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Technical Note

Duckweed (*Lemna gibba*) growth inhibition bioassay for evaluating the toxicity of olive mill wastes before and during composting

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Abstract

Two-phase olive mill waste (TPOMW) is considered the main problem confronting the modern oil extraction and processing industry. Composting has been recently proposed as a suitable method to treat TPOMW so that it is suitable for use in agriculture. In the work reported here, the *Lemna gibba* bioassay was tested to assess the toxicity of TPOMW before and during the composting process. The method was compared with the *Lepidium sativum* bioassay and with other chemical maturity indices traditionally reported in the literature. The *L. gibba* test proved to be a simple, sensitive, and accurate method to evaluate toxicity before and during the composting of TPOMW. Plant growth response was measured by two methods: counting the number of fronds (leaves) and measuring total frond area (TFA) with image analysis software. Compared to the counting of fronds (*L. gibba*) or seeds (*L. sativum*), the use of area-measuring software permitted a very rapid, unbiased and easy way of analysing the toxicity of TPOMW before and during composting. Although the accuracy of the frond count method was similar to the traditional cress seed test, data analysis showed that the TFA measurement method was statistically more accurate (significantly lower variance) than the frond count approach. Highly significant correlations were found between TFA and some important maturation indices commonly reported in literature indicating that the *L. gibba* bioassay can be a useful tool to determine the degree of maturity of TPOMW composts.

Keywords: Olive mill waste; *Lemna gibba*; *Lepidium sativum*; Phytotoxicity; Composting

1. Introduction

Olive oil production is one of the foremost agro-industries in many Mediterranean countries and its processing is traditionally linked to this geographical region. However, the olive oil output is no longer limited to this area, since many countries are becoming emergent producers worldwide (Obied et al., 2005).

Depending on the extraction method used to obtain the oil, two different types of wastes are produced. The three-phase mills use large volumes of water and the main waste produced is a liquid called olive mill wastewater (OMW). Modern two-phase mills consume little water and, in this case, the waste is a semisolid sludge called two-phase olive mill waste (TPOMW). This study focuses on TPOMW, as it is currently the system that modern mills are most widely implementing.

The phytotoxic properties of olive mill wastes are mainly associated with their high concentration of phenols (catechol, hydroxytyrosol, tyrosol, oleuropein) that are known to inhibit plant and bacterial growth (Capasso et al., 1992), although TPOMW also contains other organic

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compounds that contribute to its toxicity, such as fatty acids (butyric, acetic, stearic, oleic).

The environmental problems associated with the production of TPOMW have led to the study of a broad number of possible remediation and treatment strategies which have been recently reviewed by Roig et al. (2006). One of the most promising methods proposed for the recycling of TPOMW is through composting with other agricultural by-products. Some authors have demonstrated that composting may be a suitable low-cost strategy for the recycling of TPOMW (Filippi et al., 2002).

The study of phytotoxicity has special relevance during the composting of TPOMW because intended horticultural uses of the final compost product depends on elimination of all phytotoxic consitutents. Some studies have shown a complete detoxification of olive mill wastes during composting (Paredes et al., 2000; Cayuela et al., 2006), and thus suggest that the phytotoxic compounds have been degraded.

There are several methods for the evaluation of phytotoxicity during composting. The most widely used plant species for compost phytotoxicity bioassays is *L. sativum*, commonly known as cress seed. This plant is highly sensitive to ammonium and phenols, moderately sensitive to salinity and very fast growing. The major drawbacks to this method are the need to tediously count seeds and the high variability of the results.

Another toxicity bioassay that has received much attention recently is the duckweed growth inhibition test. The term "duckweed" commonly refers to a group of aquatic plants of the family *Lemnaceae*, a floating fast-growing plant, small and easy to cultivate. These characteristics make it an ideal candidate for toxicity testing. Hence, the duckweed bioassay has become a standard toxicity method for many certification entities and international organizations. Accordingly, the United States Environmental Protection Agency (USEPA) developed guidelines for a plant toxicity test using *Lemna* spp. (EPA, 1996). Moreover, the Organization for Economic Cooperation and Development (OECD) completed a guideline draft for the *Lemna* test (OECD, 2000) that was extended for difficult substances, i.e. suspensions and turbid or coloured test materials (OECD, 2002). It has been integrated as part of the standards for soil quality (ISO 15799, 2003). In addition, the American Society for Testing and Materials (ASTM, 1991), the APHA (APHA, 1992) and the Sweden Institute of Standards (SIS, 1995) include the *Lemna* test as a standard method for measuring toxicity.

