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Soil bioassays as tools for sludge compost quality assessment

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1. INTRODUCTION

The worldwide increase in the production of sewage sludge, as a consequence of water protection policies, has triggered a growing pressure for sludge reuse in soil (**De Brouwere and Smolders 2006**). Despite the benefits of such practices in terms of soil physical properties and fertility, the use of sludge in soils can release pathogens, produce annoying odours, contaminate groundwaters with nitrates (**Correa et al. 2006**), and pollute soil with heavy metals (**McBride et al. 1997**) and organic chemicals (**Harrison et al. 2006**).

These potential environmental risks have led to the definition of maximum permissible concentrations of heavy metals in sludge and cumulative loadings in soils receiving sludge both in the European Union (**European Commission 1986**) and the United States (**USEPA 1993**). However, some authors have suggested that these regulations are not sufficiently protective regarding the noxious effects of heavy metals in trophic chains (**De Brouwere and Smolders 2006**). In addition, none of these regulations include known organic pollutants which are present in sewage sludge (**Abad et al. 2005**), nor can they take into account new pollutants which appear every year (**Oleszczuk 2008**).

Composting is a waste management technology in which organic matter is stabilized by aerobic decomposition. This approach is mainly used for recycling organic materials into a useful product. Composting reduces the volume of sludge and transporting costs, eliminates the risk of disseminating pathogens and removes malodorous compounds (**Jakobsen 1995**). In addition, composting heightens the fertilizing properties of sludge (increased nutrient contents together with a progressive release with organic matter mineralization) (**Bernal et al. 1998**). It reduces the risk of nitrogen immobilization in the soil in microbial biomass, an effect which is associated to the use of immature composts (**Bernal et al. 1998**), and it also reduces erosion and desertification processes (**Gigliotti et al. 2005**). In addition, composting can lower the amount of some biodegradable organic chemicals, and hence decrease their potential environmental impact (**Lazzari et al. 2000, Abad et al. 2005, Sanz et al. 2006, Gibson et al. 2007, Das and Xia 2008**).

Composting involves changes in organic matter composition, in a process which in turn depends on temperature, moisture content and oxygen availability. The composting process can also be influenced by the nature of the bulking agent used. These agents lower the moisture content of sludges, increase porosity and aeration and increase the carbon content (**Field et al. 2004**). The bulking agent influences the C/N ratio and the stable organic content of sludge and other wastes, thus changing its evolution (**Pasda et al. 2005**).

Several indicators of composting effectiveness have been proposed in different studies. However, no single indicator has been found to conclusively confirm that composting of a given source stock has come to completion (**Smith and Hughes 2004**). The indicators can be classified as physical, chemical or biological and the correlation between some of them has been demonstrated in different studies (**McKinley and Vestal. 1985, Goyal et al. 2005, Tiquia et al. 2005, Zmora-Nahum et al. 2005**). Different properties have been used as physical indicators, such as lower moisture content (**Tiquia and Tam 2000**), decreased temperature (**Brewer and Sullivan 2003**), lower volatile solids content (**Huang et al. 2004**), lower pH (**Tiquia and Tam 2000, Huang et al. 2004, Miaomiao et al. 2009**), electrical conductivity or cation exchange capacity (**Wang et al. 2004**).

On the other hand, other properties have been used as chemical indicators such as low carbon or nitrogen content (**Brewer and Sullivan 2003**), lower C/N ratio (**Huang et al. 2004**), lower dissolved organic matter (**Benito et al. 2003, Huang et al. 2004, Smith and Hugues 2004, Gigliotti et al. 2005, Goyal et al. 2005, Zmora-Nahum et al. 2005**), higher nitrate/ammonium ratio (**Brewer and Sullivan 2003, Benito et al. 2003, Huang et al. 2004**) or the humic acids content (**Adani et al. 1997**).

Finally, and more rarely, biological measures have been employed as indicators, using a range of bioassays involving microorganisms to plants. In microbial bioassays, respiration rates (**Barrena et al. 2006**), changes in enzyme activities (**Smith and Hugues 2004, Goyal et al. 2005, Pasda et al. 2005**), and changes in microbial communities have been used as composting indicators (**Tiquia et al. 2005**, **Pasda et al. 2005, Nakasaki 2009**). Concerning plants, germination indices (**Smith and Hugues 2004, Gigliotti et al. 2005, Oleszczuk 2008, Ramírez et al. 2008a, 2008b)** and seedling growth (**Wang et al. 2004, Zmora-Nahum et al. 2005, Ramírez 2008b**) have been used.

However, little data on the use of biological indicators for compost quality has been published to date, despite the advantages compared to other parameters (**Crouau et al. 2002**). Concerning biological indicators, work has mainly been centered on the use of plants (**Alvarenga et al. 2007, Ramírez et al. 2008b**, **Moreira et al. 2008, Miaomiao et al. 2009, Natal-da-Luz et al. 2009**) and microorganisms (**Smith 2009**). Even more scarce are studies using soil invertebrates (**Alvarenga et al. 2007, Domene et al. 2008, Moreira et al. 2008**) or aquatic organisms (**Alvarenga et al. 2007, Oleszczuk 2007, Natal-da-Luz et al. 2009**). In addition, there is very little information on the variation in toxicity during the sewage sludge composting process, since most studies are based on evaluating the toxicity before and after composting (**Oleszczuk 2007, 2008**). In our study, we take a wider approach, assessing also intermediate phases of composting. Hence, the main aims of this study were: to report the variation in the physicochemical properties and pollutant burden of sludge along its composting; to show the sensitivity of soil invertebrate bioassays as indicators of composting quality in addition to the usual plant bioassays; and to identify the compost properties linked to the outcomes observed in the different test species.

2. METHODS

2.1. Composts

Two different wastewater treatment plants (WWTP) were selected, both carrying out composting of sewage sludge as post-treatment, but with distinct composting procedures. At Manresa WWTP (Barcelona, Spain), composting is carried out with undigested sludge mixed with pine bark and coffee grounds (1:2 v/v). The thermophilic phase of composting is carried out for 14 days in open piles placed on a conveyor belt which drives the compost slowly to the end of the line, where compost is prepared for the maturation phase. Then, compost is sieved to 1 cm and heaped up in 2.5 m high piles for the maturation phase, which lasts 3 months. At Blanes WWTP (Girona, Spain), composting is carried out with dewatered anaerobically digested sludge mixed with pine chips (1:4.5 v/v). In this case, the thermophilic phase is carried out in a closed tunnel with injected air, in a stage that lasts 28 days. After that, compost is sieved to 1 cm for the maturation stage, which is carried out identically as described for the other WWTP.

2.2. Sampling

Since the thermophilic phase differed in duration depending on the WWTP considered, sampling days were different in each plant but were taken at equivalent moments: initial mixture, intermediate and the end of the thermophilic phase and at two intermediate stages and the end of the maturation phase. By doing this, we ensured the results were comparable between composting processes. However, at Blanes WWTP, the intermediate thermophilic sampling could not be carried out because this stage was performed in a closed tunnel (**Table 1**).

The characterization of the composts suggested an accidental mixing with another compost allotment in the last sampling in Blanes WWTP. This is indicated by unexpected values in some properties when compared to the general trend observed during composting, specifically in the case of some pollutants (Cr, Zn and NPEO) and properties (electrical conductivity and total N). Mixing with other compost allotments in this WWTP was plausible because composting piles are periodically moved up and moved within the facility during the maturation stage. For this reason, the sample collected after 112 d of composting in Blanes WWTP was not considered in this study.

