extracts, Gc%: Germination percentage from control seeds exposed to distilled water, Lc: mean of root lengthening from control sample exposed to distilled water

To analyze the biodegradability of the samples, a modified methodology describes by Barrena et al. (2009) and Ponsá et al. (2010) was applied. 100 g of each compost was placed in a reactor after adjusting the humidity to 50%. Each reactor consisted of a PVC container (30 cm × 10 cm), at whose base a 2 mm diameter pore metal net was arranged to support the material and to provide an air distribution chamber. This system was continuously submerged in a water bath at 37 °C. Airflow in the reactors was adjusted by means of an air flow controller (Bronkhorst Hitec, The Netherlands). Air was passed through a humidifier at the same temperature of the reactor to avoid water losses and moisture changes. Exhaust air from the reactors was sent to an oxygen sensor (Alphasense Ltd., Essex. CM77 7AA UK) prior dehumidification in a water trap. Biodegradability of the samples was evaluated by measuring the total oxygen consumed throughout 4 days after overcoming the delay lag phase (AT_4) (Adani et al., 2001). Results were expressed as g O_2 Kg^{-1} MO h^{-1}.

2.5. Enzymatic parameters

β-glucosidase (GLUC), lipase (LIP) and phosphomonoesterase (PMN-K) activities were estimated by a colorimetric method at a 400 nm wavelength in an Eon-Biotek multiwell spectrophotometer. Method described by Tabatabai (1982) was used to determine β-glucosidase activity, by measuring p-nitrophenol released from p-nitrophenyl-β-D-glucopyranoside hydrolyzed for 1 h at 37 °C. Lipase activity was estimated by the method described by Farnet et al. (2010). In this case p-nitrophenyl laureate was hydrolyzed for 2 h at 30 °C releasing p-nitrophenol. Protocol to determine alkaline phosphomonoesterase activity was described by Tabatabai and Bremner (1969), in which p-nitrophenyl-β-D-glucopyranoside was hydrolyzed for 1 h at 37 °C releasing nitrophenyl phosphate.

Determination of cellulose (CEL), amylase (AMY) and xylanase (XYL) activities were based on the colorimetric estimation of glucose released at a 550 nm wavelength in a 96-well multiplate (Eon-Biotek). To detect cellulase activity, samples were incubated during 2 h at 37 °C in presence of substrate (1% carboxy methyl cellulose in 50 mM sodium acetate buffer, pH 5), as described Libmond and Savoie (1993); in the case of amylolytic activity the reaction lasted 24 h at 35 °C in presence of substrate (1% soluble starch in 0.5 M phosphate buffer, pH 5.5), according to the method of Mishra et al. (1979). Xylanolytic activity was determined at 30 °C for 30 min in presence of substrate (1% xylan solution in 50 mM citrate–phosphate buffer, pH 6.5) following the method described by He et al. (1993).

Ureolytic (URE) activity was measured following the method described by Bremner and Mulvaney (1978). 2 g of sample was incubated during 1 h at 37 °C in a solution of sodium hypochlorite and phenol with sodium nitroprusside as catalyst. The released ammonia was determined by measuring the chromogenic complex formed at 640 nm wavelength. In the case of protease activity (PRO), the method of Ladd and Butler (1972) was applied. 1 g of sample was incubated in sodium caseinate during 1 h at 37 °C. After reaction with the Folin Ciocalteau reagent, colorimetric estimation was performed at 700 nm in a 96-well multiplate.

2.6. Library preparation and sequencing.

DNA extraction was performed with the DNA isolation kit DNeasy PowerSoil (Qiagen). A total of 45 samples corresponding to 15 treatment plants were processed × 1 samples × 3 repetitions. Bacterial DNA for sequencing was amplified by PCR, using primers Bakt 341F and Bakt 805R, by Supreme NZYTaq 2x Green Master Mix (NZYTech) and with the following cycle conditions: 5 min at 95 °C, continued for 25 cycles from 30 s to 95 °C, 30 s to 50 °C, 30 s to 72 °C, and a final step of 10 min at 72 °C. Secondary amplification was performed to link the index sequences that were required to multiplex different libraries. It was performed with identical conditions, but only 5 cycles and 60 °C as annealing temperature.

For the preparation of the fungal library, a fragment of the ITS2 fungal region of approximately 300 bp was amplified using the ITS86F primers and ITS4 of Supreme NZYTaq 2x Green Master Mix (NZYTech) and with the following cycle conditions: 5 min at 95 °C, continued for 35 cycles 30 s at 95 °C, 30 s at 47 °C, 30 s at 72 °C, and a final step of 10 min at 72 °C.

In both cases, a secondary amplification was made to join the index sequences which were required for multiplexing different libraries. It was carried out with identical conditions, but only 5 cycles and 60 °C as annealing temperature. Negative controls were included in primary and secondary amplifications using ultrapure water and the purified PCR product from the primary amplification, respectively.

The library products previously obtained were processed in agarose gels stained with GreenSafe (NZYTech) to confirm the absence of non-specific amplification and verify the size of the library. Then, these products were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek). The final DNA concentration per sample was measured with a Qubit dsDNA HSAssay assay (Thermo Fisher Scientific). Samples were mixed in equimolar amounts and sequencing was performed in a MiSeq PE300 run (Illumina) in AllGenetics & Biology SL (La Coruña, Spain).

2.6.1. Metagenomic data and biodiversity indicators

The raw data set were separated based on the sample-specific barcodes and deleted the indices and sequencing primers. The quality of the demultiplexed FASTQ files was verified by FastQC software. Paired-end assembly of the R1 and R2 reads was performed with FLASH. CUTADAPT software 1.3 was used to eliminate sequences below 300 nt which did not contain the PCR.

Data files were analyzed using “Quantitative Insights into Microbial Ecology” (QIIME) v1.9.0 software (Caporaso et al. 2010). Low abundance of OTUs (below 0.5%) of each sample was removed to correct the index jumping phenomenon.

The sequences were taxonomically assigned using the open-reference approach in QIIME. The OTU picking was run using the Silva_128 Database for bacteria and Unite for fungi. Each OTU was assigned to a microbial taxon using the UCLUST algorithm with a confidence threshold of 97%. Singletons and OTUs with less abundance were excluded from the analysis. Low abundance of OTUs (below 0.05%) of each sample was removed to correct the index jumping phenomenon. Alpha diversity index was calculated from rarefied samples, using Shannon index for diversity and Chao1 index for richness.

