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**Application of standard statistical methods in the analysis of complex data generated from soil bioassays to assess the impacts of agrochemical-containing sludge amendments**

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**Abstract**

The use of sludge as soil amendment is widely encouraged by its high contents in organic matter and plant nutrients. Nevertheless, agrochemicals potentially present in sludge could be harmful to terrestrial ecosystems. The present work aimed to apply for suitable assessment of the ecotoxicological impacts of sludge amendments on soil, involving the following factors: the type of treated sludge, their application dose, and their contents in agrochemicals. Terrestrial Model Ecosystems (TMEs) were used to assess the effects of sludge amendments on endpoints from different trophic levels of the soil ecosystem, including an *in vitro* estrogenic bioassay on soil leachates. Here we show the significant negative effects of the highest dose of sludge in most of the soil bioassays. Thermally dried sludge increased significantly the microbial activity leading to lower contamination of leachates with endocrine disrupting molecules. Agrochemicals contents of sludge have only significant impacts on

increasing the delay of germination of plant seeds. Soil bioassays are thus complementary to sludge chemical analysis when the impacts of its application on soil should be assessed: significant negative impacts were related to the intrinsic composition of sludge rather than its agrochemicals contents. We conclude that standard statistical methods are relevant tools for the analysis of complex data generated from this type of experiments.

**Keywords:** terrestrial model ecosystem (TME), agrochemicals, soil, sewage sludge, bioassay, chemometric methods, PCA

## 1. Introduction

Sewage sludge is widely used as a soil amendment on productive (agriculture or forestry) land, because of its valuable contents in plant nutrients and organic matter. These contents have beneficial effects on soil aggregate stability, bulk density, water retention characteristics, soil functioning and soil biological activities (Dar 1997; Elsgaard et al 2001; Fließbach et al. 1994; Levi-Minzi et al. 1985; Tyler 1982). Unfortunately, sludge value is somewhat diminished by its content of potentially harmful substances including heavy metals, persistent organic pollutants and possible pathogens (Brandli et al. 2003; Fjällborg and Dave 2004; Helaleh et al. 2005; Mena et al. 2003). The occurrence of these pollutants in sewage sludge depends on both waste waters characteristics and treatment processes (Smith et al. 1998).

To avoid environmental and human health risks of sewage sludge application in agriculture, the EEC Directive 86/278 sets concentrations limits for seven heavy metals and some persistent organic pollutants such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls in sludge (Council 1986). For sustainable development, this directive is in process of revision to set concentrations limits for additional contaminants that may be found in sludge (EU 2000).

We developed previously analytical methods for monitoring the hazards of a range of chemicals in sludge, including the herbicides glyphosate and diuron, the nonionic surfactant nonylphenol, and some of their major degradation products (Ghanem et al. 2007 a; Ghanem et al. 2007 b; Ghanem et al. 2008 ; Granelli and Branzell 2007 c; Llompart et al. 2003; Petrović and Barcelò 2000). The aforementioned herbicides are widely used in urban areas for grass control or biocidal applications, whereas nonylphenol degrades from industrial/agricultural detergents and is used as formulating agent for pesticides (Hernandez-Raquet et al. 2007). The presence of these contaminants in sludge has been previously demonstrated and concentrations have been found to be of 1 mg.kg<sup>-1</sup>, 25 µg.kg<sup>-1</sup> and 100 mg.kg<sup>-1</sup> as dry weight

mean values, for glyphosate, diuron and nonylphenol, respectively (Ghanem et al. 2007 a; Ghanem et al. 2007 b). Furthermore, these contaminants are mobile and can be transferred to soil leachates and higher plants, when brought in mixture into soil *via* sludge depending on the sludge type as we demonstrated in our previous study during a 3-month incubation period (Ghanem et al. 2006). They are considered as endocrine disruptors exhibiting either estrogenic or anti-androgenic activities (Petit et al. 1997; Richard et al. 2005; Thibaut and Porte 2004).

Therefore, in-depth knowledge is required regarding the environmental, ecotoxicological, as well as health impacts of present practices of sewage sludge use on land (Abad et al. 2005; EU 2001), with a consideration of the agrochemicals contents of sludge. The ecotoxicological impacts of such pollutants should be assessed with attention to the type/origin of sludge, because the level of contamination can differ from a Wastewater Treatment Plant (WWTP) to another (municipal sewage sludge or industrial one, or sludge from storm- and run-off waters) (Alvarenga et al. 2008; Calace et al. 2005; Fent 1996; Ghanem et al. 2006; Rosa et al. 2007). Recent studies (Roig et al. 2012) shows that sludge stability (conditioned by sludge treatment) has high influence on sludge ecotoxicity. Ecotoxicological studies often focus on single species, with the disadvantage of predicting inadequately the effects of a chemical on natural systems, since they are far more complex than any laboratory testing system (Crouau et al. 2002). Despite that, Terrestrial Model Ecosystems (TMEs) are likely to assess more properly the effects of pollutants on soil and offer useful information to identify and focus on important variables (bioassays) to be monitored and studied; few studies are available using this approach (Kools et al. 2009; Weyers and Schuphan 1998). The main disadvantages of this approach are the difficulty in carrying out such complex experiments with numerous ecotoxicological tests and the complex analysis of the data generated.

Due to the complexity of this kind of experiments, the data generated should be deeply and thoroughly explored with the appropriate statistical techniques, in order to extract efficiently

most of the information lying within (Golobocanin et al. 2004; Kools et al. 2009; Skrbic and Đurisić-Mladenović 2007; Roig et al. 2012). The analysis of variance (ANOVA) using the suitable general linear mixed model (GLM) offers an effective method to assess the influence of different factors on the characteristics of TME using a reduced number of replications. Like in most ANOVA or GLM analysis the factors or independent variables are considered as fixed while the replications are considered as random. GLM with a combination of both fixed and random factor are considered as mixed models. A similar model was used in the present study. The principal component analysis (PCA) as a multivariate analytical tool is used to reduce a set of original variables by extracting a small number of latent factors (principal components). In order to interpret the significance of retained PCs in terms of the original end-points variables in TMEs, an analysis of correlations between bioassays outcomes and the principal components obtained will be carried out. According to many studies, principal components should account for approx. 75% of the total variance. The application of varimax rotation of standardized component loadings enables to obtain a clearer system as a result of the maximization of component loadings variance.

This work aimed to apply standard statistical methods in order to assess complex terrestrial ecotoxicological impacts of sludge-amended soil in TMEs. Three main factors were studied and are: the type/origin of sludge, the dose of amendment, and the sludge contamination with the mixture of agrochemicals, namely glyphosate, diuron and nonylphenol. The present study includes a multispecific approach based on different trophic levels: decomposers (litter-bag assay and phospholipidic fatty acids measurement), producers (growth performance of radish and wheat) and biomass consumers (reproduction and mortality of springtails). An *in vitro* estrogenic bioassay (MELN cells) was also performed to assess the estrogenic activity of soil leachates.