The aim of this work was to evaluate the *L. gibba* growth inhibition test as a simple, cost-effective, and accurate method for evaluating the phytotoxicity of TPOMW before and during the composting process. The method was compared with the *L. sativum* test and with other indices commonly used as maturity indicators during the composting process.

2. Materials and methods

2.1. Two-phase olive mill waste (TPOMW)

Four samples of TPOMW (A, B, C and D) were collected from an olive mill in southern Spain during the olive oil extraction period (December–February). Samples were air-dried and ground to 0.5 mm, and processed as previously described (Cayuela et al., 2004). Their main chemical characteristics are given in Table 1.

2.2. TPOMW composting process

An industrial composting pile (35 t fresh weight) was prepared by mixing two-phase olive mill waste (D) (Table 1), sheep litter (SL) (TOC/TN: 14.0) and grape stalks (GS) (TOC/TN: 43.1) in the following fresh proportions: 45% TPOMW + 45% SL + 10% GS (30:60:10 dry weight basis). The pile was aerated by mechanically turning the windrows as previously described (Cayuela et al., 2006) and irrigated regularly to maintain moisture around 40%.

Samples (500 g) were collected at four different stages of the composting process by mixing five sub-samples (100 g each) obtained from different locations of the pile: I: From the initial non-decomposed mixture (1st week of composting); T1: From the thermophilic phase (8th week of

Table 1
Main chemical characteristics of two-phase olive mill wastes (TPOMW) samples

TPOMW samples	pH	EC (dS m^{-1})	NH_4^+–N (mg l^{-1})	Carbohydrates (g l^{-1})	Phenols (g l^{-1})	Lipids (%)
A	5.23^{c}	4.97^{c}	n.d.	12.3^{ab}	1.29^{b}	16.0^{d}
B	5.28^{b}	5.31^{b}	3.0^{b}	16.4^{a}	1.50^{a}	20.8^{b}
C	5.53^{a}	5.40^{b}	5.8^{ab}	3.2^{c}	1.18^{c}	31.3^{a}
D	4.99^{d}	5.75^{a}	7.9^{a}	8.3^{b}	1.24^{bc}	18.6^{c}
	K (%)	P (g kg^{-1})	Fe (g kg^{-1})	Cu (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
A	2.7^{a}	0.93^{a}	0.46^{b}	22	14	13
B	2.9^{a}	0.93^{a}	0.17^{d}	22	13	16
C	2.2^{b}	0.81^{b}	0.33^{c}	18	11	12
D	–	0.37^{c}	1.54^{a}	18	17	19

Results represent the mean of three replicates. pH, electrical conductivity (EC), NH_4^+–N, carbohydrates and phenols represent the values for 1:10 water extracts. The rest of parameters are expressed on dry weight basis.
n.d.: not detected. –: not determined.
In a column values followed by the same letter are not significantly different according to S–N–K test ($P < 0.05$).

composting); T2: From the thermophilic phase (18th week of composting); F: From the final compost obtained (31st week of composting).

2.3. Chemical analysis

Electrical conductivity (EC) and pH were determined in a 1:10 (w/v) water-soluble extract. Phenolic substances were determined in 1:10 (w:v) water extracts by a modified version of the Folin method and water-soluble carbohydrates by the anthrone method as previously described (Cayuela et al., 2006). The total lipid content was determined using the traditional method of extraction in a Soxhlet with diethyl-ether. 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 (Lax et al., 1986). NH_4^+ was extracted from fresh samples with 2 M KCl and determined by a colorimetric method based on Berthelot's reaction (Sommer et al., 1992). K, Fe, Cu, Mn, Zn were analysed after $HNO_3/HClO_4$ digestion by atomic absorption spectroscopy (UNICAM 969 AA Spectrometer) and P spectrophotometrically as ammonium molybdovanadate phosphoric acid (Cayuela et al., 2004). 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}. The maturation indices HR (humification ratio) and HI (humification index) were calculated according to the expressions:

$$HR = C_{ext}/TOC * 100$$

$$HI = HAC/TOC * 100$$

2.4. Plant bioassays

2.4.1. Cress seed germination test (Lepidium sativum test)

TPOMW and compost water extracts were prepared according to the standard procedure developed by Zucconi et al. (1981), i.e., samples (4 g of dry material) were moistened to 60% moisture content and allowed to stand for 30 min. After that, more deionised water (54 ml) was added and the samples were stirred mechanically for 30 min, centrifuged and filtered (0.45 μm) for sterilization. Eight seeds of cress (*L. sativum*) were placed on two layers of sterile filter paper in 10 cm Petri dishes and one ml of extract was added. Sterile distilled water was used as a control. Seed germination and root length in each plate were measured after 48 h incubation in the darkness at 27 °C. Ten replicates were made for every treatment. The germination index (GI) after exposure to the extracts was calculated as follows.

