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Chemical properties and hydrolytic enzyme activities for the characterisation of two-phase olive mill wastes composting

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Abstract

Two-phase olive mill waste (TPOMW) is a semisolid sludge generated during the extraction of olive oil by the two-phase centrifugation system. Among all the available disposal options, composting is gaining interest as a sustainable strategy to recycle TPOMW for agricultural purposes. The quality of compost for agronomical use depends on the degree of organic matter stabilization, but despite several studies on the topic, there is not a single method available which alone can give a certain indication of compost stability. In addition, information on the biological and biochemical properties, including the enzymatic activity (EA) of compost, is rare. The aim of this work was to investigate the suitability of some enzymatic activities (β-glucosidase, arylsulphatase, acid-phosphatase, alkaline-phosphatase, urease and fluorescein diacetate hydrolysis (FDA)) as parameters to evaluate organic matter stability during the composting of TPOMW. These enzymatic indices were also compared to conventional stability indices. For this purpose two composting piles were prepared by mixing TPOMW with sheep manure and grape stalks in different proportions, with forced aeration and occasional turnings. The composting of TPOMW followed the common pattern reported previously for this kind of material with a reduction of 40–50% of organic matter, a gradual increase in pH, disappearance of phytotoxicity and formation of humic-like C. All EA increased during composting except acid-phosphatase. Significant correlations were found between EA and some important conventional stability indices indicating that EA can be a simple and reliable tool to determine the degree of stability of TPOMW composts.

Keywords: Olive mill wastes; Composting; Enzymatic activity; Stability; Organic matter

1. Introduction

Olive mill waste management is one of the major environmental challenges in all olive oil producing countries since they are produced in large quantities in short periods of time and their high phytotoxicity and antimicrobial properties adversely impact both soil and water qualities (Roig et al., 2006).

From the early nineties, the severe water restrictions in many Mediterranean countries has led to the creation and implementation of a new two-phase centrifugation system which has considerably reduced the amount of water needed and the wastes produced during the olive oil extraction. In spite of the environmental advantages of this new system, that has been called "ecological", olive mill waste disposal problems still remain unsolved. In fact, the semisolid sludge produced, known as two-phase olive mill waste (TPOMW), presents more disposal problems than the olive husk of the previous three-phase system and it demands for new environmental and economically viable management options.

At present, a second extraction of the remaining pomace oil prior to combustion is the most consolidate disposal option (Roig et al., 2006), but a promising alternative for

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sustainable recycling of TPOMW is through composting with other agricultural by-products (Cayuela et al., 2004; Alburquerque et al., 2006). The peculiar physico-chemical properties of TPOMW, with limited porosity, high concentration of lignin, lipids and phenols, generate a particular composting process with high temperatures and a long thermophilic phase. Several composting experiments performed in the last years revealed the high potential of the composts obtained from TPOMW for agricultural purposes (Roig et al., 2006). However, the effectiveness of compost recycling in agriculture depends mostly on the quality of the compost. Therefore the characterization of the process and the evaluation of the quality of the mature compost are crucial. Some physico-chemical properties (temperature, pH, CEC, organic C, NH_4^+, fats, phenols, humic-like substances) have been used with this purpose. However, information on the changes in biological and biochemical properties during TPOMW composting, including the hydrolytic enzymatic activity (EA), is almost inexistent (Ntougias et al., 2006). Since composting is a microbial mediated process, dynamics of microbiological properties, including EA, are expected to give valuable information on the evolution of the process and the quality of the end products.

Extracellular enzymes are known to be involved in the depolymerization of different constituents of organic wastes. Some important enzymes involved in the composting process include cellulases and β-glucosidases related to C mineralization, proteases and urease involved in N cycle and phosphatases and arylsulphatases related to P and S cycles (Mondini et al., 2004). Some authors have reported a decrease in enzymatic activity during the composting process related to the decline in microbial activity and available substrates (García et al., 1993; Benitez et al., 1999). On the other hand, the formation of stable humo-enzymatic complexes during composting explains the increase in EA found in other studies (Mondini et al., 2004; Benitez et al., 2005).