## **2. Material and Methods**

### ***2.1. Chemicals and Reagents***

All chemicals and solvents were of analytical grade. N-phosphonomethylglycine (glyphosate) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) (99.5 and 99.0% purity, respectively) were purchased from Dr. Ehrenstorfer (GmbH, Germany). 4-n-Nonylphenol (purity > 98.0%) was obtained from Lancaster, France.

### ***2.2. Sampling of sewage sludge***

Sewage sludge samples were collected at the end-point from urban plants in the vicinity of Versailles (France), in two WWTPs: Plaisir (thermally dried sludge, DS), Elancourt (limed sludge, LS), and one composting unit: Gazeran (composted sludge, CS). All the three WWTPs apply treatment processes including screening, grit removal, primary sedimentation with use of chemical coagulants, phosphorus and nitrogen elimination and conventional activated sludge treatment. The plants of Plaisir and Elancourt were each connected to a separate sewer system (SS) and an urban catchment area with moderate industrial activity (MI). The WWTP of Plaisir provided thermally dried (pelleted) sludge, whereas sludge treatment was obtained by liming at 30 % w/w in Elancourt (table 1). In the unit of Gazeran, wastewaters were collected by several sewer systems, mainly of the combined type (CS). Sludge was originated from several WWTPs, located in a residential/rural area with a mixture of agricultural (breeding of cattle) and industrial activities (AI). Sludge was then composted with wood chips as a bulking material.

From each WWTP, sewage sludge was collected in glass bottles, immediately after the final treatment. Samples were transported directly to the laboratory, where they were mixed to be homogenized. Afterwards, the samples were stored in closed glass bottles at +4 °C until use.

### ***2.3. Characterization of soil and sludge samples***

The soil was a silt loam collected in the 10-20 cm layer of a field in Versailles. It comprises 28.4% sand, 53.4% silt and 18.2% clay. Its contents in organic carbon, total nitrogen and CaCO<sub>3</sub> were 1.19, 0.12 and 1.26% respectively. Soil pH<sub>wat</sub> was 8.1 and its cationic exchange capacity was 14.4 cmol<sup>+</sup>.kg<sup>-1</sup>. The soil was roughly homogenized and immediately used for TMEs filling.

Based on the results obtained in our previous study (Ghanem *et al.* 2007 a), the characteristics of WWTPs and sludge samples, as well as their contents in glyphosate, diuron and nonylphenol are reported in Table 1 (Ghanem *et al.* 2007 a; Ghanem *et al.* 2008 ).

#### ***2.4. Terrestrial Model Ecosystem (TME) design and sludge amendments scenarios***

Experiments were conducted in TMEs (50 cm diameter, 40 cm height) filled with 40 kg of fresh soil. The TMEs were designed in order to mimic several sludge amendments scenarios on agricultural soil (figure 1). Soil in each assay was supplemented with one of the three type of sludge (thermally dried, composted or limed) to assess the impact of sludge composition - first factor- on soil ecosystem, compared to the non-amended soil as reference (figure 1a). Since the European States set maximum allowed agricultural sludge dose to be applied on soil as amendment, the sludge dose was included as a second factor that can have effects on the end-points bioassays in the TMEs. Two application doses were considered: the 6 tons (T) dw.ha<sup>-1</sup>, as a usual dose of sludge amendment in France, and the 30 tons (T) dw.ha<sup>-1</sup>, as a worst case of massive sludge amendment. The third factor studied was the agrochemicals contents in sludge, since glyphosate, diuron and nonylphenol have been found in the native studied sludge (table 1, (Ghanem *et al.* 2007 a)). In addition, some sludge samples were spiked with the mixture of glyphosate, diuron and nonylphenol at doses of 6, 17 and 7 µg.g<sup>-1</sup> dw, respectively (figure 1b). These amounts were chosen to ensure concentrations of each compound equivalent to these used by Ghanem *et al.* (2006) for experiments dealing with the fate of agrochemicals in soils, and depended on the specific activity values of the

radiochemical tracers. The glyphosate was brought into samples *via* water solution, whereas diuron and nonylphenol were brought *via* methanol solutions. After methanol evaporation at room temperature under N<sub>2</sub>, sludge samples were left 3 days at 4°C under argon for aging. They were then applied onto the soil and mixed at the surface layer of 0-10 cm. The soil moisture was adjusted by adding pure water to 80% of its moisture holding capacity. The TMEs were incubated for three months under 16 h light at 25°C and 8 h darkness at 18°C. Moisture was checked every week in each TME by conductimetry and adjusted to 80% of their moisture holding capacity. After 45 days of incubation, water (3.9 l) was sprayed onto the soils to mimic 20-mm rainfalls and to allow recovery of leachates.

### ***2.5. Litter-bag assay***

The litter-bag assay was used to assess the breakdown of organic matter and therefore the effects of sludge and chemicals on the biological activity of the soil, in real conditions of exposition (Kula and Römbke 1998). In each TME, a litter bag (10 × 10 cm size, synthetic mesh material with a mesh size of 0.5 cm) containing 4.5 g of wheat straw was placed at mid-height of the mixture sludge/soil. After 3 months, the bags were removed; the remaining straw was dried and weighted. The percent of degraded litter gave an index of soil biological activity.

### ***2.6. Phospholipidic fatty acids measurements***

Phospholipidic fatty acids (PLFAs) analysis was used to monitor the fate of fungal and bacterial communities in the soil using a modified method of Bligh and Dyer (1959) (Bligh and Dyer 1959). Firstly, 2-g soil samples were freeze-dried and ground whereas higher plant materials and gravels were withdrawn. Fatty acids were extracted in a chloroform/methanol/citrate buffer, and then extracted by solid phase extraction on a silica gel. Apolar lipids, glycolipids and PLFAs were eluted by chloroform, acetone and methanol, respectively. Then, PLFAs were trans-methylated under mild alkaline condition to yield fatty

acid methyl esters, and analyzed by gas chromatography and mass spectrometry (GC-MS) with the fatty acid methyl ester C19:0, used as internal standard. The method has been validated in several case studies (Frostegård and Bååth 1996) including grassland or cropped soils, and then used in TME experiments.

Samples were analyzed by GC-MS with a mass spectrometer (ion trap Saturn II, Varian) equipped with a VF5-MS column (50 m, 0.20 mm i.d., FT = 0.33, Varian), and helium as a carrier gas (20 psi). The temperature program was: 120°C (1 min) to 310°C at 4°C.min<sup>-1</sup>. For semi-quantitative determinations, PLFA markers used as indicators of total bacterial populations were the terminally branched, cyclic, and monenoic fatty acids 15:0, i15:0, a15:0, i16:0, 16:1w9, i17:0, a17:0, 17:0, cy17, cy19. Specific fungal PLFA assessed was the 18:2 w6,9 (linoleic acid) (Federle 1986).