Table 1

2.3. Sample preparation

Samples were immediately transported to the laboratory, where they were dried at 80ºC for 48 hours. After that, dry compost was sieved to 1 cm in order to allow results to be comparable for the different samplings (in both WWTP, composts were sieved to 1 cm at the end of the thermophilic phase). Following this the sieved compost was ground slightly and passed through a 5 mm-sieve. Finally, samples were stored in hermetic containers at 5ºC until their use in the bioassays.

The drying step at 80 ºC was unavoidable for two reasons. First, the high water content of composts, especially uncomposted sludge, prevented air-drying, something that might artificially extend the composting period. Second, grinding is only possible with dry sludge or compost. This ensured the homogeneity and the accuracy of the concentrations in soil-compost mixtures prepared for the bioassays, as well as the homogeneity in sludge composition.

Although this drying step might have volatilized some pollutants (e.g. Hg), this is not the case for most of the pollutants in sludge, which are highly adsorbed to organic matter and have reduced volatility. In addition, since the compost characterization was made with the final dry sample, we ensured that any relationships between compost composition and the biological effects observed in bioassays were true.

2.4. Compost characterization

The physicochemical properties of the composts are shown in **Table 2**. The pH and the electrical conductivity were assessed in a 1:5 (w/v) soil:water extract as described in **EN 13037 (1999)** and **EN 13038 (1999)**, respectively. Total organic matter content was measured by gravimetry after calcination at 550ºC as set in **EN 12879 (2000)**. Total nitrogen was estimated by titration after acid hydrolysis and distillation of the sample according to the Kjeldahl method, while ammonium was measured by titration after the distillation. Nitrate was measured by high performance liquid chromatography–ion chromatography (HPLC–IC) in aqueous extracts prepared according to **Huang et al. (2004**) but using a 1:5 (w/v) aqueous extract. The pollutant burden of the composts is shown in **Table 3**. Potassium, cadmium, chromium, nickel, lead and zinc were measured by ICP-MS in digests prepared in a microwave assisted digestion in aqua regia at 105ºC. Phosphorus was measured by UV-VIS in the same digests, while mercury was measured by FIAS-AAS. Ethoxylated nonylphenols (NPEO) were measured with HRGC–HRMS, polychlorinated biphenyls (PCB) by HRGC-ECD, while polycyclic aromatic hydrocarbons (PAH) and linear alkylbenzene sulfonates (LAS) were determined by HPLC with fluorescence and UV detectors, respectively. The values for each pollutant group were expressed as indicated in the third draft of the Working Document on Sludge (**European Commission 2000**): LAS correspond to total values, NPEO corresponds to the sum of concentrations of nonylphenol (NP) and nonylphenol ethoxylates with one (NPEO1) or two ethoxy groups (NPEO2), while PCB is the sum of the polychlorinated biphenyl congeners number 28, 52, 101, 118, 138, 153, and 180. LAS concentrations were complete for the different samples from Manresa WWTP, but in Blanes WWTP only the concentrations in uncomposted sludge (day 0) and compost at the intermediate maturation phase (42 days of composting) were available.

Table 2

Table 3

2.5. Compost dosages tested

The compost samples corresponding to each of the composting times, dried and sieved as described above, were mixed with a natural soil at increasing concentrations. The soil was collected in an experimental agricultural field of the Autonomous University of Barcelona campus (see **Table 4**), and had been free of pesticides for at least the last five years. Soil was air-dried and, in order to avoid any interference with the organisms used in the bioassays, it was defaunated by alternating two consecutive freezing-thawing cycles, each consisting in placing soil at -20ºC for 4 days followed by a period of 4 days at 20ºC.

Table 4

The concentrations selected to be tested in the bioassays were chosen to mimic four realistic sludge amendment scenarios (**Table 5**). These scenarios were selected according to the literature on the usual amendment rates for agricultural and reclamation purposes. Two of the scenarios corresponded to the maximum allowed agricultural amendments of the European States with legislation on this topic: 3 tons ha^{-1} (low dosage) corresponding to the median maximum annual amendment rate in these States (**Gendebien et al. 2001**); and 10 tons ha^{-1} (high dosage) which corresponded to the maximum annual amendment in Denmark (**Gendebien et al. 2001**). The remaining two scenarios simulated amendments with land reclamation purposes: 50 tons ha^{-1} (low dosage), used in several studies carried in Mediterranean Europe (**Alcañiz et al. 2008, Ojeda et al. 2008**); and 125 tons ha^{-1} (high dosage), in the range of the highest dosages used for reclamation purposes in the literature for the same region (**Barrera et al. 2001**).

The concentrations used in the bioassays (expressed as g kg^{-1}) were inferred from the field amendment rates (expressed as tons ha^{-1}) assuming a typical soil with a density of 1.25 g cm^3 and a plough depth of 20 cm. Given the higher water holding capacity (WHC) of sludges and composts compared to soils, and to provide equal moisture to all the test concentrations, moisture was adjusted to 50% of the maximum WHC of each test concentration.

Table 5

2.6. Plant emergence

Plant toxicity was assessed by germination assays, in accordance with the OECD Guideline 208 (**OECD 2006**). Commercial certificate seeds of two species were used: common mustard (*Brassica rapa* L. var. *oleifera*) and ryegrass (*Lolium perenne* L. var. *Tove*) (Semillas Fitó, Barcelona). These species are currently used as crops and are recommended by the OECD for chemical ecotoxicity testing. The dosages used were those already described in **Table 5**. Plastic Petri dishes were used as germination pots. In this case, in contrast to the remaining bioassays, water content of soil-compost mixtures was adjusted to 60% of the WHC to favour germination. Each replicate consisted of a Petri dish (9 cm diameter) filled with 20 g of the soil-compost mixture (dry weight). Four replicates per concentration were prepared and incubated in a growth chamber at 21ºC, 16/8 h (light/dark) photoperiod, and 70% air humidity. Ten seeds per replicate were sown uniformly into each Petri dish and sealed with laboratory film to avoid moisture losses. The seed germination was evaluated after 10 days and expressed as a percentage compared to germination in controls. Seeds with a radicle ≥1mm were considered to be germinated.

2.7. Earthworm growth, reproduction and survival

Earthworms were raised in our laboratory, originally provided by the Instituto do Mar of the Universidade de Coimbra (Portugal). Bioassays were carried out according to ISO Guideline 11268-2 (ISO 1998), using *Eisenia andrei* as the test species, with two small modifications; the number of earthworms per container was reduced (6 instead of 10), and we also reduced the quantity of substrate per replicate (250 g instead of 500 g). This was assumed not to impair the validity of our results, since the outcomes in controls fulfilled the validity criteria of the test. Soil-compost mixtures were moistened to 50% of the WHC. Each replicate consisted of a 1000-ml container covered with a perforated lid that allowed aeration. Four replicates per concentration were prepared. Only six clitellated individuals were used instead of the 10 indicated in the protocol due to the lower substrate volume per replicate. The individuals were of synchronized age (4 weeks difference at most), with a weight ranging from 300 to 700 mg. Worms were fed with 5 g cooked oat flakes at the start and weekly thereafter. Replicates were maintained in the dark at a constant temperature of 21±1ºC for 28 days. The moisture loss of each replicate was checked weekly by weight, and restored if necessary with distilled water. After 28 days of exposure, adults were removed from the test substrate, counted and weighed and the replicates were incubated 28 more days in order to allow juveniles to emerge and grow. This enabled assessment of the surviving adults together with their total fresh biomass. After this 28 days period, each replicate was placed in a water bath at a temperature of 60 ºC. After 25 minutes, juveniles emerged at the substrate surface and were collected and counted.