This study is based on the results previously obtained by Mondini et al. (2004) who demonstrated that the use of air-dried samples improves the reliability and the applicability of the enzymatic methods for the characterisation of the composting process. Thus, the aim of this work was to study the evolution of some important enzymatic activities during the composting of TPOMW and their comparison with other chemical and biological properties commonly used as indicators of compost quality and maturity.

2. Methods

2.1. Compost

Two composting piles were prepared by mixing TPOMW, sheep litter (SL) and grape stalks (GS) in the following proportions (dry weight basis):

Pile A: TPOMW (50%) + SL (50%)
Pile B: TPOMW (45%) + SL (45%) + GS (10%)

Both mixtures were composted in trapezoidal piles (1 m high and 2×3 m base) by the Rutgers system with occasional turnings (Cayuela et al., 2006). Water was regularly added to maintain appropriate moisture (40–60%).

Sampling was made at four different stages of the composting process by mixing five sub-samples from different locations of the pile:

- M : From the mesophilic phase (7 weeks of composting)
- T1: From the thermophilic phase (18 weeks of composting)
- T2: From the thermophilic phase (28 (pile A) and 23 (pile B) weeks of composting)
- F : From the final compost obtained (40 (pile A) and 34 (pile B) weeks of composting)

2.2. Chemical and enzymatic analyses

Chemical and enzymatic analyses were performed on air-dried samples. Total nitrogen (TN) and total organic carbon (TOC) were determined by automatic elemental microanalysis (NA 1500 Carlo Erba Instruments). The cation exchange capacity (CEC) was analysed with $BaCl_2$–triethanolamine. pH was determined in a 1:10 (w/v) water-soluble extract (Roig et al., 1988). Water soluble carbon (WSC) was determined in a 1:20 (w/v) water extract. Extractable carbon (C_{EXT}) was measured in a 0.1 M NaOH compost extract (1:20 w/v) and the fulvic acid carbon (FAC) after precipitation of the humic acid at pH 2 in the supernatant solution using a TOC analyzer (Formacs^{HT} Skalar analyzer). The humic acid carbon content (HAC) was calculated by subtracting FAC from the C_{EXT} (Sánchez-Monedero et al., 1996). Phytotoxicity, expressed as germination index (GI), was assayed by the *Lepidium sativum* test (Zucconi et al., 1981). The hydrolysis of the fluorescein diacetate (FDA) was determined according to Schnurer and Rosswall (1982). Urease was analyzed with the procedure developed by Kandeler and Gerber (1988). Arylsulphatase, β-glucosidase, alkaline phosphatase and acid phosphatase were determined according to Alef and Nannipieri (1995a,b), and Alef et al. (1995), respectively.

2.3. Statistical analysis

Data were checked for normal distribution and underwent univariate analysis of variance. Sample means were compared using the Student-Newman-Keuls test. The relationships between variables were analyzed by the Pearson correlation coefficient. All statistical analyses were performed using SPSS version 9.0 statistical package.

3. Results and discussion

3.1. Chemical evolution of the composting process

The composting process underwent the common pattern previously described for TPOMW, with a long thermo-

philic phase due to the presence of recalcitrant substances (lignin, fats, phenols) (Cayuela et al., 2004, 2006). Fig. 1 shows the evolution of the TOC/TN ratio during the composting of both mixtures. The TOC/TN decreased gradually in both composting piles starting from 25 and reaching values around 15 in the end products, consistent with well stabilized compost that would not alter the microbial equilibrium when applied to soil (Allison, 1973). The ratio (final TOC/TN)/(initial TOC/TN) was 0.60 in pile A and 0.55 in pile B, similar to that found by Alburquerque et al. (2006) in six different TPOMW composting mixtures (within the range: 0.50–0.67), showing a suitable degree of stability.

