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Phosphogypsum as a soil fertilizer: Ecotoxicity of amended soil and elutriates to bacteria, invertebrates, algae and plants

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HIGHLIGHTS

- Assessment of the impact of Tunisian phosphogypsum on soil biota was performed.
- A battery of terrestrial and aquatic species was tested.
- *E. andrei* and *D. magna* were the most sensitive species in amended soil and elutriate.
- The high levels of Ca in PG, suggest that it was responsible for the ecotoxicity.
- Serious efforts should be made to set clear limits for PG application in soils.

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ABSTRACT

Phosphogypsum (PG) is a metal and radionuclide rich-waste produced by the phosphate ore industry, which has been used as soil fertilizer in many parts of the world for several decades. The positive effects of PG in ameliorating some soil properties and increasing crop yields are well documented. More recently concerns are emerging related with the increase of metal/radionuclide residues on soils and crops. However, few studies have focused on the impact of PG applications on soil biota, as well as the contribution to soils with elements in mobile fractions of PG which may affect freshwater species as well. In this context the main aim of this study was to assess the ecotoxicity of soils amended with different percentages of Tunisian phosphogypsum (0.0, 4.9, 7.4, 11.1, 16.6 and 25%) and of elutriates obtained from PG – amended soil (0.0, 6.25, 12.5 and 25% of PG) to a battery of terrestrial (*Eisenia andrei*, *Enchytraeus crypticus*, *Folsomia candida*, *Hypoaspis aculeifer*, *Zea mays*, *Lactuca sativa*) and aquatic species (*Vibrio fischeri*, *Daphnia magna*, *Raphidocelis subcapitata*, *Lemna minor*). Both for amended soils and elutriates, invertebrates (especially *D. magna* and *E. andrei*) were the most sensitive species, displaying acute (immobilization) and chronic (reproduction inhibition) effects, respectively. Despite the presence of some concerning metals in PG and elutriates (e.g., zinc and cadmium), the extremely high levels of calcium found in both test mediums, suggest that this element was the mainly responsible for the ecotoxicological effects observed. Terrestrial and aquatic plants were the most tolerant species, which is in line with studies supporting the application of PG to increase crop yields. Nevertheless, no stimulatory effects on growth were observed for any of the species tested despite the high levels of phosphorus added to soils by PG. Given the importance of soil invertebrates for several soil functions and services, this study gives rise to new serious concerns about the consequences of PG applications on agricultural soils.

1. Introduction

The manufacture of phosphoric acid (H_3PO_4) through a wet acid process, using natural phosphate rock as raw material generates

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a major solid waste, the phosphogypsum (PG) [1]. PG is highly acidic (pH 1) when initially stored, due to residual sulfuric acid, and consists mainly of calcium sulphate dehydrated ($CaSO_4{\cdot}2H_2O$ or gypsum) or hemi-hydrated ($CaSO_4{\cdot}1/2H_2O$ or bassanite), but also contains a high levels of impurities including fluorides, sulphates, natural radionuclides, metals, and other trace elements [2–4]. As PG is dewatered and weathered in storage piles, the acidity is progressively reduced. Phosphogypsum stacks may also emit radon gas and fluorine compounds (SiF4, HF) in significant amounts into the atmosphere. It has been reported that one of the main problems of PG piles is the emanation of ^{222}Rn from the alpha-decay of ^{226}Ra [2,5]. In more windy areas the spread of fine PG particles cannot be disregarded as well.

An industrial plant of phosphate fertilizers originates about 4–6 t of PG per ton of phosphoric acid produced. Presently, the approaches used by the phosphate industry to deal with PG are: (i) discharging to water bodies; (ii) backfilling of mine pits; (iii) dry stacking and (iv) wet stacking. All the solutions have serious environmental impacts [6].

Today the biggest issue facing the Tunisian phosphate industry is the pollution of the gulf of Gabes by PG. The Sfax chemical fertilizer company, located on the south coast near Sfax city, produces a considerable and increasing amount (approximately 10 million tons) of PG per year which is stored in piles in the vicinity of the industrial plant. These piles pose serious risks to the surrounding, partly urban area, by changing air quality, as previously mentioned [5,7]. In addition, soils and ground water are affected by acidic and metal-rich infiltrations. Therefore, alternatives to PG stacking are urgently needed.