2.8. Collembolan reproduction and survival

Collembolan toxicity was assessed with the species *Folsomia candida,* in accordance with ISO Guideline 11267 (**ISO 1999**). The individuals came from our laboratory, originally obtained from individuals provided by the Institute of Ecological Science of the Vrije Universiteit (Amsterdam, The Netherlands). Five replicates per concentration were prepared consisting of sealed 100 ml plastic cups filled with 30 g of soil (dry weight) moistened to 50% of their WHC. Ten individuals aged 10 to 12 days were added to each replicate, and fed with 3 mg of yeast at the start of the bioassay and after 14 days. Replicates were placed in the dark at 21±1ºC, and were aerated twice a week. After 28 days, the number of surviving adults and juveniles was determined. Their numbers were assessed by flooding the replicate content in a 500 ml flask followed by the addition of a dark dye to allow a picture to be taken of the individuals floating on the water surface. Adults and juveniles were differentiable by their size.

2.9. Statistical methods

The variation in compost properties with the time of composting were assessed by bivariate Pearson's correlations using SPSS 15.0 for Windows (SPSS Inc., Chicago, USA). Variation in the bioassays´ outcomes at the different combinations of dosage and time of composting were assessed by repeated-measures two-way ANOVA using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA). Finally, the influence of compost properties on the outcomes of the bioassays was also assessed by a multivariate statistical procedure (principal components analysis, PCA) using SPSS 15.0. For this, we selected the most relevant compost properties with the lowest correlation with those remaining (correlation coefficient below 0.8). Namely, we discarded data on pollutant burden because firstly, the heavy metals were clearly below the concentrations expected to affect the species tested (**Domene et al. 2007**). Secondly, it was problematic to include both heavy metals and organic pollutants in the PCA for two reasons, since data on heavy metals and non-persistent organic pollutants were incomplete or highly correlated with physicochemical properties through their association with the decomposition occurring during composting (This is related to the biodegradation of organic chemicals and to the concentration of heavy metals by mass loss). In addition, data on persistent organic pollutants were incomplete, restricted to the first and the last sampling. Hence, we ended selecting the organic matter, total nitrogen, ammonium, nitrate, potassium, and non-hydrolysable nitrogen content as variables. Some of these variables were significantly correlated among each other, but none of them was highly correlated (Pearson's correlation coefficient <0.8).

Finally, correlations between bioassays outcomes and the two principal components obtained were carried out in order to find significant correlations. Since different trends were obtained in each WWTP, this was carried out separately in Blanes and Manresa WWTP datasets.

3. RESULTS

3.1. Variation in the compost properties along composting

The composting process affected physicochemical properties of the compost produced in both WWTPs similarly (**Table 2**). The initial organic matter levels, decreased significantly in both composting procedures, especially during the thermophilic phase, but also in the first part of the maturation stage. This decrease was coupled to a slight increase in total nitrogen contents, which led to a decrease in the C/N ratio from 13-18 to around 10 at the end of composting.

In addition, pH increased during the thermophilic phases in both composts, from slightly acidic to a slightly alkaline pH, remaining stable until the end of composting. Similarly, the electrical conductivity rose during the thermophilic phase and then stabilized.

As decomposition progressed, was also a tendency for slight increases in phosphorus and potassium, together with ammonium and nitrate levels. However, the ammonium increase was clearly lower in Manresa composting, coupled with a higher increase in nitrate in comparison to Blanes composting. The ammonium levels measured in this study are not total but partial levels, since ammonium was measured in dried sludge, as already presented. More precisely, it corresponded to the ammonium not easily volatilized, strongly bound to the cation exchange sites in the organic matter matrix.

Using the log-transformed values of **Table 2** and **3**, there was a significant negative correlation between the duration of composting and organic matter levels (Pearson, $p=0.04$ in Blanes), and with non-hydrolysable organic matter and nitrogen ($p=0.026$ in Blanes). On the other hand, there was a positive correlation between the length of time of composting and electrical conductivity (p=0.001 in Blanes), nitrate (p=0.014 in Manresa), and ammonium (p=0.039 in Blanes).

Concerning the pollutant burden, the composting process also affected this factor in both WWTPs (**Table 3**). More precisely, heavy metal burden increased as mass was lost with the decomposition which occurred during composting. However, this trend was not significant in any of the composting plants.

Regarding the organic pollutants, PCB were undetectable in the different samples, while PAH and DEHP levels remained stable. On the contrary, LAS and NPEO were significantly lost with composting, particularly the higher the concentrations in uncomposted sludge levels. However, this trend was only significant for NPEO, inversely correlated with the time of composting in Blanes and Manresa compost (p=0.037 and 0.019). No correlations could be found with DEHP and PAH because only initial and final concentrations were available. There were also no correlations for LAS in Blanes compost, and neither for PCB since their values were under the detection limit.

Heavy metals did not exceed the limit value for the use of sludge in soil established by the Directive 86/278/EEC (**European Commission 1986**) nor those in the 3rd Document on Sludge (**European Communities 2000**). As regards organic pollutants, no limitations exist for them in the Directive 86/278/EEC. However, based on the available draft of new Directive, some of the organic chemicals may be of concern. In particular LAS and NPEO in sludge prior to composting exceeded the limit values suggested in the draft, but levels were below this limit at the end of composting. The remaining organic chemicals were below the limits indicated in this document. This indicates that the results of the bioassays of this study are representative of wastes currently spread on land according to the current European legislation.

3.2. Variation in ecotoxicological properties along the composting process

3.2.1. Plant germination

Seed emergence was nearly complete both for common mustard and ryegrass. The repeated measures ANOVA indicated seed emergence was significantly affected by compost dosage but also by time of composting (RM-ANOVA, p<0.001), irrespective of the compost origin and species tested. Compost dosage accounted for most of the total variance observed. In Blanes compost, when ryegrass was used as test species, there was also significant interaction (p=0.0017). The higher compost dosages generally reduced the number of seeds emerged compared to controls, while lower emergence rates were generally observed in uncomposted sludge (**Figure 1**).

Figure 1

3.2.2. Earthworm growth, survival and reproduction

High survival rates were observed in controls, together with similar total biomass values and reproduction outcomes, within the requirements of the ISO Guideline 11268-2 (ISO 1998). Earthworms seemed to be the most resistant taxa to compost addition, since their survival was unaffected by dosage and time of composting (**Figure 2**). However, total biomass changed significantly with composting time (RM-ANOVA, $p<0.001$), but generally no significant effects of compost dosage were observed, despite of the significant higher or lower biomass in the highest dosages of some composts. Interaction was significant in both compost types. Earthworm biomass was higher in uncomposted sludge compared to controls in Manresa, but in Blanes composts the higher biomass compared to controls was observed at all the composting times.

Concerning reproduction, a similar pattern was observed, since both composting time and compost dosage were significant ($p>0.001$). In this case, variance was similarly accounted by both factors. Higher dosages generally led to stronger effects, depending on the time of composting, as shown by the significant interaction. In Blanes, uncomposted sludge led to higher juvenile production, while fewer offspring were produced in the more mature composts. In Manresa composting, this trend was not observed, since similar juvenile production was observed with time of composting, with a maximum juvenile production generally at 20 g kg^{-1}.