### ***2.7. Growth performance of higher plants***

Effects of factors in TMEs were assessed on radish (*Raphanus sativus*) and wheat (*Triticum aestivum*) growth. Ten seeds of each species were sowed 15 days after TMEs incubation. Several parameters were measured after 1 month (radish) and 2 months (wheat) of cultivation: rate of germination (expressed in %), delay of germination (expressed as delay index = X/Y, where X = delay in germination time over reference, and Y = germination time for reference) (Kaushik et al. 2005), biomass production (expressed the length of the seedlings and their dry weight after 24-h desiccation at 60°C).

### ***2.8. Survival and reproduction of springtails***

The test organism was *Folsomia candida* (Willem). Its breeding and synchronization, as well as the protocol for chemical testing, were performed according to published protocols (International Standards Organisation 1999) (Campiche et al. 2006). 28-Day reproduction tests were conducted in glass containers (diameter 4.5 cm, height 9.5 cm, with plastic covers closing tightly). Each container was filled with 30 g wet weight of sample, without

compressing the substrate. Ten juvenile springtails aged 10-12 days were introduced into each container. The containers were kept under high relative humidity (70-80%), constant temperature ( $20 \pm 2^\circ\text{C}$ ) and light (600 lux) with a light:dark cycle of 16h:8h. The containers were opened twice per week to allow aeration. Granulated dry yeast was used as food supply, 3 mg at the beginning of the test and 5 mg after 14 day.

Five replicate containers were used for the soil reference. Three replicates were used for each assay condition. Three additional test containers were prepared, two for pH measurement in the presence of Collembolan, and one for water content control without springtails. After 28 days, the adults and juveniles were recovered and counted to evaluate the effects of sludge and chemicals on the mortality and on the reproduction. For this purpose, the soils were poured into a vessel equipped with a sieve, placed on a second vessel, and heated in surface. After 48 h of extraction, 1-cm water was added in the lower vessel, and a picture of the surface of the water was taken with a digital camera. Pictures were transferred to a computer and the number of adults and juveniles was counted directly on the screen.

### ***2.9. In vitro estrogenicity bioassay: MELN bioassay***

Aliquots (50 mL) of the leachates obtained after 45 days of incubation were freeze-dried and dissolved in 2 ml of dimethyl sulfoxide (DMSO) to be tested on MELN cells. The MELN cell line, which enables the detection of compounds that bind to the estrogen receptor ERalpha, or interfere with its pathway, was generously provided by Dr. P. Balaguer (Balaguer et al. 2001; Balaguer et al. 1999). The line was developed from MCF-7 (Michigan Cancer Foundation -7) cells which express the estrogen receptor ERalpha that have been transfected with a plasmid containing the luciferase gene downstream from a minimum promoter and an estrogen-responsive element (Cargouet et al. 2007 ). Cells were grown in phenol red containing Dulbecco's Modified Eagle Medium (DMEM),  $1 \text{ g.l}^{-1}$  glucose supplemented with 10% v/v Foetal bovine serum, 1% L-Glutamine (200 mM) and 1% antibiotic Pen/Strep in a 5%  $\text{CO}_2$

humidified atmosphere at 37°C in 10 cm diameter plate. Three days before experiments, culture medium is replaced by phenol red-free DMEM supplemented with 10% dextran-coated charcoal treated fetal bovine serum (FBS). Calibration samples were prepared from a mother solution of 17β-estradiol in sterile DMSO for final concentration in the medium from 10<sup>-7</sup> to 10<sup>-13</sup> M.

Cells were seeded in order to obtain approximately 225 000 cells/ml of medium and distributed in 6 wells plates for a final volume of 2 ml. Samples dissolved in DMSO were added in order to avoid more than 0.1% of solvent in final volume in the wells and experiments were done in triplicate. Incubation was done for 20 h at 37°C with 5% CO<sub>2</sub> and 95% humidity.

After incubation, plates were placed on ice pellets, cells were rinsed with FBS and luciferase extracted with lysis buffer from luciferase assay kit (Roche 11814-036) and incubated on ice for 15 min. Cells were detached with a scrapper centrifuged at 4°C for 10 min at 30500 g and 50 µl of the solution was transferred in a tube read into automatic reader Luminometer Berthold Lumat LB 9507 during 15 seconds of integration after injecting automatically 100 µl of Luciferin from the Roche kit. Results are expressed as a percentage of the mean maximum value obtained for 17β-estradiol at 10<sup>-9</sup> M (100%). DMSO 5%/Milli-Q was taken as control.

### **2.10. Statistical analysis**

Statistical analysis performed in this study included descriptive analysis to determine the parameters summarizing each one of the observed end-point TME variables. The determination of Pearson coefficients of correlation to identify the significant relationship among all the studied variables was also carried out (Table 3). The ANOVA analysis and multiple mean comparisons (Duncan test) were performed to assess the effects of different factors (type of treated sludge, application dose and agrochemicals contents) on all variables (litter-bag decomposition, PLFA, plants germination and growth, *F. candida* reproduction and

mortality, luciferase activity of MELN cells). The Duncan was used as an post-hoc test when the ANOVA or GLM results indicate that at least one of the categories we are analyzing is different from the others. It was used only to establish subsets of the categories. Furthermore when the differences among the subsets of categories are highly significant, the results will not change depending on the test we may choose.

The PCA technique was used to reduce the set of the original variables and to extract a small number of latent factors (principal components). An analysis of correlation among the retained principal components and the original variables was carried out in order to associate biological significance to the PCs. The analysis of variance using the principal components as dependent variables was carried out to confirm the possible effects of some factors found in the classical ANOVA. All the statistical analyses were performed using the SPSS statistical software version 16.

### **3. Results**

#### ***3.1. Descriptive analysis of ecotoxicological variables***

The results of the descriptive analysis of the studied variables, corresponding to ecotoxicological endpoints, are shown in table 2. The effects were assessed on the growth performance of radish and wheat by measuring three main descriptors: rate of germination, delay of germination and production of biomass. These descriptors varied according to the species. It is obvious that the germination of wheat was higher (mean value 85.83 %) than this of radish (mean value 52.50 %). The minimum value for wheat germination (70 %) was close to the maximum one for radish (80 %). Furthermore, the mean value of the biomass was also higher for wheat, showing more suitable soil conditions for this specie, under the experimental conditions. Radish and wheat were chosen as plant models to determine the accumulation of contaminants by root absorption in the radish tubercle, and by root

absorption and translocation from the roots to the leaves for wheat. The physico-chemical properties (i.e. texture) of the soil limited the germination of radish seeds, thus providing low seedlings growth and biomass production. The silt loam soil used was not suitable for radish germination and growth since this plant species prefers sandy soil; this fact should be taken into consideration while interpreting the impacts of TMEs on radish descriptors.