2.7. Statistical analyses

Physico-chemical and enzymatic data obtained were subjected to statistical analysis using Statgraphics Centurion XVIII.I (StatPoint Technologies Inc., Virginia, USA). Analysis of variance (ANOVA) was performed to compare mean values for the factors analyzed (Raw materials and Facilities) (p less than 0.05). A Discriminant Analysis was used to assess the adequacy of the classification of the samples in groups of raw materials on the basis of physico-chemical and enzymatic variables. Relations among the different physical–chemical and enzymatic parameters were analyzed using principal component analyses, while correlations between those and biodiversity indexes were stablished by Pearson test at 95% confidence level.

2.8. Data availability

Sequences are stored in the MG-RAST public repository, available by Accession Number mgp94523. The whole datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Table 1
Monitoring and maturation parameters. Moisture (M), Organic Matter (OM), C/N ratio, C_{HA} / C_{FA} Ratio, Humification Ratio (HR) and Nitrification Index in final compost from 5 types of organic waste. Values followed by same letters are not significantly different (P less than 0.05) according to Fisher's LSD test. Vegetable Waste (VW), Urban Solid Waste (USW), Sewage Sludge (SS), Agro Waste (AW) and "Alpeorujo" (ALP).

Organic Waste	Moisture (%)	Organic Matter (%)	C/N Ratio	C_{HA}/C_{FA}	HR	Nitrification Index
VW	28.74ab	50.45ab	12.24a	0.97ab	14.90b	0.36ab
USW	22.64a	51.87ab	16.62b	1.30b	7.96a	0.43b
SS	31.84ab	41.15a	10.92a	1.40bc	12.57b	0.80c
AW	40.43b	58.42b	11.83a	0.71a	11.67ab	0.30ab
ALP	40.85b	58.20b	17.22b	1.85c	10.66ab	0.12a

3. Results and discussion

3.1. Physico-chemical parameters: maturity, stability and toxicity

Concerning the products obtained from the 15 composting processes surveilled in this work, Table 1 shows the main physical–chemical parameters analyzed to determine maturation level of samples. It should be noted that all the compost analyzed derived from composting processes in which a sufficient sanitation temperature was reached during thermophilic phase. The average of thermophilic temperature for each installation complied with the recommendations of the Environmental Protection Agency (EPA, 2003). The highest average temperature was reached in the process performed from plant waste (VW) (60–70 °C). In the rest of the composting facilities, the average temperature in the thermophilic phase ranged around 50 °C.

Both moisture, C/N Ratio and OM are parameters included in the Spanish regulation (BOE 999/2017) to determine basic quality compost. According to this regulation, in general, a compost suitable for use must have less than 40% humidity, a C/N Ratio less than 15–20 and an OM percentage greater than 35%. In accordance with these criteria, all the compost analyzed in this work met the basic quality parameters, with some exceptions indicated below. The USW3 and ALP3 products showed higher humidity values than expected (50.95 and 52.68%, respectively). Only the SS2 sample showed values below 35% organic matter (26.09%), while USW3 was the only compost that showed a C/N Ratio higher than 20 (data not shown).

When the products were analyzed statistically according to the nature of raw materials, the results revealed a greater homogeneity of the samples (Table 1). Therefore, no significant differences were observed in terms of moisture and organic matter content, although the USW and ALP compost stood out in relation to the C/N Ratio, which could be due to the characteristics of the original materials, or even to the hasty completion of composting processes.

In addition to the parameters included in BOE 999/2017 (humidity, organic matter and C/N Ratio), other parameters affecting the quality of final product have been studied. Stabilization and humification of composted material occurs during the maturation phase of the process. In order to be useful in agriculture, the product obtained must show low toxicity and high humification level (Jurado et al., 2015).

The humification process could be reflected through the C_{HA}/C_{FA} ratio, which has traditionally been proposed as an important indicator of compost stability (Iglesias-Jiménez et al., 2008). C_{HA} are derived mainly from organic materials with a higher maturity level, in contrast to C_{FA}, which predominate in more immature materials (Martínez-Salgado et al., 2019). According to Iglesias-Jiménez et al. (2008), C_{HA}/C_{FA} values greater than 1 indicate that a proper humification process is occurring, while C_{HA}/C_{FA} values greater than 1.6 indicate that product is already mature. Additionally, Humification Rate (HR) is considered an important indicator of the formation of humic substances during composting, since it can be established with precision if the humification has been carried out successfully (Tittarelli et al., 2002). According to Roletto et al. (1985) the minimum value of HR for a mature compost should be 7.

Based on the results shown in Table 1, the compost obtained from ALP showed the highest C_{HA}/C_{FA} ratio (1.85), followed by the compost made from USW (1.30) and SS (1.40). However, when the total humic content was calculated in relation to total carbon (Humification Rate, RT), the highest values corresponded to materials made of VW and SS (14.90 and 12.57, respectively) although no real significant differences were observed depending on the raw materials.

On the other hand, some authors consider that compost maturity is related to the ratio between $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ (Nitrification Index) (Bernal et al., 1998; Onwosi et al., 2017). The classification of the compost according to this parameter is carried out based on some guidance values: fully mature compost (less than 0.5), mature compost (between 0.5 and 3.0) and immature product (greater than 3.0). Based on this classification, none of the compost analyzed could be considered immature, although a different degree of maturity could be established (Table 1). Thus, while compost from ALP was considered statistically more mature (0.12), the product obtained from SS was considered the least mature (0.8) (based on Nitrification Index).

In addition to maturity level, in order to stablish quality compost some authors consider of great relevancy several parameters of biological stability and phytotoxicity (Adani et al., 2001; Barrena-Gómez et al., 2006; Barrena et al., 2009; Cesaro et al., 2019). In this sense, compost could be classified on the basis of Germination Index (GI). GI values less than 50% are considered a sign of phytotoxicity and immaturity. Phytotoxicity level is considered moderate when GI is around 50–80%, while a compost could be considered non phytotoxic and mature when GI is between 80 and 100%. GI values higher than 100% are signs of phytostimulant effect in compost. On the other hand, the accumulated oxygen consumption during 4 days (AT_4) could be taken into account as an adequate stability parameter directly related to the microbial activity inside the composting piles. Excessively high AT_4 values could influence the toxic nature of the samples and adversely affect the GI. This effect has also been described when the content in phenolic compounds is too high (Said-Pullicino et al., 2007).