Figure 2

3.2.3. Collembolan survival and reproduction

Collembolan survival was high in controls, and only changed significantly with composting time in both composts procedures, but also with compost dosage (RM-ANOVA, $p<0.05$). More precisely, survival rates are lowest in uncomposted sludge compared to more mature composts (**Figure 3**).

Regarding reproduction, both composting time and dosage were significant ($p<0.001$), most of the variance being accounted for by dosage. In Blanes reproduction was inhibited in the higher dosages tested, and especially in uncomposted sludge. In Manresa compost, this association is less clear but significant.

Figure 3

3.3. Correlation of outcomes between species and endpoints

The effect of composts on seed emergence was similar in both plant species, since they showed a positive correlation (Pearson, $p<0.001$) (**Table 6**). This is also generally consistent with the reproduction of collembolans, positively correlated with common mustard and ryegrass germination ($p<0.001$). On the other hand, ryegrass emergence was positively correlated with earthworm reproduction ($p<0.001$), while no other correlations appeared with the remaining invertebrate endpoints.

Concerning invertebrates, there was a significant positive correlation between earthworm survival and reproduction ($p<0.05$), a trend not observed in collembolans. In addition, in earthworms, survival and reproduction was also correlated with the total biomass ($p<0.001$). No similarities in the outcomes were found between earthworms and collembolans with the exception of the positive correlation between earthworm reproduction and collembolan survival.

Table 6

3.4. Influence of compost properties on the biological effects

The PCA reduced the explanatory variables selected (organic matter, total nitrogen, ammonium, nitrate, potassium and non-hydrolysable nitrogen) to two principal components, explaining 47.9% and 38.1% of the variance, hence explaining together 86% of the variability (**Figure 4**). Organic matter (-0.926), total nitrogen (0.880) and ammonium (0.840) were mainly associated to the first component, while non-hydrolysable nitrogen (0.891) and nitrate (0.874) were mainly associated to second component. This association with time of composting is meaningful since all these compost properties reflect the decomposition of sludge and the release of decomposition endproducts as composting progresses there.

Figure 4

After that, Pearson's correlations between the days of composting and the PCA factor scores in both components of each one of the composted materials were carried out to identify their association with the composting process. Similarly, Pearson's correlations were also carried out between the observed outcomes of bioassays and the factor scores in order to relate these outcomes with a set of compost properties represented by a PCA component. In this case, and given that these relationships might change with compost dosage, we did this analysis separately for the outcomes in the lowest dosages (1.2 and 4 mg kg^{-1}) and for those in the highest dosages (20 and 50 mg kg^{-1}), as shown in **Table 7** and **8.**

The factor scores of the PCA analysis of compost properties indicated that the length of time of composting was positively and significantly correlated with the first component ($r=0.725$, $p<0.001$), and also with the second ($r=0.399$, $p<0.001$). Blanes composting was mainly explained by the first component, while Manresa composting was explained similarly by both components (**Figure 4)**.

Seed emergence in both plant species was not clearly associated with either of the PCA components at low compost dosages. At high dosages, only common mustard presented a positive association with the first component both in Blanes and Manresa composting, but also with the second component in Manresa composts (**Table 7 and 8**), agreeing with the lower emergence rates in uncomposted sludge observed in this species (**Figure 1**).

Concerning soil invertebrates, earthworm and collembolan survival was uncorrelated with any component, either in low or in high compost dosages, indicating that these endpoints were not sensitive to the degree of composting of the sludge at the dosages tested (**Table 7 and 8**).

Concerning reproduction**,** different responses were observed depending on the taxa and endpoint assessed. In earthworms, no significant associations were found at low compost dosages, with the exception of the negative association of earthworm reproduction with the first component in Blanes compost and its positive correlation with the second component in Manresa composts (**Table 7 and 8**). At higher compost dosages, more clear and consistent results were observed. More precisely, reproduction was negatively associated to both components in Blanes compost, while no correlations were found in Manresa compost. In addition, total biomass was negatively associated with the first component in both compost types. This agrees with the higher reproduction and biomass observed in uncomposted sludge in Blanes composting, and not observed in Manresa composting (**Figure 2**).

In collembolans, no associations were found for survival nor for reproduction, with the exception of the higher dosages of Manresa compost (**Table 7 and 8**), where reproduction was correlated with both components, agreeing with the higher reproduction observed in mature composts compared to uncomposted sludge.

Table 7

Table 8

4. DISCUSSION

4.1. The need of bioassays for the quality assessment of composts

The benefits of composts and other organic wastes in terms of fertility for agricultural and forestry purposes has been widely reported in literature**.** However, not all wastes are suitable for this purpose. Generally only the chemical composition is taken into account to assess their quality, despite the fact that a wide variety of bioassays could be used for this purpose (**Kapanen and Itävaara 2001**).

Bioassays are basic for the assessment of the quality and ecotoxicological risks of wastes because chemical data are generally insufficient. The physicochemical properties of composts and total concentrations of pollutants usually say nothing about their overall effects on exposed organisms. The reason is that there is only partial information about the composition of the wastes. In addition, in the specific case of pollutants, knowledge of their total concentrations in wastes does not enable us to predict their bioavailability, degradation products, or to know potential synergisms and antagonisms among them (**Crouau et al. 2002**). More importantly, bioassays are useful because it is impossible to measure all the potential noxious properties of wastes, and especially in the specific case of pollutants, since new chemicals appear continuously (**Farré et al. 2008**). For all these reasons, data on the suitability of bioassays for waste quality assessment purposes are particularly useful and needed (**Kapanen and Itävaara 2001**). However, the few studies available in the literature have been mainly restricted to the use of plants (**Alvarenga et al. 2007, Ramírez et al. 2008b**, **Moreira et al. 2008, Miaomiao et al. 2009, Natal-da-Luz et al. 2009**) and microorganisms (**Smith 2009**). Only recently, the soil invertebrates have been also used in bioassays of wastes (**Alvarenga et al. 2007, Domene et al. 2008, Moreira et al. 2008, Natal-da-Luz et al. 2009**).

Observing the results from our study as a whole, the ecotoxicological properties of the composts differed depending on the test organism observed, and also on the type of compost. Plant emergence showed consistent patterns in the two species tested, but this was not true for the invertebrate species assessed, generally showing uncorrelated responses (**Figures 1** to **3**). Nevertheless, seed emergence response was positively correlated with collembolan reproduction. On the contrary, earthworm endpoints were generally uncorrelated with plant emergence or collembolan performance, with the exception of the positive correlation between earthworm reproduction and ryegrass emergence and collembolan survival.

4.2. Changes in compost properties along the composting process

Both for physicochemical properties and pollutant concentrations, there were evident changes during composting. More precisely, there was a clear loss in total organic matter and the C/N ratio, consistent with other studies (**Benito et al. 2003, Brewer and Sullivan 2003, Huang et al. 2004, Goyal et al. 2005)**. On the other hand, a significant increase in total nitrogen was observed, in accordance with **Hua et al. (2009)**, and indicating a minor loss of nitrogen as ammonia. Also nitrate, phosphorus and potassium increased significantly with composting.