The general recommendations for managing PG indicate valorization as the main way for minimizing storage costs and for reducing the negative public health and environmental impacts caused by this waste [2]. Indeed, Tunisian PG has been incorporated in construction materials, giving rise to products with good mechanical properties and very low levels of radionuclides [8,9]. One of these products was cement made with low quantities of PG, instead of natural gypsum [10]. Other usages of PG in constructions have also been proposed [11–15].

The use of PG as an agriculture fertilizer has been practiced in many parts of the world for decades [16,17]. It has being applied in agricultural soils as a calcium, phosphorus and sulfate supplement to enhance crop production and to recover acidic soils reducing Al-toxicity [18–21] and sodic soils [22,23]. It was also used alone or in combination with other synthetic organic polymers for preventing runoffs and erosion in agricultural soils exposed to heavy rainstorms [24]. The amount of PG recommended for amending agriculture soils varies between 500 and 1000 kg ha^{-1} [25]. Therefore, and based on the announced benefits of PG to increase crop yields more recently, and only based on ^{226}Ra limits [26] or on total metal concentrations considered individually [27], PG from Brazil and Jordan, respectively, were recommended for fertilization purposes and as a soil conditioner.

Soil organisms are essential for the provision of several soil functions (and the related ecosystem services) due to their abiotic (e.g., improving soil structure by burrowing) and biotic (e.g., regulating organic matter decomposition) activities [28]. Hence, any change occurring in soil properties is expected to affect them, as well as the self-organized system to which they belong [29]. To the best of our knowledge, and despite the current use of PG in agriculture in several regions of the world (e.g., Spain, Brazil, India and USA) [17,22,23], the impacts of soil amendments with PG on soil biota bacteria, plants, invertebrates were never tested. Only Nayak et al. [30] found that a 10% PG amendment had a positive effect on microbial (fungal and bacteria) growth and on cellulose and amylase activities in an agriculture soil.

In this context and considering that agriculture could be the main sink of PG [23], the aim of the present study was to assess the impact of PG amendments on the soil habitat (i.e., soil as a place to live) and retention (i.e., soil as a cleaning medium for groundwater) functions, before recommending the use of Sfax PG as a soil fertilizer. To attain this purpose OECD artificial soil, amended with different proportions of PG (up to 25%) was tested to assess its toxicity for terrestrial plant *species* (*Lactuca sativa* L. and *Zea mays* L.) and for soil invertebrates (*Eisenia andrei* Bouché, *Folsomia candida* Willem, *Enchytraeus crypticus* Westheide & Graefe and *Hypoaspis aculeifer* Canestrini). In addition, elutriates of OECD artificial soil amended with PG were tested for their toxicity to aquatic organisms, namely the bacteria *Vibrio fischeri* (Beijerinck) Lehmann & Neumann, the green unicellular algae *Raphidocelis subcapitata* (Korshikov) Nygaard, Komanék, Kristiansen & Skulberg, the aquatic plant *Lemna minor* L. and the microcrustacean *Daphnia magna* Straus.

2. Materials and methods

2.1. Mineralogical and physico-chemical analysis of PG and of PG-amended soil samples

The phosphogypsum (PG) used in this study came from the industrial plant located in the city of Sfax, Tunisia. PG was sieved to discard the >2 mm fraction before use. The mineralogical components of the PG were identified by X-ray Diffraction (XRD) using a Bruker (D8 advance) powder diffractometer with a tube Cu anode (40 kV, 40 mA). The Total Organic Carbon TOC (%) was extracted from PG with KNO_3 [31] and analysed by an Analytic Jena-Analyzer multi N/C 2100 S. The soluble sulfates were analysed in a water:PG suspension (1:5 w/v) by ionic chromatography (HIC-6A Shimadzu type) equipped with a conductivity detector and Shim-pack column. The conductivity of PG was measured according to the method described in FAOUN [32] in a (1:5 w/v) soil water suspension. Soil pH was measured in a soil-KCl (1 M) suspension (1:5 w/v) according to, the method described by the ISO guideline 10390 [33] by using a pre-calibrated WTW330/SET-2 pH meter. Organic matter content was determined on the same samples by loss on ignition after 8 h, at 450 °C [34]. Water holding capacity (WHC) of the amended soil samples was determined according to the ISO guideline 11274 [35]. All the measurements were made in triplicate.