Based on the results shown in Fig. 1a, 1b and 1c, the most phytotoxic compost was those obtained from VW and USW (average GI less than 50%). Compost from SS and ALP were considered as mature showing GI average values between 80 and 100%, while the best phytostimulant effect was reported in the case of AW-based compost (GI average higher than 100%). In general terms, the best GI values (SS, AW and ALP) matched with the lowest average of phenolic compounds content and AT_4 values. However, in view of the results obtained in this work, this last correlation is not clear and it could be influenced by other different factors such as the content of organic acids, volatile compounds, heavy metals, salts, as well as the differences in the chemical structure or the hydrophobic character of the different phenolic compounds present in the samples (Barral and Paradelo, 2011; Pinho et al., 2017).

3.2. Enzymatic characterization of the final products

The great influence of the starting materials could affect the standardization of the enzymatic activity at the end of composting process (Jurado et al., 2014; Estrella-González et al., 2019).

In this work, enzymatic characterization of the samples was very

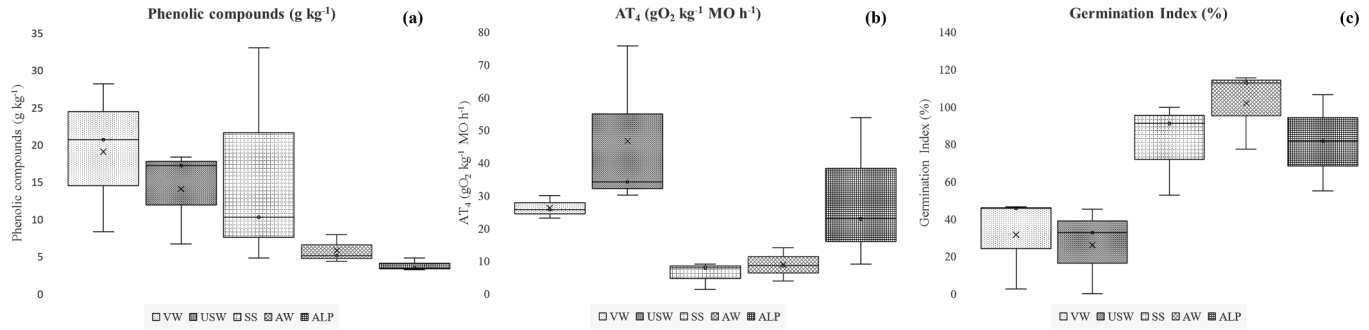


Fig. 1. Toxicity and stability parameters. (a) Phenolic compounds (g kg^{-1}); (b) Accumulated respiration activity after 4 days, AT_4 (g O_2 kg^{-1} MO h^{-1}) and (c) Germination Index (%) in compost samples from industrial scale composting facilities (Box plots). Vegetable Waste (VW), Urban Solid Waste (USW), Sewage Sludge (SS), Agro Waste (AW) and "Alpeorujo" (ALP).

different depending on the raw material and the enzymatic activity analyzed in each case. In general terms, enzymatic activity observed in the sewage sludge samples (SS) was comparatively lower than that observed in the rest of the compost (Fig. 2a, 2b and 2d). However, the same did not happen in the case of urease activity (Fig. 2c), since, in this case, the highest activity values were detected in the SS compost. As expected, GLUC, CEL, XYL and AMY activities stood out in the samples composed of an important plant fraction. This was the case of the VW, AW and ALP compost (Fig. 2a and 2b). LIP activity was more notable in the case of samples from "alpeorujo" (ALP), which was directly related to the composition of the starting mixtures (Fig. 2d). Otherwise, PMN-K activity was higher in the case of the USW and AW samples (Fig. 2d), while PRO activity stood out in the samples made from USW (Fig. 2c).

The differences observed in relation to the different enzymatic activities analyzed could be due not only to the nature of the materials but also to the duration of the processes. In this sense, one of the main problems related to composting on an industrial scale is the heterogeneity of the treatments and the maturity criteria and stability of the final compost. Previous works support that the differences at the enzymatic level of industrial composting processes are mainly due to the material complexity, the duration of the processes and the entry of fresh material in advanced phases of the organic matter biotransformation (Tiquia, 2002; Estrella-González et al., 2019). Therefore, composting strategies and raw materials could influence the speed of composting, the time needed to complete stability and maturation degree and, consequently, the enzymatic activity in the final products.

The results published in previous works with respect to final compost show comparatively lower β-glucosidase, xylanase and lipase activities than those obtained in this work. On the contrary, the final urease activity in this work turned out to be much lower than that obtained by Jurado et al. (2014).