A decrease in ammonium should be observed during composting due to volatilization and nitrification processes, coupled with an increase in nitrate (**Garcia et al. 1992**), but we observed the opposite effect (ammonium increase). Since we dried samples before analysis, most ammonium was lost during drying. Thus, the observed ammonium mainly corresponds to the least volatile fraction attached to cation exchange sites. Since cation exchange capacity increases with the degree of composting (**Saharinene et al. 1996**), an increase in ammonium content might be expected. In Manresa, despite their higher initial organic matter levels, we observed lower ammonium levels than that in Blanes, together with a much higher rate of nitrate production. These facts suggest that Manresa had an increased ammonia loss by volatilization or nitrification rather than a reduced decomposition, since total nitrogen content increased similarly in both composting plants. This might also explain the lack of influence of the time of composting on the outcomes of bioassays carried out with Manresa composts, since test organisms are less exposed to ammonium.

Concerning electrical conductivity, we did not observe the substantial increases with composting reported in other studies (**Wang et al. 2004**). In addition, we did not observe a decrease in pH (**Tiquia and Tam 2000, Huang et al. 2004, Miaomiao et al. 2009**), but an increase, in accordance with **McKinley and Vestal (1985)**, who attributed this pattern to an increase in ammonia content, also observed in our study. According to **Cai et al. (2007)**, the increase with composting time in the relative concentrations of non-degradable pollutants, like heavy metals or persistent organics, is the dark side of a process which generally improves the quality of sludge as an organic matter amendment. This increase in such pollutants is due to the loss of mass with decomposition, but also a decrease can be observed if leaching occurs (**Amir et al. 2005, Oleszczuk (2007**). In our study, some heavy metals concentrations rose significantly with time of composting, but the increase was not significantly correlated with time of composting. Concerning organic pollutants, the concentrations of persistent organic pollutants were below the detection limits (PCB) or remained stable (PAH and DEHP). These results contradict the slight degradation of PAH reported for sludge composting in some studies (**Lazzari et al. 2000, Hua et al. 2008**), as well as the significant degradation of DEHP shown in other studies (**Abad et al. 2005, Gibson et al. 2007, Das and Xia 2008**). On the contrary, the less persistent organics (LAS and NPEOs) decreased considerably with composting time, and as already reported by other authors (**Abad et al. 2005, Sanz et al. 2006, Gibson et al. 2007, Das and Xia 2008, Pakou et al. 2009**), but in our case this effect was only significant with nonylphenol.

4.3. Variation in ecotoxicological properties along composting and the influence of compost properties

The noxious effects of composts reported in the literature have been mainly related to the low organic matter stability of immature composts. Hence, it has been shown that phytotoxicity generally increases with composting time (**Bernal et al. 1998, Ramírez et al. 2008b, Miaomiao et al. 2009**). Also **Huang et al. (2004)** linked specifically the inhibition of *Lepidium sativum* germination with DOC, ammonia and the C/N ratio. The toxic effect of immature composts has been specifically linked to their high decomposition activity in soil through (1) a retard in plant growth due to nitrogen starvation (immobilization in soil as microbial biomass) associated to organic substrates with high C/N ratios, which promote the use of soil nitrogen by microorganisms (**Bernal et al. 1998**); (2) the direct toxicity of chemicals, mainly ammonia (NH_3) and short-chain organic acids, but also soluble salts, released in significant amounts with decomposition of wastes with high C/N ratios (**Kapanen and Itävaara 2001, Huang et al. 2004, Ramírez et al. 2007a, Ramírez et al. 2007b**); (3) the anaerobic conditions in potting media due to O_2 consumption (**Brinton and Evans 2001**); or (4) nitrogen immobilization in soil caused by the immature compost (**Bernal et al. 1998**).

The results from our study are consistent with these studies, since germination increases with degree of composting in the highest dosages tested (20 and 50 g kg^{-1}), as shown by the RM-ANOVA and the PCA analysis. This is reflected in **Figure 1**, where germination is clearly inhibited at all dosages of uncomposted sludge. However, this was more apparent in common mustard, and less clear in ryegrass, where the PCA failed to show any association with composting degree. The lack of clear results for ryegrass might agree with other studies that have indicated the low sensitivity of seed emergence for this purpose (**Benito et al. 2003, Brewer and Sullivan 2003)** compared to root length **(Kapustka and Reporter 1993, Fuentes et al. 2006, Oleszczuk 2008).** On the other hand, our results disagree with studies that have shown increased phytoxicity for germination as composting advances, something that has been related to increased soluble salts contents (**Brewer and Sullivan 2003**) or increased concentrations of persistent pollutant contents due to the mass losses with decomposition (**Oleszczuk 2008**).

Regarding the effects of composts on soil invertebrates, most of the studies available in the literature have been carried out in field conditions, while laboratory bioassays have been rarely used. Semi-field and field studies have generally shown stimulatory effects of compost applications on soil invertebrates. In a microcosm experiment with introduced mesofauna, **Larsen et al. (2007)** reported increased abundance of enchytraeids and microarthropods with amendment by anaerobically digested sludge and municipal waste compost, as compared to controls. However, enchytraeid abundance was higher in digested sludge than in compost due to the former's more labile compounds, bacteria and microfauna. Conversely, microarthropod abundance was higher in soil amended with composted waste than in that amended with dewatered sludge. In a field study, **Reinecke et al. (2008)** reported that in an organically managed vineyard, which involved the application of compost as fertilizer, there were increased feeding activities of soil invertebrates, measured according to the bait-lamina method, in comparison to a conventional management. They also found this trend in a microcosm experiment.

As regards laboratory studies, in a previous study we reported that composted sludge decreases toxicity compared to the parental sludge using earthworms (*E. andrei*), enchytraeids (*Enchytraeus crypticus*), and collembolans (*F. candida*), and that toxicity increased with decreasing organic matter stability and with increasing ammonia contents of the wastes (**Domene et al. 2008**). The only exception to this pattern was collembolan reproduction.

In the current study, earthworm survival was unaffected by the compost dosage and its degree of composting, in accordance with studies that have used the closely related species *Eisenia foetida* for sludge vermicomposting (**Domínguez et al. 2000**). Despite of that fact, we found effects on survival in *E. andrei* in a previous study at very higher sludge and compost dosages (**Domene et al. 2008**). On the other hand, earthworm total biomass was higher in soil amended with sludge composts compared to controls, with generally the highest values occurring in the highest dosages of uncomposted sludge, as also shown by the PCA analysis. This pattern seems to contrast with that reported by **Moreira et al. (2008**) and **Domene et al. (2008)** who found no significant changes in individual fresh weight compared to controls at two different dosages of sludge compost (6 and 12 tons ha^{-1}). Concerning earthworm reproduction, different patterns were observed depending on the composting procedure. In Blanes compost, higher reproduction was observed in uncomposted sludge, and lower outcomes were found in mature composts, decreasing below the control outcomes at the highest dosages. This was observed both at low and high dosages. However, in Manresa composting, reproduction increased compared to the controls irrespective of the degree of composting. This might be related to the reduced release of decomposition endproducts in Manresa composting and thus the likely reduced toxic effect of uncomposted sludge compared to Blanes composting.

Concerning collembolans, survival was also not affected by compost addition, therefore in accordance with **Moreira et al. (2008)** who did not find significant effects on survival in this species in comparison to control soil at 6 tons ha^{-1}, although they did find inhibition at 12 tons ha^{-1}. However, in previous studies, we reported survival inhibition at higher dosages, higher in sludge than in its corresponding compost (**Domene et al. 2007**).