Table 1 shows five maturation indices widely reported in literature that were selected for the evaluation of the composting process (Bernal et al., 1998). Both piles showed high initial values for the ratio water soluble C/total N (WSC/TN) that noticeably decreased during composting. The final values were in the range reported by other authors for olive mill wastes derived composts with good degree of stability (Alburquerque et al., 2006). Values of this ratio for compost prepared from olive mill wastes are, in general, higher than those obtained for other materials (<0.5), such as municipal solid waste or sewage sludge (Sánchez-Monedero et al., 2001).

The *L. sativum* test has been recognized as a simple and reliable method to evaluate maturity during TPOMW composting (Alburquerque et al., 2006; Cayuela et al., 2007). In the present work, low germination indices (GI) were observed during the mesophilic period in both piles, showing the high toxicity in the early composting stages. During the thermophilic phase GI exceeded 50%, threshold value established by Zucconi et al. (1981) for mature compost. GI was highly correlated with most of the stability and humification indices studied (Table 2).

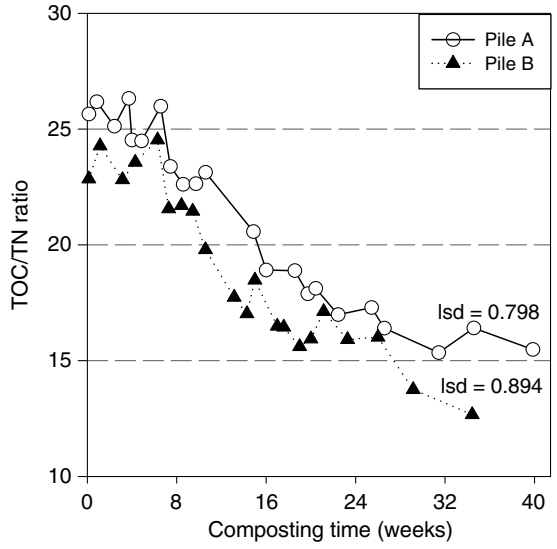

Fig. 1. Evolution of TOC/TN ratio during TPOMW composting in piles A and B. Values are the mean of three replicates.

Table 1
Chemical properties selected for the characterisation of TPOMW composting

Pile	Composting stage	WSC/TN	GI (%)	C_{HA}/C_{EXT} (%)	C_{HA}/C_{FA}	CEC/TOC (mEq g^{-1})
A	M	4.6^{a}	9^{a}	33.5^{a}	0.50^{a}	1.4^{a}
	T_1	3.6^{b}	0^{a}	27.7^{a}	0.38^{a}	2.1^{ab}
	T_2	3.0^{c}	61^{b}	68.8^{b}	2.20^{b}	2.1^{ab}
	F	1.7^{d}	74^{c}	73.1^{b}	2.71^{c}	2.4^{b}
B	M	4.3^{a}	5^{a}	36.3^{a}	0.57^{a}	1.8^{a}
	T_1	1.6^{b}	61^{b}	72.3^{b}	2.61^{b}	2.6^{b}
	T_2	1.6^{b}	98^{c}	73.5^{b}	2.77^{b}	2.7^{b}
	F	1.5^{b}	88^{c}	76.8^{c}	3.32^{c}	3.6^{c}

WSC: water soluble carbon.
TN: total nitrogen.
GI: germination index.
C_{HA}: humic-acid C.
C_{EXT}: extractable C.
C_{FA}: fulvic acid C.
CEC: cation exchange capacity.
TOC: total organic C.
Results are the mean of three replicates. For each pile, values in the same column followed by the same letter are not significantly different according to the S-N-K test ($P < 0.05$).