PLFA analysis showed significant bacterial and fungal biomass in the TMEs, with an increase of the later consistent with the proliferation of fungi observed on several TMEs.

### ***3.2. Correlation coefficients among ecotoxicological variables***

Table 3 shows a significant correlation among all the observed variables. The results show that, as expected, a positive correlation between length and weight biomass of both plant ( $r=0.774$ ;  $p<0.002$ ). A positive correlation is also obvious for the percentage of germination between radish and wheat ( $r=0.624$ ;  $p<0.017$ ). A negative correlation is observed between the bacterial PLFA and the biomass of wheat expressed as length ( $r=-0.533$ ;  $p<0.050$ ). A negative correlation ( $r=-0.553$ ;  $p<0.04$ ) also is between the bacterial PLFA and the MELN test. As supposed, *F. candida* reproduction is positively and strongly correlated to the survival of adults ( $r=0.861$ ;  $p<0.000$ ).

### ***3.3. Effects of sludge amendments on ecotoxicological variables***

ANOVA results are shown in Table 4. The biological activity of soils was assessed through wheat straw decomposition using the litter-bag assay. Mesh size allowed the action of microfauna, mesofauna and macrofauna, e.g. earthworms. When dried sludge is applied, the activity of the decomposers was significantly decreased, compared to other types of sludge or to the non-amended soil ( $F=3.797$ ;  $p<0.05$ ). This inhibition of the biological activity of the soil can be explained by the supply of degradable organic matter from the sludge, which may compete with wheat straw and lead to a decrease in its decomposition. Whatever the sludge type is at the higher dose ( $30 \text{ T dw.ha}^{-1}$ ), inhibitory effects are observed ( $F = 20.859$ ;

$p < 0.002$ ). That effect may be due to an increase of the concentrated salts of the soil provided by sludge. The concentrated salts such as sodium chloride, and other soluble salt content of  $\text{Ca}^{2+}$  (mainly in limed sludge),  $\text{Mg}^{2+}$ , etc. enter to the sludge during the WWT either from the waste waters contents or after the addition of different coagulant/flocculants reagents during sludge treatment (Cai et al. 2010). This increase in the salinity at 30 T dw.ha<sup>-1</sup> decreases the biological activity of soil.

Regarding spiked samples, no significant difference was observed by the mean of the litter-bag test. It is therefore not obvious to outline a toxic effect of the indirect application of chemicals mixture through sludge.

Semi-quantitative data concerning bacterial and fungal biomass have been obtained using PLFA measurements. The stimulating effect of dried sludge on bacterial PLFA is clear ( $F = 8.200$ ;  $p < 0.012$ ). Globally, sludge addition stimulated the bacterial PLFA with no evident dose-response relationship with sludge or impact of studied chemicals. Despite that situation, a dramatic proliferation of fungal hyphae was observed at the soil surface in the presence of the dried sludge. However, the value for specific fungal PLFA was not the highest in that case, and the effects of all the studied factors remain no significant for this variable.

Otherwise, the effects of the three main factors (type of treated sludge, application dose and spiking with agrochemicals) were also assessed on the growth performance of radish and wheat. Table 4 shows that there is no significant effect of the type of sludge (DS, CS or LS) on the radish and wheat growth (three descriptors), except for the delay index of wheat ( $F=4.718$ ;  $p < 0.05$ ). The dried sludge dramatically decreases the DI of wheat germination, because of providing available organic matter and nutrients for plants. The DI, for radish and wheat, is also affected by the presence of pesticides in the TMEs. The delay index is increasing in spiked TMEs, vs non spiked TMEs, ( $F=4.950$ ;  $p < 0.05$  and  $F=4.718$ ;  $p < 0.05$ ) for radish and for wheat, respectively. Otherwise, the application dose of sludge affects the

growth of plants with inhibiting effects of the 30 T.ha<sup>-1</sup> dose of sludge applied compared to the 6 T.ha<sup>-1</sup>, on the germination percentage of radish (root vegetable with less favourable soil conditions) (F=6.900;  $p<0.03$ ), and on the biomass of plants correlated to the seeds germination. This result demonstrates the fertilizing potential of sludge application at low dose for radish and wheat growth. The delay index at high dose decreases, showing a faster germination of wheat, even if the biomass and germination rate are lower.

The effects of the studied factors were assessed on the reproduction and survival of *F. Candida*, as biomass consumers. Standardised requirements should be fulfilled to validate the carried out experiments: soil pH ( $6 \pm 0.5$ ), adult mortality (< 20 %), reproduction rate (100 %) and relative standard deviation (< 30 %). With the studied soil, these values were 7.8 for pH, 28 % for *F. candida* mortality, 87 % for reproduction rate with a relative standard deviation of 8 %. Nevertheless, these conditions were considered acceptable to develop the assays. It is also important to notice that salts and concentrations of ammonium nitrogen measured in the TMEs were lower than those able to induce deleterious effect on collembolla reproduction (data not shown). The spiking levels were below the EC50 values calculated concerning the reproduction of *F. candida*, which are 71 mg.kg<sup>-1</sup> dw for nonylphenol and 19.7 mg.kg<sup>-1</sup> for diuron (Campiche et al. 2006; Gejlsbjerg et al. 2001).

The reproduction and mortality of adults were not significantly modified by the addition of agrochemicals to the TMEs or by the type of sludge applied (Table 4). Nevertheless, the 30 T dw.ha<sup>-1</sup> of sludge applied to the soil decreases strongly the juvenile number (F=6.073;  $p<0.039$ ) and the adults survivals (mortality increase) (F=3.849;  $p<0.05$ ). The survival of *F. candida* seemed more sensitive to sludge addition than its reproduction. Negative effects of the high dose observed with LS could be related either to medium alkalinity (lime addition) or to the presence of toxic pollutants in the sludge; whereas in DS sludge, the mortality of *F. candida* was due to fungal proliferation observed in the soil-sludge mixture, which

immobilized collembolla and caused their indirect mortality. A pH decrease (0.5-1.3 units) was observed in TMEs where fungi proliferated after 1 month of incubation. The estrogenic potential of water used to moisten the TMEs was assayed on MELN cells. No significant response has been evidenced (Table 4) using the different leachates, whatever the dose of sludge application is or the TMEs are spiked or not with agrochemicals. However, significant effects were related to the sludge type (intrinsic contamination of sludge). For instance, estrogenic effects of the composted sludge were higher than these of the dried one. This was obvious with lower estrogenic effects of the leachates when dried sludge is applied *vs* composted one (table 4), probably because of the excess contents of organic matter provided by the DS, which stabilize more the agrochemicals and decrease their mobility (Ghanem et al. 2006).