On the other hand, both in Blanes and Manresa compost, collembolan reproduction was inhibited by uncomposted sludge, but the PCA analysis only indicate this point clearly in Manresa compost. This trend is also in agreement with the previous study mentioned (**Domene et al. 2007**). Regarding the effect of dosage, there is inhibition in both composting procedures, less clear in Manresa compost but still significant, agreeing with **Moreira et al. (2008)**, who reported a decrease in reproduction with the addition of sludge compost at a dosage of 12 tons ha^{-1}.

CONCLUSIONS

Composting involved a substantial decrease in total organic matter, C/N ratio, and an increase in total nitrogen content, nitrate, phosphorus and potassium, indicating a clear increase in the degree of composting and its stability. On the other hand, there was an increase in pH and the relative content of some heavy metals due to the mass loss with decomposition. There were no significant changes in the electrical conductivity nor in non-persistent pollutants (DEHP and PAH), while the non-persistent organic pollutants were substantially lost (LAS and NPEO).

The association between the low organic matter stability of composts and noxious effects on soil organisms found in the literature has been generally confirmed in our study, with the exception of earthworm species tested, which were favoured in uncomposted sludge.

The results from this study indicate the usefulness of bioassays for the assessment of the quality and ecotoxicological risks of wastes, since different outcomes were obtained in two similar composting procedures. In addition, we also found that the different organisms used in the bioassays have specific responses to composting degree, in that earthworm biomass and reproduction are favoured by uncomposted materials, while seed emergence and collembolan performance were inhibited by these materials.

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Composting phase	Manresa WWTP	Blanes WWTP
Initial mixture	0	0
Intermediate termophilic phase	7	-
End of the termophilic phase	14	28
Intermediate maturation stage (2 weeks)	28	42
Intermediate maturation stage (8 weeks)	70	84
End of the maturation stage (12 weeks)	98	112

Table 1. Composting stages and sampling dates expressed as days of composting.

WWTP	Time of composting	pH	Electrical conductivity	Organic matter	Total N	N-NH_4	N-NO_3	C/N	P	K
	days		*dS m^{-1} 25ºC*	*%*	*%*	*%*	*mg kg^{-1}*		*%*	*%*
Blanes	0	6.7	7.06	66.3	2.49	0.92	25	13	2.73	0.28
	28	7.1	8.02	54.2	2.36	1.25	60	12	3.57	0.35
	42	7	8.54	51.7	3.43	1.51	55	7.5	4.1	0.4
	84	7.2	9.31	50.2	3.26	1.54	180	7.7	3.92	0.38
Manresa	0	6.2	3.26	80.2	2.18	0.59	25	18	0.94	0.32
	7	7	4.18	67.1	2.21	0.8	30	15	1.44	0.38
	14	7.2	4.07	62.2	2.93	0.81	310	11	1.69	0.39
	28	7.2	4.25	59.2	2.94	0.78	705	10	1.83	0.4
	70	7.2	4.05	53	3.06	0.84	795	8.7	2.14	0.38
	98	7.1	4.28	59.5	2.99	0.89	1438	9.9	1.84	0.43

Table 2. Physico-chemical properties of the composts along the composting process. All the values are expressed as dry weight with the exception of ammonium, expressed as a percentage of the fresh weight.

WWTP	Days of composting	Cd	Cr	Cu	Hg	Ni	Pb	Zn	DEHP	PAH	PCB	LAS	NPEO
Blanes	0	1	59	331	1.12	28	40	616	45.3	0.5	<0.1	5386	124
	28	1	130	288	1.31	23	41	775	-	-	-	-	62
	42	1.1	124	338	1.08	26	52	838	-	-	-	1191	36
	84	1.2	112	358	1.24	30	59	840	-	-	-	-	27
Manresa	0	0.5	41	127	0.46	20	23	361	27.3	0.7	<0.1	1127	83
	7	0.6	54	125	0.83	21	25	473	-	-	-	910	66
	14	1	70	200	1.19	32	43	768	-	-	-	832	73
	28	0.9	63	186	1.15	28	39	648	-	-	-	651	41
	70	1.1	88	215	1.24	29	52	836	-	-	-	794	42
	98	0.8	66	181	1.19	24	37	697	14.2	1.3	<0.1	617	38

Table 3. Pollutant content of the composts along the composting process. All the values are expressed as dry weight in mg kg^{-1}.

WHC	**pH**	**EC 25ºC**	**Sand**	**Silt**	**Clay**	**C**	**N**	**C/N**	**CEC**	**Cd**	**Cu**	**Cr**	**Ni**	**Pb**	**Zn**
%		dS m^{-1}	%	%	%	%	%		meq(+)/100g	ppm	ppm	ppm	ppm	ppm	ppm
55.3	8.3	0.2	36.4	44.9	18.7	2.63	0.18	14.6	13.9	<0.1	121	25	19	35	104

Table 4. Soil properties of the soil used in the experiments; *WHC* = water holding capacity, EC = electrical conductivity, OM = organic matter, OC = organic carbon, N = total nitrogen.

Species/Endpoint	*B. rapa* germination	*L. perenne* germination	*E. andrei* survival	*E. andrei* biomass	*E. andrei* reproduction	*F. candida* survival
L. perenne **germination**	0.487**					
E. andrei **survival**	0.009	0.047				
E. andrei **biomass**	0.007	-0.040	0.651**			
E. andrei **reproduction**	0.090	0.191**	0.411*	0.206**		
F. candida **survival**	0.110	0.058	0.059	0.128	0.142*	
F. candida **reproduction**	0.232**	0.273**	-0.040	-0.068	0.117	0.014

Table 5. Pearson's correlation coefficients between the outcomes of the different species and endpoints using the log-transformed values. Significant correlations are indicated by (*) p<0.05, (**) p<0.001.

	Low compost dosages		High compost dosages	
	Component 1	*Component 2*	*Component 1*	*Component 2*
B. rapa germination	0.129	-0.137	0.382*	-0.021
L. perenne germination	0.092	0.049	0.217	-0.034
E. andrei survival	0.239	0.222	0.145	0.297
E. andrei biomass	-0.028	-0.129	-0.382*	-0.152
E. andrei reproduction	-0.439*	-0.288	-0.706*	-0.438*
F. candida survival	0.171	0.129	0.028	0.030
F. candida reproduction	0.227	0.279	-0.117	-0.221

Table 6. Pearson's correlation coefficients between the PCA factor scores for each component and the outcomes of the bioassays at low (1.2 and 4 g kg^{-1}) or high (20 and 50 g kg^{-1}) compost dosages in Blanes WWTP. (*) $p<0.05$, (**) $p<0.01$.

	Low compost dosages		High compost dosages	
	Component 1	*Component 2*	*Component 1*	*Component 2*
B. rapa germination	0.045	0.098	0.335*	0.292*
L. perenne germination	-0.143	-0.176	0.092	0.123
E. andrei survival	-0.153	-0.218	-0.107	-0.128
E. andrei biomass	-0.127	-0.025	-0.290*	-0.272
E. andrei reproduction	0.267	0.319*	0.187	0.188
F. candida survival	0.030	0.080	-0.218	-0.148
F. candida reproduction	-0.205	-0.174	0.307*	0.385*

Table 7. Pearson's correlation coefficients between the PCA factor scores for each component and the outcomes of the bioassays at low (1.2 and 4 g kg^{-1}) or high (20 and 50 g kg^{-1}) compost dosages in Manresa WWTP. (*) $p<0.05$, (**) $p<0.01$.