2.2. Soluble metal contents of elutriates obtained from PG amended soils

Soluble concentrations of metals (Cd, Cu, Ni, Zn and U) as well as calcium (Ca) and phosphorus (P) in soil elutriates (preparation described in Section 2.3) were quantified by ICP-MS [Inductively Coupled Plasma Mass Spectrometry; Thermo X-Series quadrupole ICP-MS apparatus (Thermo Scientific), equipped with Ni cones and a Burgener nebulizer, refrigerated with a Peltier system].

2.3. Ecotoxicological assays

The OECD artificial soil was prepared by mixing 10% of sphagnum peat, 20% of kaolin clay, 70% air-dried quartz sand and calcium carbonate ($CaCO_3$) to adjust pH to 6.0 ± 0.5 [36]. The percentage of PG mixed with the OECD artificial soil [36] and tested with the different species varied between 25.0% and 4.9% separated by a factor of 1.5 for the tests with the terrestrial species. After the amendment and a stabilization period of 48 h, the water content and the maximum water holding capacity of the soil samples were analysed (please see previous section for methods).

For the evaluation of the effects of PG on the soil retention function, four batches of OECD were mixed with 25, 12.5, 6.25 and 0% of PG. After a 48 h stabilization period, elutriates were obtained

by preparing suspensions of 1:4 (w/v) of the four OECD amended-soils with the respective test media of aquatic test species, namely: ASTM hard water for *D. magna* assays, Woods Hole MBL medium for *R. subcapitata* assays, Steinberg medium for *L. minor* assays and deionised water for the Microtox® test. After shaking mechanically for 12 h at room temperature, and left for settling for another 12 h, the overlying water (elutriate) and settled material were separated by decanting. Elutriates were then centrifuged (3000 rpm, 15 min) to remove suspended particles, and stored at 4 °C in dark until further use, for no more than one week. Sub-samples of each elutriate were acidified (pH 2) with nitric acid Suprapur® 65% from Merck, for chemical analysis by ICP-MS. Each elutriate was then tested individually, after being diluted to obtain a range of five concentrations separated by a factor of 1.25, varying between 100% and 41.0% (except for the Microtox® test). For this last assay 81.9% is the highest elutriate concentration tested in a total of 9 (1:2) serial dilutions of the sample [37].

2.3.1. Tests with invertebrates and terrestrial plants (soil habitat function)

E. andrei reproduction tests were performed following the standard protocol ISO 11268-2 [38]. Adult worms with a well-developed clitellum and weighing between 300 and 600 mg were selected for the test from synchronized laboratorial cultures. Ten worms were incubated in pots with 500 g of the PG-amended soils plus the control (5 replicates per treatment) in polyethylene test containers, after moisture adjustment to 45% of the maximum WHC. Every week, the moisture content of each pot was checked and adjusted if necessary and food (defaunated horse manure) was replenished. After 28 days of exposure under controlled conditions (photoperiod 16^{L}:8^{D}; temperature 20 ± 2 °C; light 400–800 lux), adult earthworms were gently extracted from the test vessels by hand sorting. The pots were then incubated for four additional weeks under the same test conditions. At the end of the assay, the juveniles hatched in the replicates were counted and validity criteria were checked.

E. crypticus reproduction tests were performed following the standard guideline ISO 16387 [39]. Animals from stock cultures were transferred to a Petri dish filled with tap water, and adult worms with visible eggs (white spots) in the clitellum region and with approximately the same size were chosen for the assays. The animals were exposed to thirty grams of the PG-amended soils plus the control (5 replicates per treatment), with soil moisture adjusted to 45% of its WHC_{max}, in 100 mL polyethylene cups covered with a perforated lid. The cups were incubated at the same conditions already described for *E. andrei* assays. Animals were fed once a week with approximately 50 mg of sterilized rolled oats to avoid growth of fungi. After 28 days, test vessels were filled with ethanol and Bengal red solution (1% in ethanol). After 12 h, the potworms stained with reddish color were easily counted while lying on the surface of the substrate and validity criteria established by the standard protocol were checked.