3.3. Biodiversity indexes

The values of Chao1 and Shannon indexes for bacterial and fungal

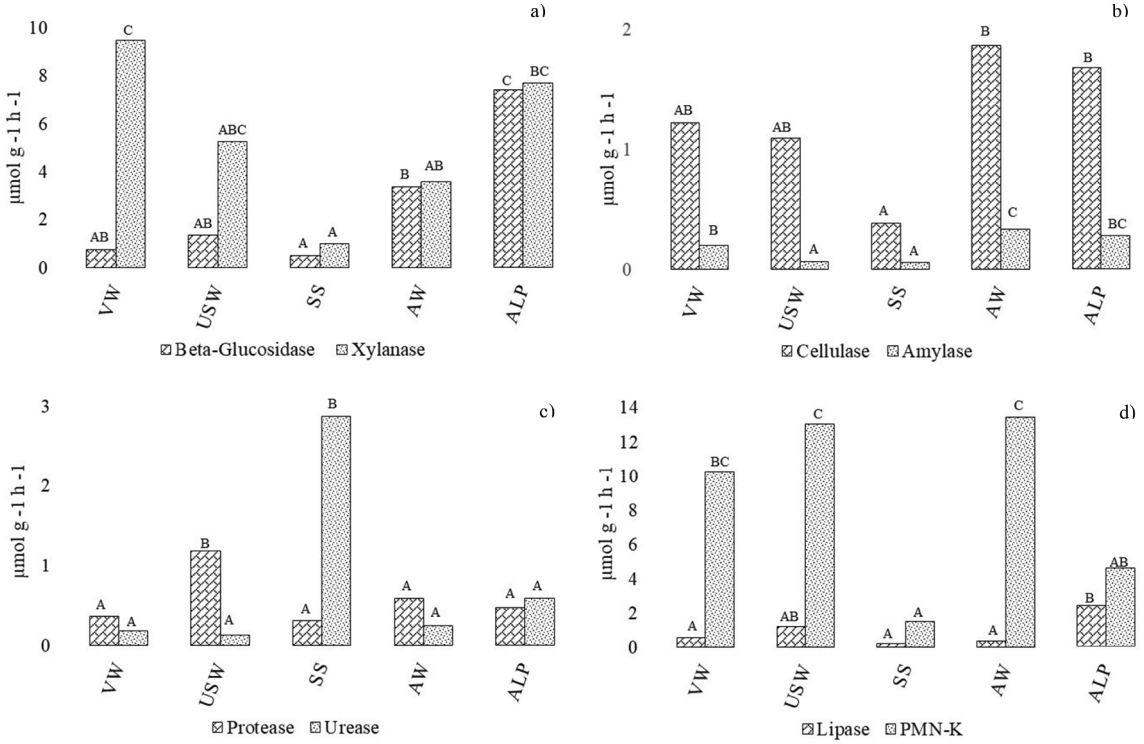


Fig. 2. Quantification of enzymatic activities (µmol $g^{-1}h^{-1}$) in composts samples from different industrial scale composting facilities. a) β-Glucosidase and Xylanase; b) Cellulase and Amylase; c) Protease and Urease; d) PMN-K and Lipase. Vegetable Waste (VW), Urban Solid Waste (USW), Sewage Sludge (SS), Agro Waste (AW) and "Alpeorujo" (ALP).

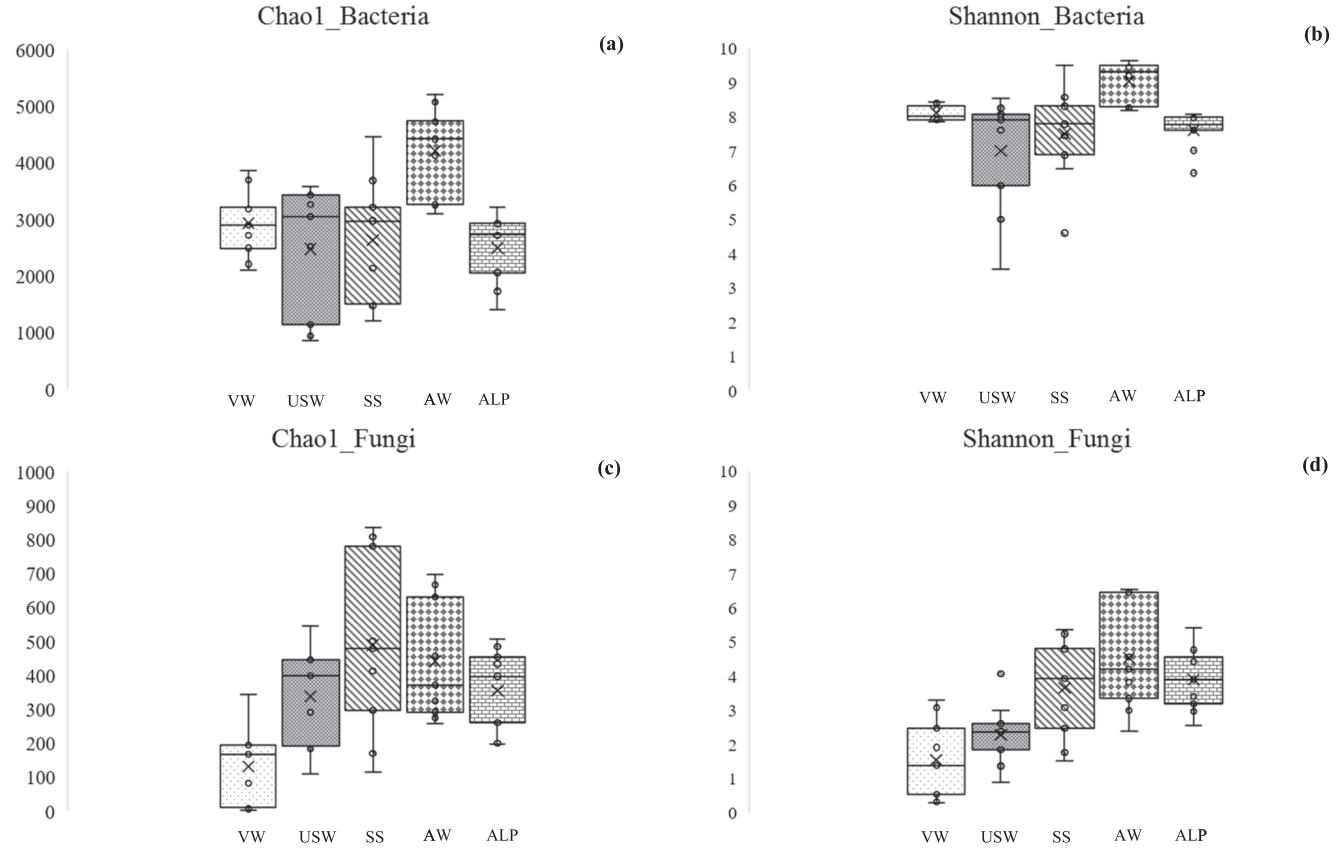


Fig. 3. Characterization of biodiversity indexes in compost samples from industrial scale composting facilities (Box plots). Chao1 and Shannon Bacterial indexes (a, b); Chao1 and Shannon Fungal indexes (c, d). Vegetable Waste (VW), Urban Solid Waste (USW), Sewage Sludge (SS), Agro Waste (AW) and "Alpeorujo" (ALP).

community are represented in Fig. 3. These values were corresponding to raw data without chimeras and singletons. The biodiversity Chao1 index is generally useful to know species richness, while Shannon index is more related to abundance. During the last decade numerous authors have used both indexes to know the biodiversity of compost samples (Liu et al., 2018; De Corato et al., 2019; Zhao et al., 2019). Traditionally, biodiversity has been based on cultivable microbiota (López-González et al., 2015), though deeper analysis closer to the reality consider the total microbiota by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) (Wang et al., 2015) or more recently by metagenomic analyses.