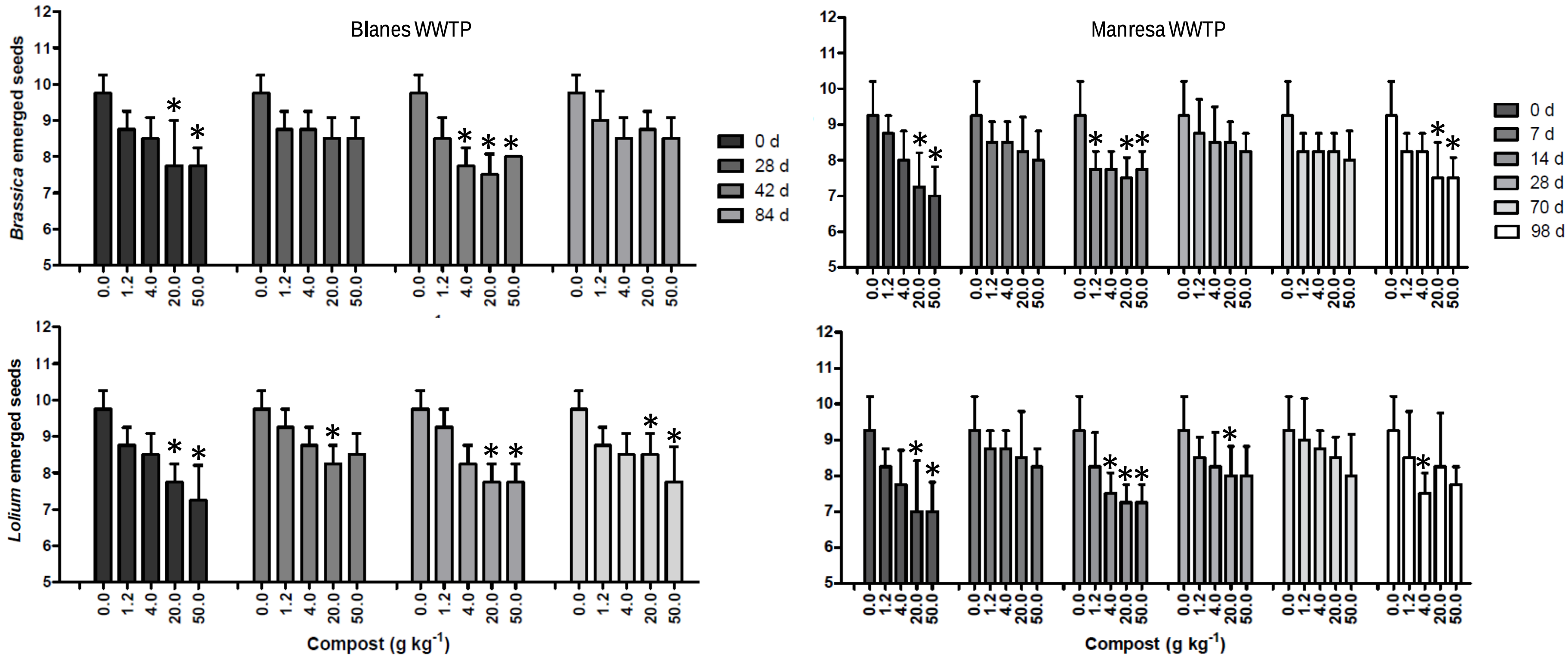

Figure 1. Seed emergence in the plant species *Brassica rapa* and *Lolium perenne* when exposed to increasing concentrations of sludge composts at different degrees of composting, obtained from two different WWTPs. (*) indicates significant differences compared to controls (Bonferroni, $p<0.05$) at a specific composting time; n=4.

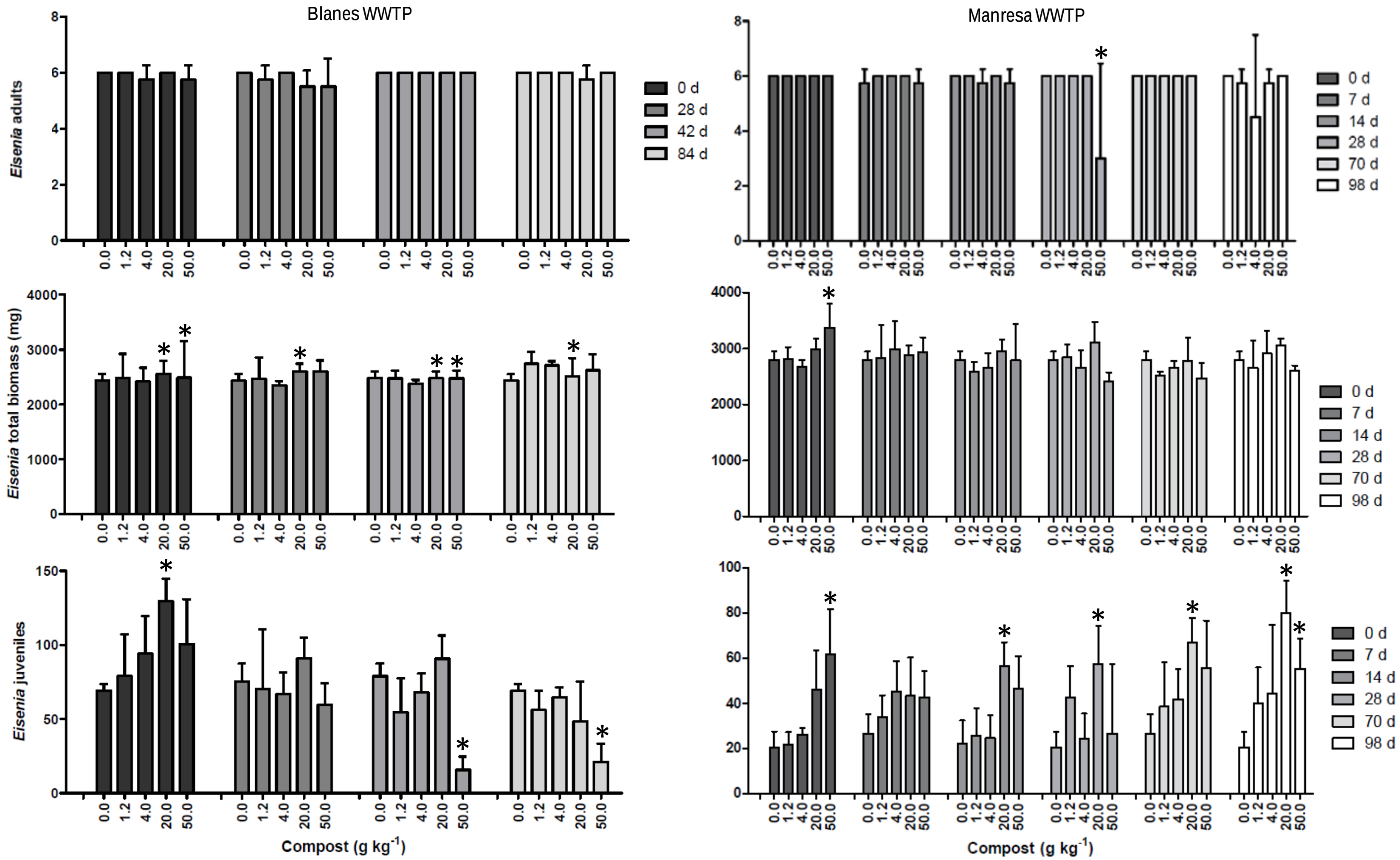

Figure 2. Survival, biomass and reproduction of the earthworm *Eisenia andrei* when exposed to increasing concentrations of sludge composts at different degrees of composting, from two differents WWTPs. (*) indicates significant differences compared to controls (Bonferroni, p<0.05) at a specific composting time; n=4.