Stability was also confirmed by the remarkable increase in the indices based on the humification process such as the percentage of humic acids ($C_{HA}/C_{EXT} \times 100$), the polimerisation degree (C_{HA}/C_{FA}) and the humification degree (CEC/TOC). The values obtained were comparable to those reported for sewage sludge and sorghum bagasse composts and higher than for municipal solid waste composts (Sánchez-Monedero et al., 1999). In pile A the disappearance of toxicity and the formation of humic-like compounds were delayed respect to pile B, probably because, in the latest, the addition of grape stalks improved the structure and therefore the aeration of the mixture (Cayuela et al., 2006).

A slight initial decrease in pH was observed after five weeks, in pile A, and two weeks, in pile B, as a result of the release of organic acids from decomposition of the most labile organic fractions (Fig. 2). After that, a marked increase in pH was observed in both piles, keeping a steady value (around 9.5) from week 20 onwards. This sharp increase in alkalinity has been previously reported during the composting of TPOMW (Cayuela et al., 2004; Alburquerque et al., 2006) and explained as an intrinsic outcome of the mineralization of these specific materials. However, the chemical mechanisms for such a high increase have not yet been elucidated.

The evolution of pH during composting varies greatly depending on the starting materials. For example, no significant changes in pH were reported for different manures (Wang et al., 2004) and sorghum bagasse (Sánchez-Monedero et al., 2001); a decrease in pH was observed for sewage sludge (Benitez et al., 1999) and pig manure (Tiquia and Tam, 2000), while increases were monitored in pruning wastes (Benito et al., 2003) and rice straw (Shi et al., 2006). The increase in pH during the most active phases

Table 2
Pearson correlation matrix between chemical and biochemical parameters during TPOMW composting

	TOC/TN	WSC/TN	pH	GI	PHA	PD	HD	FDA	AlkP
WSC/ TN	0.837**								
pH	−0.796*	−0.882**							
GI	−0.786*	−0.907**	0.757*						
PHA	−0.751*	−0.907**	0.767*	0.963**					
PD	−0.809*	−0.940**	0.783*	0.970**	0.747*				
HD	−0.761*	−0.857**	0.743*	0.778*	0.986**	0.838**			
FDA	−0.813*	−0.735*	0.735*	0.842**	0.811*	0.832*	0.679		
AlkP	−0.562	−0.499	0.588	0.626	0.626	0.641	0.564	0.911**	
ArylS	−0.651	−0.671	0.545	0.703	0.669	0.760*	0.887*	0.785*	0.787*

TOC: total organic carbon.
TN: total nitrogen.
WSC: water soluble carbon.
GI: germination index.
PHA: percentage of humic acids ($C_{HA}/C_{EXT} \times 100$).
PD: polymerisation degree (C_{HA}/C_{FA}).
HD: humification degree (CEC/TOC).
FDA: fluorescein diacetate hydrolysis.
AlkP: alkaline phosphatase.
ArylS: arylsulphatase.
(Correlation points = 8); **, *: significant at $p < 0.01$ and $p < 0.05$, respectively.
No significant correlations were found for β-glucosidase, urease and acid phosphatase.

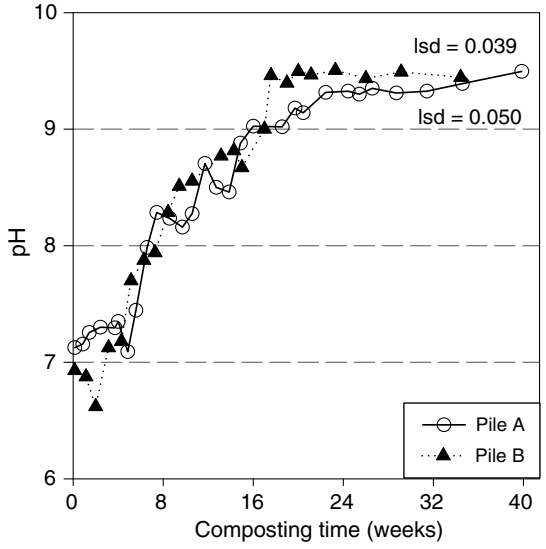

Fig. 2. Dynamics of pH during TPOMW composting in piles A and B. Values are the mean of three replicates.

of composting has been associated to the formation of NH_4^+ as a consequence of the mineralization of proteins. During maturation process, nitrifying bacteria transform this NH_4^+ into NO_3^- with the consequent acidification of the composting pile (Sánchez-Monedero et al., 2001).