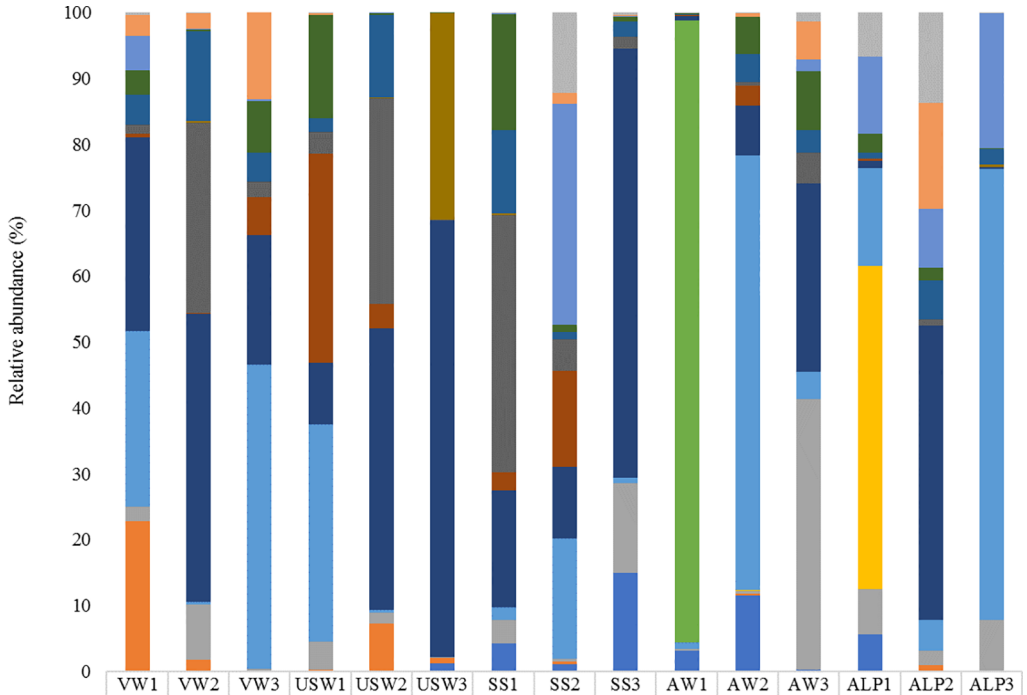
Fig. 3a and 3b show the biodiversity indexes of bacteria for Chao1 and Shannon, respectively. On the one hand, highest values were obtained in the AW samples, showing a mean Chao1 index around 4000 and a mean Shannon index near of 9 (Fig. 3a and 3b). By the other hand, mean values of diversity in the rest of the samples analyzed were not noticeably different, ranging between 2500 and 3000, in the case of Chao1 index, while Shannon index mean fluctuated between 7 and 8. Here, it is possible to emphasize a greater dispersion of the data in the case of the USW, SS and AW samples, which could be related to a greater heterogeneity in the composition of the composting piles, as well as in the operating protocols (overall in the case of USW samples). Despite the differences detected according to the different processes and materials analyzed, the results revealed a very diverse bacterial community in all cases. Shannon values were even higher than those obtained by Liu et al. (2018) after 22 days from the beginning of the composting process. These authors determined that the biodiversity in the compost samples may be affected by the initial particle size, as well as by the application of commercial cellulose or specific microbial inoculum.

The indexes of Chao1 and Shannon for fungal communities are shown in Fig. 3c and 3d, respectively. In general terms, fungal diversity was noticeable lower than bacterial diversity. Regarding to Chao1 index, values were approximately ten times lower than those corresponding to bacterial communities. Mean Chao1 Index was lower in VW samples (around 150), while these values ranged between 400 and 500 in the rest of samples. On the other hand, the highest average Chao1 values were observed for the SS and AW samples, although the data range was very wide in both cases. Mean of Shannon Index in fungi were higher in SS, AW and ALP, ranging from 4 to 5 and matched the range established by other authors (De Corato et al., 2019; Zhao et al., 2019), while USW and VW samples showed a very low level of diversity.

Bearing in mind the heterogeneity of the different processes, results concerning the metagenomics profile were practically unique and exclusive for each process. Despite that disadvantage, several previous works support the results here obtained respect to bacterial and fungal metagenomic from similar composted raw materials (Langarica-Fuentes et al, 2014; Silva et al., 2016).

Figs. 4 and 5 show the bacterial and fungal families after filtering the metagenomic results at a 0.5% abundance. Globally, the most representative bacterial Phyla were Firmicutes (44.8%), Bacteroidetes (28.7%), Proteobacteria (15.2%) and Actinobacteria (11.3%) (data not shown), being *Rhodospirillaceae, Bacillaceae, Flavobacteriaceae* and *Nocardiopsaceae* the most widely distributed bacterial families (Fig. 4). These were present in all type of samples but at different proportions. On the contrary, the *Chitinophagaceae* family was exclusively present in samples from AW1 process (94.5%), *Marinilabiaceae* family was only detected in samples from ALP1 process (49.06%) and *Lactobacillaceae* family almost exclusive in samples from USW3 process (31.21%).