### ***3.4. PCA analysis of ecotoxicological variables***

The PCA analysis resulted in two components explaining 73% (approx. 75%) of the total variation. The relationship between these two components and the analysed variables can be shown in Figure 2. The component 1 describes the biomass (weight and length) of radish and wheat, PLFA (bacterial and fungal), and the number of juveniles and adults survivals of *F. Candida*, with a positive loading. In the performed experiments, when the soil is fertile, both collembolan and plants will have more suitable conditions for their growth. The radish germination is also positively and significantly correlated with juveniles numbers of *F. Candida* ( $r=0.629$ ;  $p<0.016$ ) and can be also described by the component 1. MELN is negatively loaded on the component 1, and is negatively correlated to the last aforementioned parameters. This correlation agrees with the results of Table 3 ( $r=-0.553$ ;  $p<0.040$ ).

The component 2 shows a high, positive loading that occurred for litter-bag, wheat germination, radish and wheat delay index (Figure 2). The parameters are related to decomposers and producers of biomass that are well correlated ( $r=0.536$ ;  $p<0.05$  for litter and

delay index radish) and ( $r=0.716$ ;  $p<0.006$  for litter and delay index wheat) (Table 3). However, radish germination can be less obviously described by the component 2.

Figure 3 shows the scatter diagram using the two PCA factors. An obvious and clear separation can be noticed between the doses of sludge application: 6 and 30 T.ha<sup>-1</sup>, which is in agreement with the results obtained by ANOVA. The other scatter diagrams using the two PCAs factors do not show a clear discrimination between TMEs spiked and unspiked with agrochemicals. A classification by the effects related to the type of sludge was also not obvious (data not shown).

## **4. Discussion**

### ***4.1. Relevance of ecotoxicological assays for quality assessment of sludge amendment***

The present work shows that the analysis of physico-chemical properties and agrochemicals contents in sewage sludge is not enough to assess the quality of sludge amendment. All the sludge samples used in this study fulfilled the French and EEC Directive 86/278 requirements for sludge amendments. However, numerous and unpredictable pollutants can be present in sludge, depending on the waste waters composition, and waste waters and sludge treatments (Abad et al. 2005). Agrochemicals including glyphosate, diuron and nonylphenol have been determined in the studied sludge samples at  $\geq 0.8$  mg.kg<sup>-1</sup> dw for glyphosate,  $\geq 8.6$  µg.kg<sup>-1</sup> dw for diuron and  $\geq 78.9$  mg.kg<sup>-1</sup> dw for nonylphenol (Ghanem *et al.* 2007 a) (table 1). Since the analytical determination of pollutants is not a sufficient tool to provide suitable assessment of the sludge quality, complementary ecotoxicological assays should be performed (Domene et al. 2011; Moreira *et al.* 2008). When ecotoxicological bioassays are carried out using TMEs, the effects of the sludge amendments on soil are better assessed (Domene et al. 2011; Singh and Agrawal 2008) where the toxicity, concentrations, interactions among pollutants, and fate of pollutants (Dean and Suess 1985; Ghanem et al. 2006) are taken into consideration.

Despite that the studied sludge samples are allowed to be applied on agricultural soil, some negative effects were observed on litter-bag decomposition, radish germination, wheat delay index and biomass, *F. candida* reproduction and survivals (table 4). The application of statistical analysis is crucial in the determination of the significant effects and therefore of the most relevant bioassays for such studies. The effects of sludge can differ depending on the target organisms and their trophic levels in the TMEs. Our study is a first step to evidence ecotoxicological impacts on several species toward sludge amendment quality. Nevertheless, one of the challenges of ecotoxicological studies remains the assessment of impacts on communities, including interspecific relationships.

Length and weight biomass are positively correlated (table 3) showing that one of them can be used alone to assess impacts of the TMEs factors on plant growth. Plants growth, *F. candida* reproduction and survival are very well correlated (tables 3 and 4) and can be used as indicators of soil pollution (Domene et al. 2011; Murillo et al. 1995; Oleszczuk 2008). When soil fertility increases, more nutrients are available in the soil ecosystem, which is healthier for collembolan reproduction and growth. To assess soil health and therefore fertility, *F. candida* test could be sufficient if performed alone and expectation on radish and wheat growth (biomass producers) could be thus done based on the collembolan (biomass consumers) reproduction and survival. Impacts observed on radish and wheat (growth and delay index) were similar (tables 3 and 4) independently on the plant species; even if soil conditions were more suitable for wheat than radish germination. When available nutrient are provided to the soil, the soil fertility increases and is reflected by higher percentage of germination and better growth of both radish and wheat. Under these favourable conditions, nutrients are also available for the reproduction and survival of soil arthropods, used as bioassays of soil quality (Domene et al. 2007; Kaneda and Kanedo 2002). Table 3 shows a positive correlation between wheat biomass and *F. candida* juvenile number ( $r=0.551$ ;

$p < 0.041$ ) and between wheat biomass and *F. candida* survival ( $r = 0.584$ ;  $p < 0.028$ ). When high concentrations of toxic constituents or/and high salinity are introduced to the soil through sludge, soil fertility decreases. These hard conditions threaten in the same manner the *F. candida* reproduction and survival, the radish and wheat germination, and both plants growth (tables 3 and 4). The toxic organic matter decreases the biomass consumers (*F. candida*) survival and reproduction; less degradation of the organic matter consequently occurs leading to lower amounts of available nutrients for plants growth. Based on these results, soil pollution could be assessed by only one of these bioassays (collembolan or plants growth), as both bioassays are positively and significantly correlated.

Correlations between litter-bag and plant growth (table 3) can be explained by the fact that when the litter-bag decomposition increases, the soil activity is high and more organic matter is faster degraded, providing thus more available nutrients to the plant germination. The delay index increases, reflecting thus the faster germination of plants.

Negative correlation between bacterial PLFA and MELN test (and other parameters) (table 3 and figure 2) show that the increase in the bacterial population, leads to a decrease in the endocrine disruptors molecules contents and decreases therefore estrogenic potential of leachates obtained from the corresponding TMEs.

The negative impacts of the high dose of application of sludge ( $30 \text{ T} \cdot \text{ha}^{-1}$ ) were determined by most of the assays: litter-bag, *F. candida* reproduction and survival, and plants growth (table 4, figure 1). It is obvious that PLFA assays are not sufficiently informative regarding the quality of the amendment, since the negative impacts of the  $30 \text{ T} \cdot \text{ha}^{-1}$  were not observed on bacterial and fungal PLFA. The litter-bag might be more suitable assay than PLFA to assess the impacts of several factors on soil biomass decomposers. However, litter-bag assay has the disadvantage of requiring a long time of incubation (3 months) which is not practical for a routine assessment of sludge quality.