The explanation for the high pH increase in TPOMW compost could be the decarboxilation of organic anions during the aerobic decomposition of TPOMW. Plant residues generally contain more cations than inorganic anions and the internal charge balance is maintained by organic anions (Xu et al., 2006). The decarboxilation of organic anions during microbial decomposition of plant residues requires one proton per carboxylic group decarboxilated according to the equation:

$$R{-}CO{-}COO^- + H^+ \rightarrow R{-}CHO + CO_2$$

According to this, the microbial decomposition of abundant organic anions present in TPOMW (Arienzo and Capasso, 2000) could lead to the high increase in pH during the most active phase of the composting process. Once the composting mass has achieved a high degree of stability, the low concentration of NH_4^+ and high pH inhibit the nitrification reactions during the maturation period and therefore the typical decrease in pH. In fact, pH evolution correlated with all the maturation indices studied (Table 2). Hence, the stabilisation of pH may be used as a stability index in TPOMW composts.

3.2. Evolution of hydrolytic enzyme activity

Changes in the six hydrolytic EAs evaluated throughout the composting of TPOMW are shown in Fig. 3. All the enzymes, but acid phosphatase, showed very low activity in the initial TPOMW samples and during the first stages of composting. Both piles showed a parallel evolution of EA throughout the process.

Fluorescein diacetate (FDA) hydrolysis increased with composting time, showing the highest increment during the thermophilic phase between T1 and T2 (Fig. 3). Results obtained were in agreement with those reported by Ntougias et al. (2006) who observed an increase in FDA activity during the composting of olive press cake and olive leaves. FDA activity has been proposed in recent studies to estimate non-specific enzyme activity during the composting process (Garcia-Gómez et al., 2003; Ntougias et al.,