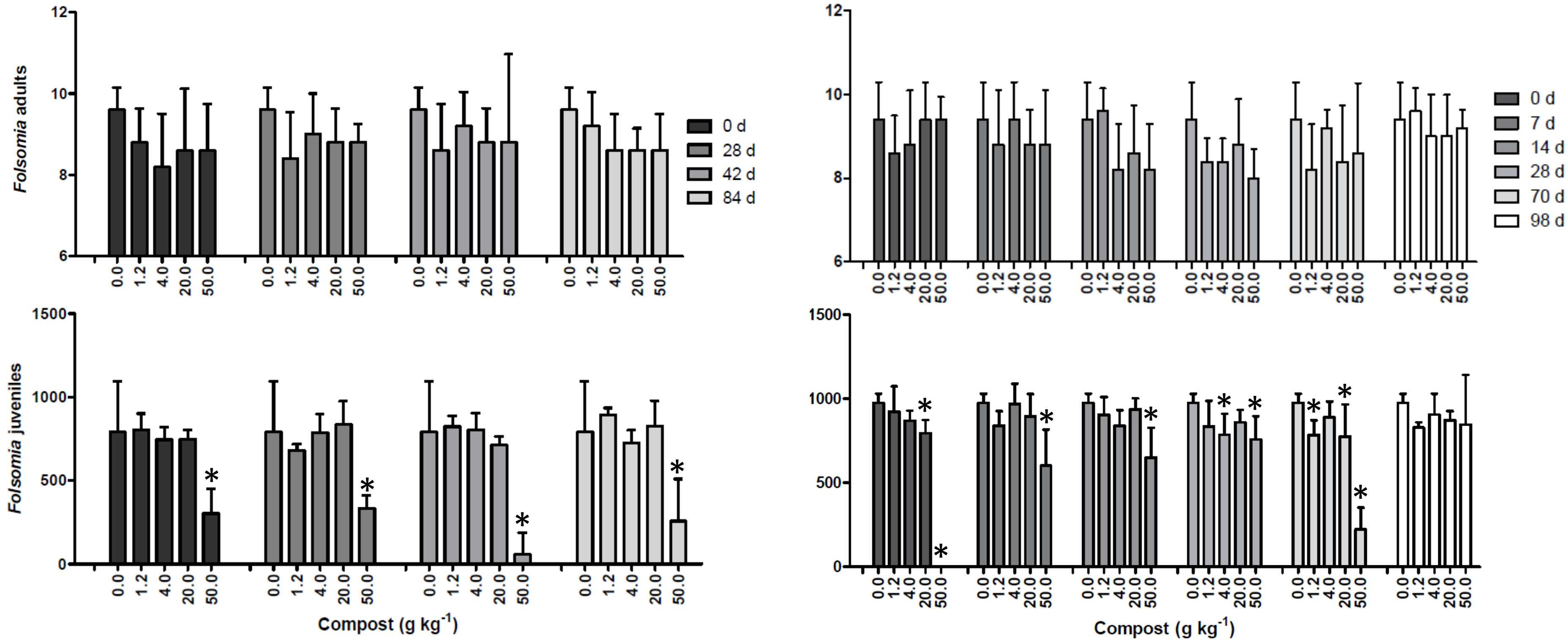

Figure 3. Survival and reproduction of the collembolan *Folsomia candida* when exposed to increasing concentrations of sludge composts at different degrees of composting, from two different WWTPs. (*) indicates significant differences compared to controls (Mann-Whitney, p<0.05) at a specific composting time; n=5.

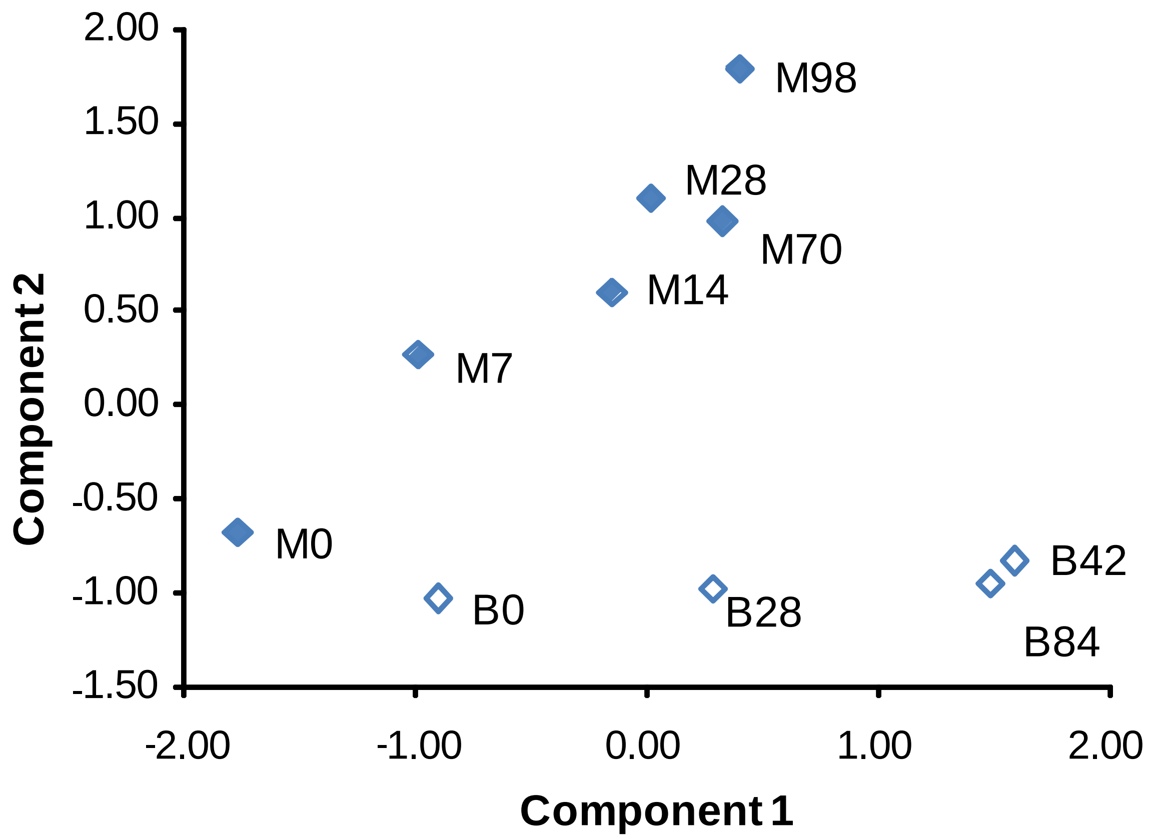

Figure 4. PCA factor scores for the two main components summarizing the physicochemical properties of the composts, arranged by origin (B=Blanes compost, M=Manresa compost) and by days of composting (0 to 98 days).