Besides the determination of the ecotoxicological impacts on terrestrial ecosystem, the *in vitro* estrogenic bioassay (MELN cells) was carried out on soil leachates (aquatic bioassay) (Loureiro et al. 2005), as a prediction of the potential contamination of the ground water with estrogenic molecules that are bioavailable and can interfere with the endocrine system in humans and animals. However, the MELN test on leachates was also not able to distinguish significant effects related to the sludge amendment dose. Only one negative correlation ( $r = -0.553$ ;  $p < 0.04$ ) was determined between MELN and bacterial PLFA that can be explained by the fact that when microbial activity increases, the degradation of the organic pollution and estrogenic compounds increases leading to low estrogenic activity in the leachates (table 3). NP has an EC50 value of 11  $\mu\text{M}$  (2.42 mg/L) with the MELN test (Pillon et al. 2005). When the mobility of the NP is reduced (formation of bound residues), the estrogenic potency of the leachates is also low. The agrochemicals (glyphosate and diuron) are also known to be endocrine disrupting compounds (McKinlay et al. 2008). In this study, the impacts of the agrochemicals are the global impacts resulting from mixture, taking into account the possible interactions/competition between glyphosate/diuron and nonylphenol. These impacts can be correlated with the fate of these agrochemicals under similar conditions, where higher microbial activity leads to higher bound residues formation and therefore to lower estrogenic activity of the leachates (Ghanem et al. 2006). No correlation between MELN test and any other bioassay was determined. The potential risks of the sludge amendment on groundwater quality cannot be predicted by terrestrial bioassays alone. Aquatic ecotoxicological test such as MELN assay should be carried out to provide the realistic assessment.

#### **4.2. Impacts of the sludge application dose**

Sludge benefits when applied on soil at low dose as organic fertilizer are well known (Singh and Agrawal 2008). However, sludge amendment dose should be controlled since it can have

negative impacts on soil ecosystem and disturb the soil properties (Domene et al. 2011). When the sludge amendment dose increases, the concentration of nutrients, organic content, but also agrochemicals and toxic constituents provided to the soil increase (Moreira et al. 2008). An increase in the biomass of plants has been reported in our study, as well as by other authors (Singh and Agrawal 2008) with low sludge amendment dose. By contrast, sludge amendment benefits decrease at higher doses (30 T.ha<sup>-1</sup>), and results (table 4, figure 3) show toxic effects including a decrease in the litter-bag decomposition, a decrease in the germination percentage of radish, a decrease in the plants biomass, an inhibition of the *F. candida* reproduction and an increase in their mortality (figure 2, table 4). The decrease in the litter-bag decomposition could be related to: i) increase in soil organic matter providing available nutrients to the microbial biomass; this compete with litter-bag decomposition, ii) increase in the toxic constituents contents in soil brought through sludge that reduce microbial biomass. Litter-bag is thus not a relevant assay to assess the ecotoxicity of sludge amendment. However, the decrease observed on plants germination and growth, as well as the inhibition of collembolan reproduction and the increase in their mortality are likely related to negative impacts of sludge amendment dose (Murillo et al. 1995). It is assumed that when high dose of sludge amendment is applied, the concentrations of nitrogen increase in the soil, and thus the available ammonium threatens collembolans life and inhibits their reproduction (Domene et al. 2010). However, the sludge toxicity observed in this study at 30 T.ha<sup>-1</sup> cannot be attributed to the ammonium concentrations, since they were within the acceptable range in all TMEs. The strong correlation of the effects of sludge amendments on *F. candida* with sludge doses is in agreement with the correlation described previously using other microarthropods species (Ai-Assiuty et al. 2000). A high content of toxic pollutants (e.g. polychlorobiphenyls, other agrochemicals, heavy metals, etc.) brought to the soil at the high sludge amendment dose is strongly suspected (Roig et al. 2012).

### ***4.3. Impacts of the sludge type***

Despite that authors (Andrés et al. 2011; Ramírez et al. 2008; Tarrasón et al. 2010) described the effects of sludge treatment on sludge amendment quality, the difference observed in the effects related to the type of sludge (dried, composted or limed) was less important than those related to the amendment dose. Significant difference was determined for bacterial PLFA between the dried sludge amendment and all the other factors (table 4). Thermal drying (at 110°C) is applied to stabilize sludge by eliminating water that is essential for microbial life. Only a low destruction of organic matter occurred by comparison to what happened when higher temperatures are applied. After soil amendment, and sludge re-hydration, the microbial activity seems to be very fast re-established. Similar statistical approach was applied in recent studies (Roig et al., 2012) involving five categories of treated sludge with different properties (28 different samples) and demonstrates that sludge treatment and therefore sludge stability might have high influence on sludge ecotoxicity. Bacterial PLFAs are therefore in higher amounts in the TMEs amended with the dried sludge as it was shown by the ANOVA results (table 4). In addition, dried sludge provides excess contents of organic matter to the decomposers in soil (table 1), and therefore the need to decompose the litter as nutrients source decreases. Similar observation was described in the literature (Andrés et al. 2011; Tarrasón et al. 2010), where organic matter and nitrogen levels decreased after a period of soil amendment with thermally dried sludge. This decrease is related to the increase in the microbial biomass, microbial basal respiration and microbial metabolic quotient (Tarrasón et al. 2010), compared to other types of sewage sludge. The stimulation of the microbial activity after dried sludge application can be likely explained by providing the soil with a lower percentage of stable organic matter than other sludge samples (Tarrasón et al. 2010).

The ANOVA results (table 4) concerning the collembolan bioassay did not show any significant effect that depends on the sludge type. Application of dried and composted sludge on soil did also not modify the total abundance of collembolans in others studies (Andrés et al. 2011). The most relevant effects were only related to the sludge dose on both reproduction and mortality of the *F. candida*.

Regarding the plants bioassays, only the wheat delay index was decreased when dried sludge is applied, showing an accelerated germination due to the availability of organic matter more easily decomposable (Marando et al. 2011). The effects of the type of sludge on the delay of germination are only observed on wheat, showing the importance of the choice of plant species to assess phytotoxic effects (Oleszczuk 2008).

The increase in the soil microbial biomass (bacterial PLFA) may lead to an increased activity due to the dried sludge application and contributes thus to a lower leaching of contaminants (including endocrine disruptors) to the ground water by increasing their degradation (Ghanem et al. 2006). The contamination of the leachates by estrogenic contaminants is likely related to the molecules coming from the wastewaters treated in Gazeran WWTP (parent or by-products), mainly because of the agricultural activities nearby. The mobility of studied chemicals is lower in TMEs with dried sludge than those with the composted one, but their levels ( $\mu\text{g.L}^{-1}$ ) in leachates are relatively considerable (Ghanem et al. 2006).