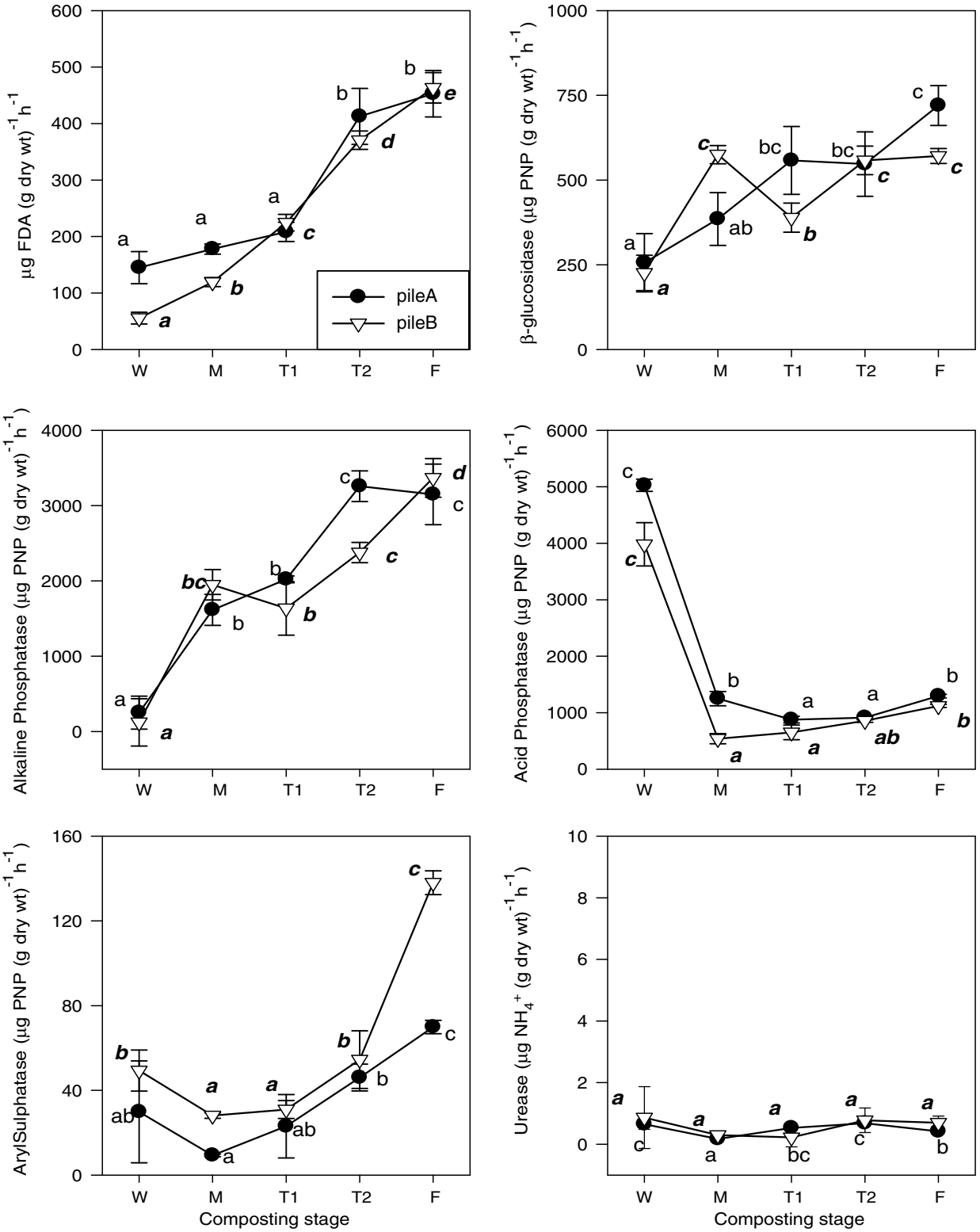

Fig. 3. Dynamics of enzymatic activity during the composting of TPOMW. W: TPOMW before composting; M: mesophile stage; T1, T2: thermophile stage; F: final compost. Vertical bars represent standard deviation ($n = 3$). Different letters indicate significant differences according to S-N-K test ($P < 0.05$).

2006). It has been also used as a measure of microbial activity to predict suppressive capacity of compost on plant pathogens (Inbar et al., 1991). However, the effectiveness of this parameter for the evaluation of stability in compost is controversial, since different trends have been found depending on the starting composting materials. In this

study, FDA showed significant correlations with most of the maturation indices traditionally used in literature (Table 2).

An increase in β-glucosidase was also observed in both piles (Fig. 3). Comparing to other materials such as pig slurry (Ros et al., 2006) and yard or cotton wastes (Mondini et al., 2004), TPOMW composts showed lower β-glucosidase activity. This enzyme catalyses the hydrolysis of cellobiose and thus plays a major role in the decomposition of organic C compounds. The peculiar chemical composition of TPOMW, characterized by high content of compounds with low degree of degradability (i.e. lignin), could explain the low values of enzymatic activity since it is known that β-glucosidase activity is reduced during lignocellulose hydrolysis, by interaction with lignin or lignin–carbohydrate complexes (Berlin et al., 2006).

Some authors have found a decrease of β-glucosidase activity throughout the composting of different materials (Benitez et al., 1999; García et al., 1993). Nevertheless, other studies have reported an increase of this enzymatic activity during composting (Mondini et al., 2004). Benitez et al. (2005) reported an increase in β-glucosidase activity measured in a pyrophosphate extract during the vermicomposting of dry olive cake and found the maximum activity at the seventh month tending to stabilise from then onwards. In another recent study, Ros et al. (2006) found a gradual increase in the synthesis of this enzyme during composting of pig slurry with a slight decrease at the end.