Members of *Flavobacteraceae* family (Bacteroidetes phylum) participate in the degradation of macromolecules such as cellulose and chitin. According to Fracchia et al. (2006) this group of bacteria is dominant in mature compost and vermicompost. Likewise, members of



	VW1	VW2	VW3	USW1	USW2	USW3	SS1	SS2	SS3	AW1	AW2	AW3	ALP1	ALP2	ALP3
Pseudomonadaceae	0.31	0.02	0.00	0.02	0.00	0.00	0.06	12.22	0.35	0.06	0.03	1.27	6.65	13.73	0.06
Halomonadaceae	3.25	2.41	13.11	0.35	0.00	0.00	0.01	1.55	0.14	0.00	0.60	5.82	0.00	16.01	0.00
Idiomarinaceae	5.22	0.07	0.28	0.03	0.05	0.01	0.20	33.57	0.20	0.00	0.00	1.78	11.76	8.91	20.46
Rhodospirillaceae	3.62	0.34	7.89	15.62	0.30	0.03	17.50	1.14	0.66	0.18	5.64	8.96	2.88	1.96	0.17
Uncultured bacterium	4.58	13.66	4.34	2.08	12.58	0.11	12.63	1.08	2.37	0.06	4.26	3.45	0.84	5.87	2.31
Lactobacillaceae	0.01	0.20	0.01	0.04	0.03	31.21	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.49
Thermoactinomycetaceae	1.33	28.84	2.38	3.30	31.21	0.24	39.03	4.78	1.68	0.03	0.53	4.65	0.07	0.95	0.00
Planococcaceae	0.55	0.11	5.74	31.65	3.68	0.01	2.77	14.63	0.04	0.13	3.01	0.00	0.31	0.05	0.00
Bacillaceae	29.48	43.78	19.66	9.43	42.82	66.15	17.75	10.86	65.14	0.70	7.56	28.57	1.08	44.65	0.29
Chitinophagaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	94.50	0.00	0.00	0.00	0.00	0.00
Flavobacteriaceae	26.61	0.37	46.19	33.00	0.37	0.01	1.93	18.22	0.85	0.94	66.07	4.12	14.90	4.69	68.46
Marinilabiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	49.06	0.00	0.00
Nocardiopsaceae	2.25	8.42	0.34	4.26	1.70	0.15	3.58	0.40	13.57	0.24	0.52	41.08	6.80	2.15	7.75
Corynebacteriaceae	22.78	1.74	0.05	0.20	7.21	0.80	0.00	0.42	0.06	0.00	0.20	0.00	0.02	0.90	0.00
OM1 clade	0.01	0.03	0.01	0.02	0.04	1.28	4.26	1.12	14.95	3.15	11.56	0.30	5.62	0.07	0.01

Fig. 4. Relative abundance of bacterial family in compost samples from industrial scale composting facilities. Vegetable Waste (VW), Urban Solid Waste (USW), Sewage Sludge (SS), Agro Waste (AW) and "Alpeorujo" (ALP). The figure represents the percentage of presence of each bacterial family in the 15 sampled composting plants.

Actinobacteria play an important role in the later stages of the composting process (Tian et al., 2013), so it was atypical to found it at low level in several compost samples (*Nocardiopsaceae* and *Corynebacteriacea* families). Proteobacteria phylum was represented by *Rhodospirillaceae, Idiomarinaceae, Halomonadaceae* and *Pseudomonadaceae* families. Most of them are typical of composting processes, and they could be detected at different maturation stages (Silva et al., 2016).

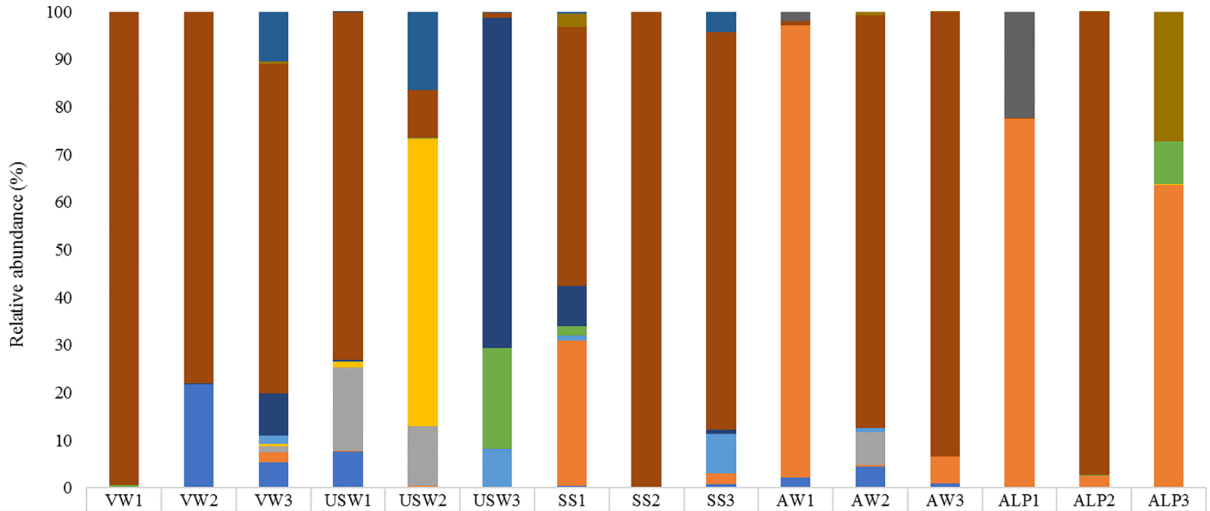
Regarding the abundance of fungi (Fig. 5), Ascomycota was the most representative phylum (97.2%). The majority family was *Microascaceae*, being detected in all the samples analyzed. *Trichocomaceae* was the second family most abundant but it was not globally detected. The other fungal families do not represent more than 24.8% each. These results agree with those found by Langarica-Fuentes et al. (2014, 2015). On the other hand, the phylum Basidiomycota only represented 2.1% of the total, being *Trichosporonaceae* the only representative family.

3.4. Statistical analyses

Taking into account the great diversity of parameters analyzed in this work (monitoring, maturation, stability, toxicity and enzymatic parameters), it could be of interest to determine the influence of all of them when obtaining different composting products. On one side, Fig. 6a shows a discriminant analysis that helps to verify how different the compost samples from each process were, on the basis of the raw material used. The discriminant analysis revealed two discriminant functions responsible for more than 80% of the variability observed between the data. In this case, data corresponding to the compost elaborated from Agri-food Waste (AW) and "Alpeorujo" (ALP) stand out as being the most distant groups in relation to the rest, while compost made from USW, SS and VW were more nearby to each other. On the other hand, the compost made from sewage sludge occupies an intermediate place in the analysis of main components shown in the Fig. 6a.