#### ***4.4. Impacts of the agrochemicals contents in sludge***

The agrochemicals (glyphosate, diuron and nonylphenol) contents in spiked-sludge have the lower impacts, compared to the two aforementioned factors. Agrochemicals contents in sludge increase only the delay index of plants germination as indicator of these molecules toxicity to the seeds (table 4). This effect may be due to the high level of the herbicide diuron present in the spiked sludge. It indicates that this assay (an indicator of early plant growth) is

a suitable tool for determining amendment sludge toxicity related to its agrochemicals contents. Once the seed germination occurs and plant emergence is observed, the germination percentage and plants growth are not significantly affected by the agrochemicals concentrations (Mosse et al. 2010). The degradation and mobility of the studied agrochemicals, after a period of time, might explain their lack of inhibiting activity on plant growth (Ghanem et al. 2006).

## 5. Conclusions

The study confirms the relevance of TMEs for short to mid-term ecotoxicological studies since they allow the impacts assessment of sludge amendments on soil that are variables depending on the biological species exposed. However, the standard statistical methods applied showed reliable correlations between the variables, providing thus useful information to select relevant bioassays for ecotoxicological assessment. Plant growth can be assessed by either length or weight biomass that are positively correlated. Plants growth, *F. candida* reproduction and survival are very well correlated and one of them can be used as indicator of soil pollution. Bacterial PLFA are negatively correlated to MELN showing that the first bioassay can predict the impacts of the TMEs on the estrogenic potential of the leachates.

Regarding the impacts of the three studied factors, the results obtained showed high negative impacts of the application dose on the biomass decomposers (litter-bag), producers (plant germination and growth) and consumers (*F. candida* reproduction and survival). The application dose of sludge amendment should be strongly controlled in respect to the EC regulations to avoid any abuse on soil ecosystem. The variability in sludge treatment and therefore composition has less important impacts than those related to the aforementioned factor. During 3-months incubation, the thermally dried sludge (Plaisir WWTP) increased the microbial activity of the TMEs, and showed lower estrogenic effects of the leachates

compared to the composted sludge (Gazeran WWTP). No specific effects of the agrochemical-contaminated sludge were outlined, except in an increase in the delay index for both radish and wheat, which is likely due to the high spiking level with diuron in the corresponding TMEs.

Altogether, the results showed that ecotoxicological studies are complementary to the analytical determination of a wide range of pollutants that are unpredictable, to assess the sludge quality and impacts. This conclusion is supported by the fact that the studied sludge are allowed to be applied on soil in respect to the EEC Directive 86/278, but negative impacts were observed in TMEs (e.g. estrogenic effect of leachates). More realistic environmental risk assessment requires in the future longer-term studies with the involvement of modeling of the ecosystem responses to the different factors in TMEs.

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Figure 1. Scenarios of sludge amendments assessed through designed TMEs to assess their impacts on soil ecosystem.

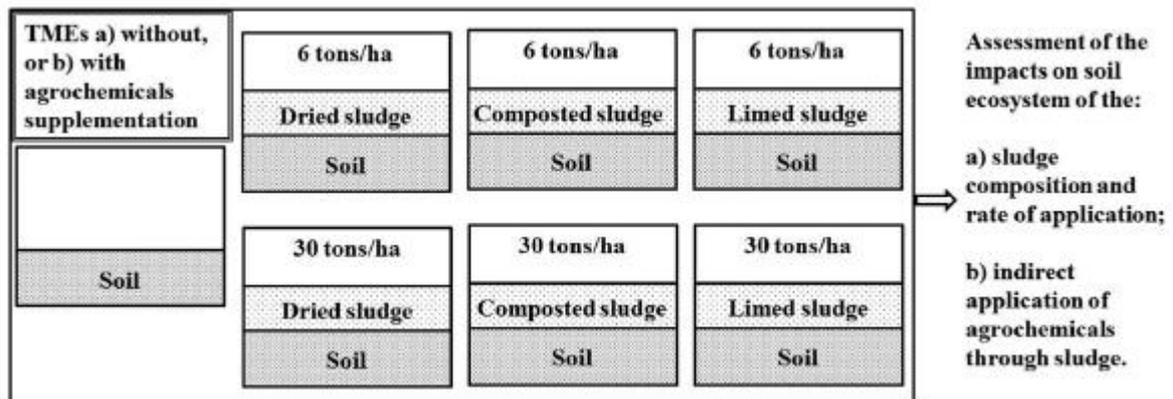


Figure 2. PCA analysis of the different ecotoxicological variables of agricultural soils in TMEs. Each test is designed by its abbreviation. For plant growth, each abbreviation is followed by R for radish, or W for wheat. PLFA is followed by either F for fungal or B for bacterial.

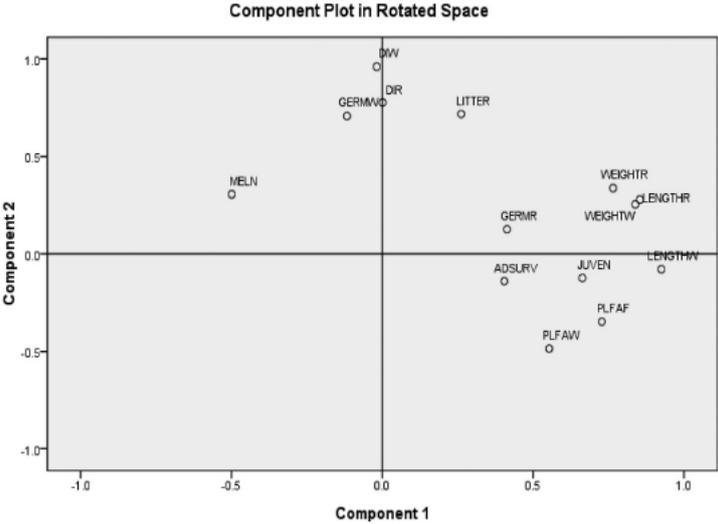


Figure 3. Scatter diagram showing the distribution of doses according to the factors obtained from PCA analysis.

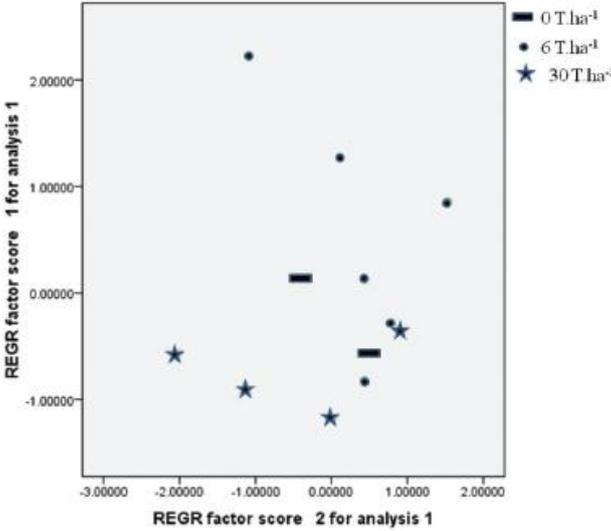


Table 1. Characteristics of wastewater treatment plants and sludge samples used in the study.