Alkaline phosphatase was the enzymatic activity that showed the largest increase during TPOMW composting. This enzyme is particularly relevant for the evaluation of the composting process, since it is only synthesised by microorganisms and does not originate from plant residues (Burns, 1982). Different patterns have been found for the evolution of this enzyme for different composting materials. Tiquia (2002) observed a gradual increase during composting of manure followed by stabilisation at the end of the process. On the other hand, Ros et al. (2006) found an initial increase in different pig slurry composting piles reaching maximum activity after three weeks of composting and declining slightly afterwards. The values obtained for final TPOMW composts are in the range of those obtained by Mondini et al. (2004) for composts made from cotton wastes. In this study, alkaline phosphatase did not correlated with common stability indices, but did with FDA and arylsulphatase activities (Table 2).

Acid phosphatase activity was very high in TPOMW samples (4000–5000 μg PNP g dry weight^{-1} h^{-1}). However, values were very low during the composting process, and no significant changes were observed. The increase in pH with composting time, that reached values above 9, was much higher than the optimum (between 4 and 6.5) reported for this enzyme and this could explain the low values recorded for this activity with the ongoing of the process.

Studies on arylsulphatase during composting are particularly scarce. This enzyme, that catalyses the hydrolysis of organic sulphate esters, increased in both TPOMW composting piles within time showing a higher increase in pile A (without grape stalks). This EA also correlated with important maturation indices such as C_{HA}/C_{FA} and CEC/TOC (Table 2).

The urease activity, which catalyses the hydrolysis of urea to CO_2 and NH_4^+, was very low during all the experiment, probably because of the low concentration of N and the low level of available substrates released during the mineralization of TPOMW. These results are in agreement with those reported by Benitez et al. (2005), who found little significant changes in urease activity during the vermicomposting of dry olive cake.

From all the EA studied in the present experiment, FDA, alkaline phosphatase and arylsulphatase showed an unambiguous increasing trend during the process and the ability to significantly discriminate compost samples with different ages (Fig. 3). Moreover, FDA and arylsulphatases showed significant correlation with common indices of stability (Table 2). Therefore dynamics of FDA, alkaline phosphatase and arylsulphatases indicate that these activities could be a promising tool for the characterization of TPOMW composting. However, time course of urease, acid phosphatase and β-glucosidase activity did not show a clear trend during the process and therefore these EA do not represent an effective indicator of compost stability for TPOMW.

The increase in activity during the process could be attributed to the protection of extracellular enzymes due to the formation of complexes with humic-like substances. Formation of such complexes is indicated by the significant correlations between some humification indexes and specific enzymatic activity (i.e. EA for unit of organic C) (Table 3).

The formation of complexes between humic substances and enzymes in soil is well documented (Burns, 1982; Nannipieri et al., 1996). The ecological significance of the formation of humo-enzymatic complexes in soil is relevant as extracellular enzymes are usually short-lived unless they are adsorbed by soil colloids (Ladd, 1985) as they are prone to denaturation, inactivation and degradation processes. Formation of complexes with humic substances is known to stabilize and protect the enzymes towards adverse environmental conditions. Conversely to soil, information about the occurrence of humo-enzymatic complexes in compost is relatively scarce. Nevertheless, significant correlation between enzymatic activity and humic substances were described by Rad Moradillo and Gonzalez Carcedo (1996), Garcia et al. (1992), Mondini et al. (2004). The formation of humo-enzymatic complexes should be considered as a process with a direct link with compost stability, as it is closely related to changes in compost matrix, i.e. formation of humic-like substances, which is among the main purposes of the composting process.