Two Principal Component Analyses were performed bearing in mind the groups of physico-chemical and enzymatic parameters as independent way (Fig. 6b and 6c). The cumulative contribution rate of two principal components (PC1 and PC2) in the case of physico-chemical parameters reached 51.3% (Fig. 6b), while both PCs contributed around 48.4% to the variability of enzymatic data (Fig. 6c).

In Fig. 6b, PC1 had a weight of 29.8%, being the most influential variables C/N Ratio, OM and HR. PHEN compounds, GI, M and AT_4 were too significant parameter that contributed to the variability of samples. It should be noted that those parameters that have a greater



	VW1	VW2	VW3	USW1	USW2	USW3	SS1	SS2	SS3	AW1	AW2	AW3	ALP1	ALP2	ALP3
Trichosporonaceae	0.00	0.00	10.53	0.02	16.40	0.18	0.40	0.00	4.22	0.00	0.00	0.00	0.00	0.00	0.00
Sordariaceae	0.00	0.00	0.44	0.00	0.00	0.00	2.78	0.00	0.14	0.00	0.69	0.15	0.00	0.04	27.16
Chaetomiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	1.93	0.00	0.00	22.37	0.00	0.00
Microascaceae	99.44	78.15	69.30	73.14	10.18	1.10	54.47	99.96	83.42	0.93	86.77	93.28	0.07	97.31	0.04
Saccharomycetales	0.00	0.02	8.77	0.25	0.04	69.31	8.35	0.01	0.77	0.00	0.00	0.00	0.00	0.00	0.00
Pichiaceae	0.28	0.00	0.00	0.00	0.01	21.25	1.99	0.01	0.00	0.00	0.00	0.05	0.00	0.18	9.05
Debaryomycetaceae	0.00	0.02	1.75	0.04	0.00	8.09	0.99	0.00	8.40	0.00	0.86	0.00	0.00	0.00	0.00
Pyronemataceae	0.00	0.00	0.44	1.34	60.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15
Gymnoascaceae	0.00	0.00	1.32	17.46	12.54	0.00	0.00	0.00	0.00	0.00	6.87	0.00	0.00	0.00	0.00
Trichocomaceae	0.28	0.00	2.19	0.07	0.25	0.02	30.62	0.02	2.37	95.03	0.34	5.59	77.56	2.46	63.60
Cladosporiaceae	0.00	21.80	5.26	7.68	0.19	0.06	0.40	0.00	0.67	2.11	4.47	0.94	0.00	0.00	0.00

Fig. 5. Relative abundance of fungal family in compost samples from industrial scale composting facilities. Vegetable Waste (VW), Urban Solid Waste (USW), Sewage Sludge (SS), Agro Waste (AW) and "Alpeorujo" (ALP). The figure represents the percentage of presence of each fungal family in the 15 sampled composting plants.

weight during the bioxidative phase were grouped, such as C/N Ratio, OM, M and AT_4; while PHEN compounds, HR, GI and NI appear as representatives of more advanced stages of maturation.

Regarding PC2 (21.5%), M and GI had a significant negative weight while phenolic compounds and AT_4 had a significant positive weight. Highlighted the opposite weight detected between AT_4 and GI, which would imply that, in theory, at a lower respiration rate, higher germination rates are expected in the final compost.

On the other hand, a second analysis of main components was carried out taking into account parameters of an enzymatic nature (Fig. 3c). PC1 (27.2%) was useful to determine the different and opposite behavior of urease activity with respect to the rest of the enzymatic parameters. Likewise, GLUC, AMY and PMN-K activities showed closeness (activities more related to the degradation of the most readily bioavailable fractions), as did the CEL and XYL activities (activities more related to the degradation of the more complex fractions). In any case, all of them can be considered as biological indicators of earlier or more advanced phases of the process, respectively.

A Pearson's correlation between physical–chemical-enzymatic parameters and the biodiversity indexes for fungi and bacteria (Shannon and Chao1) was carried out ($p < 0.05$). This analysis corresponds to a correlation study in which differences between raw materials were not taken into account. Therefore, the information that arises from it has a global nature, not exclusive to the materials. In this way, correlations have been established that can be useful regardless of the type of starting material. Regarding the monitoring parameters, a significant negative correlation was detected between bacterial biodiversity indexes and C/N Ratio (Chao1 B = −0.4349 and Shannon B = −0.5504), while Moisture was positively correlated with fungal biodiversity (Chao1 F = 0.3408 and Shannon F = 0.5220). Organic matter only correlated as significant way Shannon F (0.3316). In relation to the parameters traditionally considered as indicators of maturity, it should be noted the strongly negative correlation observed between Nitrification Index and bacterial biodiversity (Chao1 B = −0.5002 and Shannon B = −0.5035).

Regarding the block of parameters related to stability and, the results showed a significantly positive correlation between microbial biodiversity and germination index, in all cases. Surprisingly, the respirometric index AT_4 was negatively correlated with Chao1F (−0.3441), but not with the rest of bioindicators. Highlighted the negative correlation between phenolic compounds and fungal bioindicators (Chao 1F = −0.5618 and Shannon F = −0.6293).

On the other hand, the enzyme parameters most related to bacterial biodiversity were amylase (Chao1 B = 0.3593 and Shannon B = 0.3661) and PMN-K (Chao1 B = 0.4174 and Shannon B = 0.3917). In both cases, strong positive correlations were stablished. Respecting fungal biodiversity, GLUC and AMY activities showed a positive correlation with fungal biodiversity (Shannon F), while did negative in the case of XYL (−0.4614). Urease activity was positively correlated exclusively with Chao1 F (0.4369).

In order to obtain a formula capable of predicting the degree of microbial biodiversity of a compost, two regression analyses were established using the parameters most closely correlated with the bacterial and fungal Shannon indexes. Thus, the predictive approximations obtained were:

$$Shannon\ B = 10.5836 + 0.0071 \times GI - 1.9014 \times NITRIF - 0.1756 \times C/N\ Ratio$$

$$Shannon\ F = 3.6611 - 0.0898 \times XYL - 0.0837 \times PHEN + 0.0155 \times GI$$

Although the equations obtained did not show a high level of prediction (64% and 62%, respectively), it was possible to establish a way of comparing the degree of biodiversity between different compost samples using a little number of physical–chemical-enzymatic parameters.