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

where $G(\%)$ and $L(\%)$ are the germination and elongation percentages relative to the control sample. The threshold value for mature composts according to Zucconi et al. (1981) is GI > 50%.

2.4.2. Duckweed growth inhibition test (Lemna gibba test)

Sterile cultures of *L. gibba* G-3 (parental line) were maintained on E medium (Slovin, 1997) at 25 °C. Several colonies were transferred and grown on 50 ml of liquid E medium in 125 ml Erlenmeyer flasks at 25 °C in preparation for the bioassay tests. Flasks were illuminated with an equal mixture of cool and warm-white fluorescent lamps providing 80 μmol m^{-2} s^{-1}.

The TPOMW and compost water-extracts were prepared as 1:10 and 1:100 (w:v) dilutions. They were kept in continuous agitation for two hours at room temperature. Extracts were centrifuged (3000 rpm) and filtered (0.45 μm) for sterilisation.

Sterile 12-well culture plates (BD Falcon, New Jersey, USA) were used for the bioassay. One two-frond colony was planted in every cell containing 2.5 ml of the test solution. Six replicates were made for every treatment. Tests were carried out on lighted carts with overhead fluorescent lighting of 80 μmol m^{-2} s^{-1}, in a growth chamber at 25 °C. The frond number (FN) and total frond area (TFA) were measured after 7 d of incubation.

Area measurement was performed by means of digital image capture (Nikon Digital Sight DS-5Mc) software (Nikon ACT-2U, Nikon Corp., Tokyo, Japan) and data analysis system able to interpret frond area depending on colour intensity (Assess. Image Analysis Software for Plant Disease Quantification. American Phytopathological Society Press, St. Paul, MN). This is the first reported use of this image analysis software for this purpose. FN and TFA were expressed as percentages relative to the control sample.

2.4.3. Statistical analysis

One-way ANOVA tests were used to analyse the differences between samples. Pairwise comparison of means was performed using the "post hoc" Student-Newman–Keules test. Bivariate Pearson correlations were calculated (statistical software Sigmastat 9). The accuracy of the results obtained by the *L. sativum* and the *L. gibba* bioassays was compared using the *F* test (Miller and Miller, 1993).

3. Results and discussion

3.1. Phytotoxicity of TPOMW

TPOMW toxicity is due to organic chemicals (polyphenols and low molecular weight organic acids). Table 1 shows some of the most important characteristics of TPOMWs used in this study. The characteristics of TPOMW may change during the crushing period due to differences in the quality of the consignment of olives, ripeness, process efficiency, etc. These changes in their chemical

composition influence their phytotoxic properties. In our study, the concentration of water-soluble phenols ranged between 1.18 and 1.50 g l^{-1} in the 1:10 (w:v) extract. Barber et al. (1995) demonstrated the toxicity of phenol to *L. gibba* and reported an EC_{50} of 57 mg l^{-1}: therefore both 1:10 and 1:100 extracts contain polyphenol concentrations that are within the range expected to be toxic. The concentration of NH_4^+ was relatively low, and according to the findings of Caicedo et al. (2000), it would not greatly affect the growth of *Lemna*. The latter studied the effect of NH_4^+/NH_3 on the growth rate of duckweed depending on pH and found the lowest inhibition at low concentrations of ammonium (3.5–20 mg l^{-1}) and acidic to neutral pH.

The pH of the extracts was in the range 4.99–5.53, which is close to the optimum pH for the growth of *Lemna* (5.6–7.5), and therefore unlikely to inhibit plant growth. Nevertheless, low pH can reveal the presence of low molecular weight organic acids that can exert a toxic effect. EC was substantially high (4.97–5.75 dS m^{-1}), which could have contributed to inhibition of *L. gibba* growth according to Oron and Willers (1989), who reported negative effects on growth when EC exceeded 4 dS m^{-1}.