Not all EA present similar effectiveness as indicator of compost stability. In the present work the most reliable indicator seems to be FDA and this could be related to

Table 3
Pearson correlation matrix between specific EA (i.e. EA/TOC) and humification degree

	PD	PHA	HR	HD	FDA/TOC	AlkP/TOC
PHA	0.986**					
HR	0.978**	0.966**				
HD	0.838**	0.747*	0.791*			
FDA/TOC	0.877**	0.821*	0.871**	0.836**		
AlkP/TOC	0.758*	0.697	0.760*	0.801*	0.953**	
ArylS/TOC	0.722*	0.621	0.733*	0.891**	0.880**	0.922**

PD: polymerisation degree (C_{HA}/ C_{FA}).
PHA: percentage of humic acids ($C_{HA}/C_{EXT} \times 100$).
HD: humification degree (CEC/TOC).
HR: humification ratio ($C_{EXT}/TOC \times 100$).
FDA: fluorescein diacetate hydrolysis.
AlkP: alkaline phosphatase.
ArylS: arylsulphatase.
TOC: total organic carbon.
(Correlation points = 8); **, *: significant at $p < 0.01$ and $p < 0.05$, respectively.

the broad specificity of this EA. Hydrolysis of FDA can be carried out by a number of non-specific enzymes such as esterases, proteases and lipases all of them involved in the transformation of organic matter in compost and produced by different groups of microorganisms. Therefore, hydrolysis of FDA is a better indicator of the whole microbial hydrolytic activity in compost with respect to more specific EA and is likely to present a better capacity to reflect the overall compost behaviour and the changes in the quality of organic matter. The different behaviour recorded for urease and acid phosphatase and, partially, for β-glucosidase could be attributed to environmental constraints that limited the enzymatic activity (i.e. unfavorable pH after the initial stages of the process, low level of inducing substrate, interaction with lignin) and prevented consequently the formation of stable humo-enzymatic complexes.

It is also important to consider that EA measurements were carried out on air dried samples. Measurement of hydrolytic enzymes is likely to measure mainly extracellular activity because it is generally assumed that polar esterified compounds used as enzymes substrates in the determination of EA are not capable to diffuse freely into intact cell (Breeuwer et al., 1995). Main categories of extracellular enzymes are free enzymes in the compost solution and enzymes complexed with humic substances. Drying of samples is known to cause the inactivation of free extracellular enzymes, while stabilized enzymes are protected from the adverse effects of low water content (Dick, 1994). Measurements performed on air dried samples are likely to measure mostly the fraction of extracellular activity that is associated with humic substances and, as a consequence, they are indirect indicators of the stabilization process occurring in the composting matrices. Mondini et al. (2004) comparing EA activity performed on moist and air dried compost samples showed that measurements performed on the latter samples enhanced the reliability of EA as a parameter for the characterization of the composting process.

In addition, the use of air dried samples increases the versatility of methods based on EA, since their analysis in moist samples is not always possible and compost storage at low temperature (2–4 °C) is not sufficient to inhibit microbial activity, maintaining unchanged the chemical properties of the sample (Wu and Ma, 2001).

4. Conclusion

The analysis of chemical properties during TPOMW composting (TOC/TN, WSC/TN, GI, pH, humification indices) showed the peculiar nature of this substrate with values often different from the data reported for compost derived from other materials. However, the evolution of these parameters was in accordance with previous works on TPOMW composting and indicated the achievement of a good degree of stability in the end products. Urease and acid phosphatase activities showed no significant changes during the composting of TPOMW because of the low level of N in the organic matrix and the high pH evolved during composting. Dynamics of these enzymes underline the sensibility of EA to changes in environmental conditions (pH) or their effectiveness as indicators of available substrate (nitrogen). FDA, alkaline phosphatase and arylsulphatase increased during the composting of TPOMW and were able to classify compost samples with different degree of stability. In addition FDA, alkaline phosphatase and arylsulphatase correlated with many important stability indices, including humification parameters, and therefore can be considered as valid parameters for establishing the degree of biological stability of the composting material. The relationship between the increase in FDA, alkaline phosphatase and arylsulphatase activity and organic matter stability could be attributed to the protection of extracellular enzymes by formation of complexes with humic-like substances.

The analysis of EA dynamics in composting air-dried samples represents a rapid, simple method and could be used as a reliable index for the characterization of TPOMW compost stability.

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