Although the work presented does not study the temporal evolution of the physical–chemical-biological parameters throughout the different composting processes analyzed (only the product is analyzed at

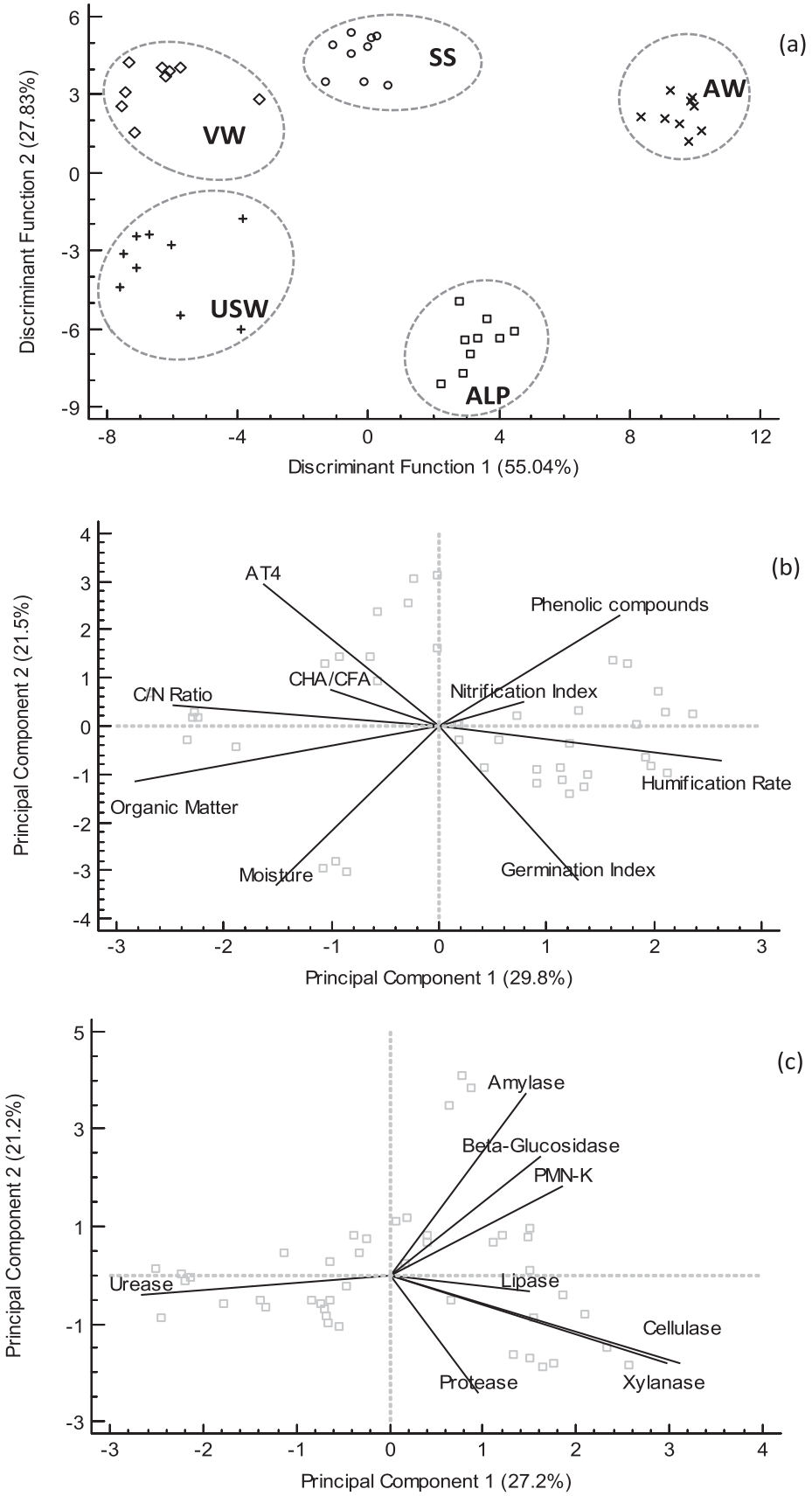


Fig. 6. Statistical analyses. a) Discriminant analysis based on composting of different processes, taking into account the monitoring, enzymatic, stability and maturation parameter. Differences were stablished between different raw materials. VW: Vegetable Waste; USW: Urban Soild Waste; SS: Sewage Sluges; AW: Agrifood Waste; ALP: Alpeorujo. b) Principal Components Analyses taking into account the monitoring, stability, maturation and c) enzymatic parameters.

the end time), a certain increase in fungal and bacterial biodiversity is intuited to as the process progresses. This intuition is based on the apparently found relationship between the biodiversity indexes derived from the analyzed compost samples and some parameters related to phytotoxicity and biological stability.

4. Conclusions

The problem derived from the lack of homogeneity and repeatability of composting processes on an industrial scale is evident. Although numerous attempts have been made to predict the quality of compost based on different traditional parameters, the latest metagenomic advances reveal the complexity of this prediction, since microbial biodiversity can be almost exclusive to each process. We could conclude with this work that the quality of a compost can not only be measured in physicochemical terms, but it is also necessary to consider other biological parameters, as well as the "biodiverse" nature of each compost pile.

CRediT authorship contribution statement

M.J. Estrella-González: Formal analysis, Investigation, Writing - original draft, Visualization. **F. Suárez-Estrella:** Formal analysis, Investigation, Writing - review & editing, Visualization. **M.M. Jurado:** Investigation, Resources. **M.J. López:** Conceptualization, Methodology, Resources. **J.A. López-González:** Investigation, Resources. **A.B. Siles-Castellano:** Investigation, Resources. **A. Muñoz-Mérida:** Supervision, Formal analysis. **J. Moreno:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing - original draft.

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.

Acknowledgements

This work has been funded by the Ministry of Economy and Competitiveness through project AGL2015-64512R. This research group wishes to thank the enormous help provided by the doctors Teresa Gea and Dr. Raquel Barrena (Autonomous University of Barcelona), and Javier Noguera, Computer Engineer, without whom it would have been impossible to carry out this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123557.

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