TPOMWs generally have a high concentration of lipids that can vary among samples depending on the extraction efficiency. Although the bioassay was performed in aqueous extracts, some lipids were observed in the extraction liquids in the form of an emulsion. Thus, lipids could have had an inhibitory effect on the growth of *Lemna* as previously reported for other plants (Buta, 1983).

Regarding heavy metals, *Lemna* spp. is especially sensitive to Cd, Ni, and Cu (Wang, 1990). In TPOMW, the concentration of heavy metals is very low and they are mainly bound to the organic polymeric organic fractions (Capasso et al., 2004). Thus, olive mill solid residue has been proposed to remove heavy metals from aqueous solutions with a particular affinity for copper (Pagnanelli et al., 2002). Toxic heavy metals such as Pb, Cd, Ni or Cr were not detected at concentrations exceeding the detection limits (5, 1, 4 and 2 μg g^{-1} respectively) in any of the TPOMW samples analysed in this study.

Table 2 shows TPOMW phytotoxicity by both *L. sativum* and *L. gibba* tests. The cress seed test performed as described by Zucconi et al. (1981) showed a high sensitivity to the TPOMW water extracts. In all cases, the GI approached 0% and the method was not able to distinguish differences in phytotoxicity among the TPOMW samples. In the case of the duckweed assay, two water extract dilutions were evaluated (1:10 and 1:100 (w:v)) in order to select the most suitable for these kinds of materials. Furthermore, two methods for the measurement of growth are reported: one based on FN counts and other based on TFA measurement.

The duckweed assay also showed a high sensitivity to the 1:10 extracts and, after 7 d, almost all the fronds died (Fig. 1). However, when measuring TFA, different patterns were found. Statistically significant differences were found for the four TPOMW samples, with toxic responses higher for sample D and lower for sample C. For the latter, an interesting phenomenon was observed. Although sample C exhibited less phytotoxicity than the other TPOMWs, the *Lemna* plants underwent a rapid separation and did not form the usual 3–4 frond colonies. Such frond abscission has been observed in the presence of known toxic compounds (Slovin, 1997).

Table 2
Phytotoxicity of TPOMW and TPOMW composting samples according to *Lepidium sativum* and *Lemna gibba* bioassays

	Lepidium test GI (%)	*Lemna* test			
		Extracts 1:10 (w:v)		Extracts 1:100 (w:v)	
		FN (%)	TFA (%)	FN (%)	TFA (%)
TPOMW sample					
A	0	0	28^{b}	60	69^{a}
B	0	0	25^{b}	43	46^{bc}
C	0	<2	34^{a}	56	60^{ab}
D	0	0	19^{c}	37	39^{d}
ANOVA	NS	NS	**	NS	**
Composting sample					
I	24^{b}	31^{d}	26^{d}	62^{b}	66^{b}
T1	34^{b}	55^{c}	45^{c}	73^{b}	83^{b}
T2	106^{a}	72^{b}	61^{b}	75^{b}	85^{b}
F	99^{a}	100^{a}	96^{a}	101^{a}	114^{a}
ANOVA	***	***	***	***	***

In *Lepidium* test, TPOMW an composting extracts were prepared according to the standard procedure developed by Zucconi et al. (1981).
GI: germination index; FN: Frond number; TFA: Total frond area.
GI, FN and TFA are expressed in % with respect to the control sample (water).
In a column results followed by the same letter are not significantly different according to the S–N–K test. ***,**$P < 0.01$, $P < 0.05$, respectively. NS: not significant.

The 1:100 extracts showed lower phytotoxicity than the 1:10 extracts. In this case, frond counts and area measurements showed similar results, but the TFA method gave significantly different means whereas the FN could not statistically distinguish between samples. Probably this is because not only the rate of frond reproduction was inhibited by TPOMW, but also their size and health. All the data indicated that sample D was the most phytotoxic and A or C less so.

No simple linear correlation was found between TFA and some general chemical characteristics of the TPOMW, indicating that different compounds are responsible for TPOMW phytotoxicity. A multiple linear regression was found between TFA in 1/100 extracts, total phenols and ammonium concentration ($r^2 = 0.919$; $P < 0.05$).