	Plaisir	Gazeran	Elancourt
Separate sewer system (%)	98	–	100
Catchment area	Urban with moderate industrial activities	Rural with agricultural and industrial activities	Urban with moderate industrial activities
Equivalent inhabitant connected	34,000	–	34,000
Treatment process	<ol style="list-style-type: none"> <li>1. Thickening of primary sludge</li> <li>2. Flotation of secondary sludge</li> <li>3. Anaerobic digestion (37°C) of combined sludge</li> <li>4. Centrifuge dewatering and addition of polymers</li> <li>5. Thermal drying at 110°C</li> <li>6. Compression to form pellets</li> </ol>	<ol style="list-style-type: none"> <li>1. Thickening of primary sludge</li> <li>2. Flotation of secondary sludge</li> <li>3. Mechanical dewatering of combined sludge</li> <li>4. Composting with wood residues (maturation during 15 days)</li> </ol>	<ol style="list-style-type: none"> <li>1. Thickening of primary sludge</li> <li>2. Flotation of secondary sludge</li> <li>3. Anaerobic digestion (37°C) of combined sludge</li> <li>4. Centrifuge dewatering and addition of polymers</li> <li>5. Treatment with lime (CaO) at 30% w/w</li> </ol>
Treated sludge	Dried	Composted	Limed
Organic carbon (g kg <sup>-1</sup> )	396	336	231
Organic matter (g kg <sup>-1</sup> )	685	582	399
Nitrogen (N) total (g kg <sup>-1</sup> )	70	41	37
C/N ratio	5.6	8.1	6.1
pH <sub>water</sub>	5.95	7.7	12.5
Dry weight %	94	75	30
Glyphosate (mg kg <sup>-1</sup> dw)	1.8	0.9	0.8
Diuron (µg kg <sup>-1</sup> dw)	8.6	28.0	11.4
Nonylphenol (mg kg <sup>-1</sup> dw)	78.9	173.2	102.5

Table 2. Descriptive statistics: mean, maximum, minimum and standard error for the analyzed variables.

Parameter	Mean	Minimum	Maximum	Standard error
Litter-bag (decomposition %)	61.67	36	71	2.77
Radish				
Germination (%)	52.50	20	80	5.38
Delay index*	0.67	0	1	0.09
Biomass (g)	0.019	0	0.052	0.005
Length (cm)	15.04	8	21	1.15
Wheat				
Germination (%)	85.83	70	100	3.78
Delay index*	0.77	0	1	0.07
Biomass (g)	0.124	0.039	0.35	0.02
Length (cm)	43.44	34.95	53.69	1.80
PLFA				
Bacterial	108.25	62	180	11.3
Fungal	1403.92	100	5666	457.74
(relative abundance)				
<i>F. candida</i>				
Reproduction (juvenile number)	74.23	2.0	145.0	12.36
Number of Adults survival	6.41	0.0	10.0	0.78
MELN				
Luciferase activity (luciferase units LU*10 <sup>-3</sup> )	3585	161	10559	765

Note: \*Delay index = X/Y, where X = delay in germination time over reference and Y = germination time for reference.

Table 3. Coefficients of correlation found among all the observed variables.

		Radish				Wheat				PLFA		<i>F. candida</i>		Luciferase activity (MELN)
		Delay index	Germination %	Biomass length	Biomass weight	Delay index	Germination %	Biomass length	Biomass weight	Bacterial	Fungal	Juveniles	Adults survivals	
Radish	Litter-bag	0.536*	0.656*			0.716**	0.564*	0.555*		-0.544*				
	DI					0.842**								
	Germination (%)						0.624*	0.683**				0.629*		
	Biomass (length)				0.774**		0.737**	0.861**	0.669*			0.616*		
	Biomass (weight)			0.774**				0.599*	0.795**		0.603*	0.600*		
Wheat	DI	0.842**					0.720**							
	Germination (%)		0.624*	0.737**		0.720**		0.758**		-0.894**		0.537*	0.576*	
	Biomass (length)		0.683**	0.861**	0.599*		0.758**		0.695**	-0.533*		0.551*	0.584*	
	Biomass (weight)			0.669*	0.795**			0.695**			0.714**			
PLFA	Bacterial						-0.894**	-0.533*					-0.563*	-0.553*
	Fungal				0.603*				0.714**					
<i>F. candida</i>	Juvenile		0.629*	0.616*	0.600*		0.537*	0.551*					0.861**	
	Adults survivals						0.576*	0.584*		-0.563*		0.861**		

Notes: \* Significant at 0.05.  
 \*\* Significant at 0.001.

Table 4. Results of the ANOVA analyses revealing the significant effects of factors (type of sludge, spiking with pesticides and application dose of the sludge) on all the observed variables. Results of multiple mean comparison using Duncan test are also shown.

Variables	Factors		
	Type of post-treated sludge	Application dose (T ha <sup>-1</sup> )	Spiking with agrochemicals
Litter-bag	3.797 <sup>ab</sup> (1)(2-0-3) <sup>d</sup>	20.859 <sup>c</sup> (30)(0-6)	NS
PLFA	Bacterial 8.200 <sup>c</sup> (3-2-0)(1)	NS	NS
Radish	Fungal Germination (%)	NS 6.900 <sup>b</sup> (30-6)(6-0)	NS NS
	Delay index	NS	4.950 <sup>b</sup> (2)(1)
Wheat	Biomass (weight)	NS	4.524 <sup>b</sup> (0-30)(6)
	Biomass (length)	NS	6.690 <sup>b</sup> (0-30)(6)
	Germination (%)	NS	NS
	Delay index	4.718 <sup>c</sup> (1-2)(2-0)(0-3)	12.503 <sup>c</sup> (30)(0-6)
<i>F. candida</i>	Biomass (weight)	NS	3.399 <sup>b</sup> (30)(0-6)
	Biomass (length)	NS	5.083 <sup>b</sup> (30)(0-6)
	Reproduction	NS	6.073 <sup>b</sup> (30)(0-6)
MELN	Adults survival	NS	3.849 <sup>b</sup> (30)(6-0)
	Luciferase activity	5.188 <sup>b</sup> (1-0-3)(3-2)	NS

Notes: The results of multiple mean comparison using Duncan test are also shown.

NS: not significant

Application dose: 0-6-30 T ha<sup>-1</sup>.

Sludge type: 0 (soil not amended), 1 (soil amended with DS), 2 (soil amended with CS), and 3 (soil amended with LS).

Spiking with pesticides: 1 (spiked) and 2 (not spiked).

<sup>a</sup>F-value.

<sup>b</sup>Significant at 0.05.

<sup>c</sup>Significant at 0.001.

<sup>d</sup>Clusters of levels for each factor based on Duncan test results; levels are presented in increasing order and when within brackets are not significantly different.