3.2. Phytotoxicity during TPOMW composting

The composting of TPOMW followed the common pattern reported previously for this kind of material, with high temperatures (around 60 °C) and a long thermophilic phase (Cayuela et al., 2004, 2006). Table 3 shows some of the main chemical characteristics at four representative

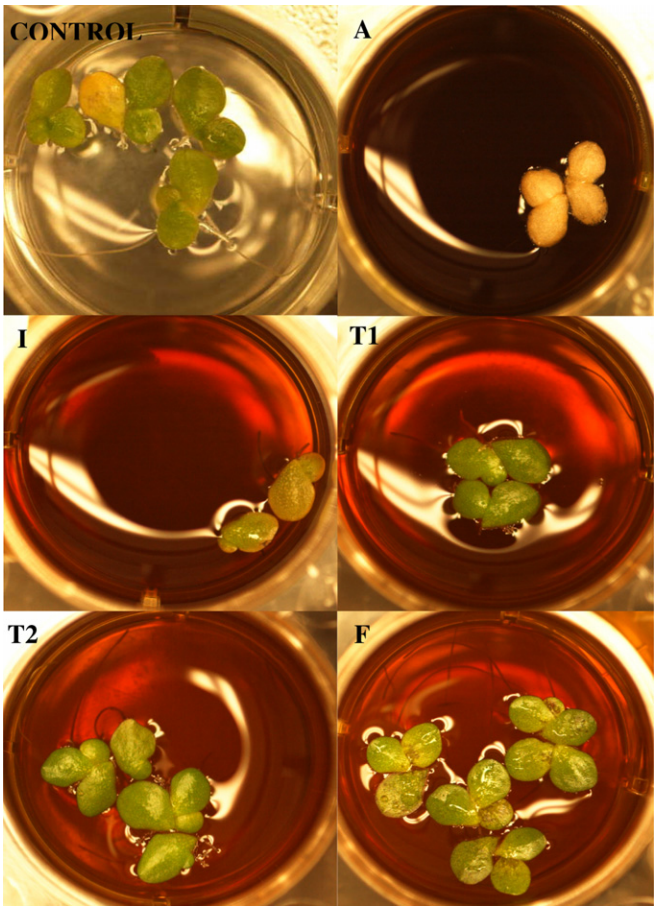

Fig. 1. Results of *Lemna gibba* bioassay for control with water, 1:10 (w:v) water extracts of two-phase olive mill waste (sample A) and four samples (I: initial, T1, T2: thermophilic and F: final) taken during the composting of TPOMW after a 7 day incubation at 25 °C.

Table 3
Changes in chemical characteristics and maturation parameters during two-phase olive mill waste composting

Stage	Weeks of composting	pH	EC (dS m^{-1})	NH_4^+–N (mg l^{-1})	Phenols (mg l^{-1})	Lipids (%)
I	1	7.35[d]	4.95[a]	9.7	298[a]	6.4[a]
T1	8	7.69[c]	3.03[b]	11.2	191[b]	2.7[b]
T2	18	8.73[b]	1.78[c]	10.4	139[c]	1.4[c]
F	31	8.85[a]	1.91[c]	8.6	117[d]	0.5[d]
		WSC (%)	WSC/TN	HR (%)	HI (%)	CEC/TOC
I	1	4.48[a]	3.31[a]	36.0[d]	22.5[d]	1.94[c]
T1	8	3.14[b]	2.52[b]	37.4[c]	26.1[c]	2.31[b]
T2	18	1.27[c]	1.23[c]	40.0[b]	34.3[b]	2.48[b]
F	31	1.17[c]	0.81[d]	43.6[a]	37.4[a]	3.08[a]

Results are the mean of three replicates. pH, electrical conductivity (EC), NH_4^+–N and phenols represent the values for 1:10 water extracts. The rest of parameters are expressed on dry weight basis.
WSC: water soluble carbon; TN: total nitrogen; HR: humification ratio; HI: humification index; CEC: cation exchange capacity; TOC: total organic carbon.
In the same column values followed by the same letter are not significantly different according to the S–N–K test ($P < 0.05$).

stages of the composting process. The pH significantly increased during composting as usual for this type of material (Cayuela et al., 2004, 2006). The main chemical compounds responsible for TPOMW phytotoxicity (phenols and lipids) were high at the initial stages and decreased substantially during the composting process. Several maturation indices usually applied to compost such as the ratio water soluble carbon/total nitrogen (WSC/TN), HI and HR showed that the final composted TPOMW achieved considerable stability.

While maturity is an important factor in evaluating compost quality because it relates to the disappearance of phytotoxic substances, stability, which is associated with microbial activity, must also be considered (Hue and Liu, 1995; Wu et al., 2000). Frequently, both terms are used interchangeably due to their parallel development during the composting process. A universally accepted method to evaluate stability and maturity does not exist despite the need to assess both parameters jointly. Some maturation indices reported by other investigators include WSC/TN (Hue and Liu, 1995), GI (Zucconi et al., 1981), and indices based on humification processes such as HR and HI (Paredes et al., 2000).

L. gibba is sensitive to a wide range of pollutants, which is an advantage in toxicity evaluation. It is able to grow across a wide range of pH, from pH 3.5–10.5. Thus, it has been used extensively for eco-toxicology studies reacting non-specifically to a number of xenobiotics such as herbicides, pesticides and heavy metals (Wang, 1990; Lewis, 1995). In spite of all these advantages, the *Lemna* bioassay is not widely used for elucidating toxicity during composting (Juvonen et al., 2000).

Fig. 1 shows the results of *L. gibba* test at four stages of the composting process (I: initial, T1, T2: thermophilic and F: final). The disappearance of toxicity at T2 and F stages is remarkably unambiguous. Results of phytotoxicity tests using *L. gibba* (Table 2) show that the 1:100 extracts could not be used to evaluate toxicity because the toxins were diluted extensively and counteracted the effect of nutrients. The 1:10 dilutions of the extracts were more appropriate for the samples in this assay.

Highly significant inverse correlations were found between the maturation indices and the phytotoxicity analysed with both *L. sativum* and *L. gibba* assays, indicating their ability to predict the maturity degree in TPOMW composts (Table 4).

The accuracy of *L. gibba* method was compared to the *L. sativum* test developed by Zucconi et al. (1981) using the F test (Miller and Miller, 1993). When phytotoxicity was calculated by FN, $F = 2.018$, which is lower than the critical value ($F = 9.277$) for a one-tailed test ($P < 0.05$) (3 degrees of freedom). Therefore, there is no statistical evidence that the *L. gibba* test is more accurate than the *L. sativum* test.

However, when measuring TFA, $F = 12.740$ and therefore, the precision of the *L. gibba* method with area measurement is significantly greater than that of the traditional *L. sativum* test.

Table 4
Correlation matrix between the main chemical characteristics and maturation indices during the composting of TPOMW

	GI (%)	TFA (%)	pH	EC (dS m^{-1})	Lipids (%)	Phenols (%)	WSC/TN	HR (%)	HI (%)
TFA (%)	0.843								
pH	0.985**	0.916*							
EC (dS m^{-1})	−0.898	−0.819	−0.932*						
Lipids (%)	−0.842	−0.871	−0.907*	−0.981**					
Phenols (%)	−0.871	−0.876	−0.928*	−0.989**	0.998*				
WSC/TN	−0.959*	−0.941*	−0.993**	−0.950*	0.945*	0.958*			
HR (%)	0.872	0.996**	0.932*	−0.810	−0.849	0.858	−0.946*		
HI (%)	0.962*	0.953*	0.994**	−0.923*	−0.919*	0.935*	−0.997**	0.963*	
CEC/ TOC	0.786	0.989**	0.879	−0.826	−0.896	−0.893	−0.920*	0.972*	0.925*

GI: germination index (*Lepidium sativum* test); TFA: total frond area (*Lemna gibba* test; 1:10 (w:v) extracts); EC: electrical conductivity; WSC: water soluble carbon; TN: total nitrogen; HR: humification ratio; HI: humification index; CEC: cation exchange capacity; TOC: total organic carbon. *,**Significant at $P < 0.05$ and 0.01, respectively.

The advantage of the duckweed assay over the germination and growth test lies in the highly homogeneous plant material. While all duckweed plants are clones, in the seeds, different weight distribution and the heterogeneity of genetic make-up leads to a large standard deviation in results. However, toxic substances in TPOMW do not only influence frond reproduction, but their vigour and dimensions, which are better evaluated by measuring area than by counting the number of fronds produced.

4. Conclusions

The use of the *L. gibba* bioassay with frond area measurement is a suitable method to evaluate TPOMW phytotoxicity before and during composting. The *L. sativum* test according to the procedure developed by Zucconi et al. (1981) is appropriate to estimate toxicity during TPOMW composting, however to make a distinction between toxicity in different TPOMW samples, another waste-to-water dilution ratio should be tested. In the *L. gibba* test, the effects of toxic substances can be analysed with image analysis software that saves time and gives unbiased and reliable results. The *L. gibba* bioassay with area image analysis is significantly more precise than the *L. sativum* test, but when frond counting is used there is no significant difference between variances in both methods. Both methods are simple, cost-effective and can be used as complimentary tests for evaluating TPOMW compost quality.

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