F. candida reproduction tests were performed following the standard protocol ISO 11267 [40]. Polyethylene test containers with a volume of 100 mL were filled with 30 g of amended soil or control soil, after the adjustment of the moisture content to 45% of the maximum WHC. Twenty animals aged 10 to 12 days were placed in each replicate and were collected from a synchronized laboratorial culture after being examined under a stereomicroscope to discard damaged individuals. In each test vessel 30 mg of baker's yeast was spread on the soil surface before the jars were covered with the perforated lids. The test containers were incubated at the same conditions described for the previous assays with invertebrates, regularly weighed and soil moisture adjusted. After 21 days, adults and juveniles were extracted by adding approximately 50 mL of deionized water to the test containers. A few dark ink drops were added, providing a higher contrast between the white individuals and the black background. The suspension was gently stirred to make adult and juvenile springtails float on the water surface. The organisms were counted through the use of the Image J software (online available: http://rsb.info.nih.gov/ij/download.html) and validity criteria were checked.

H. aculeifer reproduction assays were performed according to the OECD guideline 226 [41] with animals from cultures maintained at ECT GmbH. Ten adult female mites (aged between 28 and 35 days), obtained from a synchronized cohort were exposed to 20 g of all the PG-amended soils plus the control (5 replicates per treatment), with WHC adjusted to 45% of its maximum value. Glass test vessels of 3–5 cm diameter (height of soil $\geq$1.5 cm), covered by gauze for direct gaseous exchange were used. The moisture content of each test vessel was controlled every three days and adjusted if necessary. Further, at the same time cheese mites (*Tyrophagus putrescentiae* (Schrank, 1781)) were added to feed the animals. The test vessels were incubated at the same conditions described for *E. andrei* during 14 days.

At the end of the testing period, the surviving mites were extracted from the soil via heat/light extraction and the number of juveniles and the number of surviving females per test vessel was counted, after being preserved in ethanol (70%) and by using a binocular stereomicroscope. Adult mites not found at this time were recorded as dead. Validity criteria were checked.

Terrestrial plants emergence and growth tests were performed following the standard protocol ISO 11269-2 [42]. Seeds were obtained from a local supplier. For each species twenty seeds, visually analysed for their good conditions, were placed in contact with 200 g_{dw} of each PG-amended soil plus the control soil (five replicates per treatment) after soil saturation with water. The tests were carried out in plastic pots with a hole in the bottom to let a cotton rope pass trough. The rope was always in contact with another pot filled with deionized water that was placed under the test pot to guarantee a constant water supply to the soil by capillarity. After soil saturation the seeds were randomly placed in the soil surface and gently covered with soil. The test started and was validated after 50% of seeds have emerged in the control replicates. Afterwards seedlings were grown for 14 days in a growth chamber under controlled conditions (16^{L}:8^{D} photoperiod; 25,000 lux of light intensity with cool-white lamps; temperature: 21 ± 1 °C). The location of the pots inside the growth chamber was changed randomly every 2 to 3 days to provide uniform light exposure. Pots were daily checked and adjusted for water content if necessary. At the end of the assays the total number of emerged seeds was counted and the fresh and dry biomass above soil was determined for each replicate. After weighing for the determination of fresh biomass the plant material from each replicate was dried at 70 °C, till weight stabilization, and dry biomass was obtained.

2.3.2. Tests of soil elutriates with aquatic species (soil retention function)

Elutriates obtained for each batch of OECD soil amended with PG (25%, 12.5%, 6.25% and 0.0%) with the corresponding medium of each test species, were diluted with the same medium to obtain a range of concentrations to be tested (as above described, except for the Microtox® assay, which tests a particular range of concentrations separated by a factor of 2).

The Microtox® assay (also called the *V. fischeri* bioluminescence assay) was performed to assess the toxicity of soil elutriates after 5, 15 and 30 min of exposure, following the 81.9% basic test protocol and using a Microtox 500 Analyzer [37]. The EC_{20} and EC_{50} values and the corresponding 95% confidence intervals were computed for each elutriate using the Software MicrotoxOmni [37].

The *R. subcapitata* growth inhibition assay was performed according to the standard OECD protocol 201 [43]. The algae were obtained from axenic batch cultures maintained in Woods Hole MBL medium at continuous light exposure (cool white fluorescent illumination of 100 μE/m^2/s) and a temperature of 24 ± 1 °C. Algae were exposed in 24-well sterile plates in three replicates per each elutriate dilution plus the control. In each well, 900 μL of the elutriate dilution were inoculated with 100 μL of algae inoculum, with an initial cell concentration of 10^4 cells mL^{-1}. The tests were incubated at the same conditions of illumination used for culturing and at 24 ± 1 °C. Algae from each well were re-suspended by gentle pipetting twice a day. After 72 h, the algae growth inhibition rate was calculated, after measuring optical density at 440 nm, of algae suspensions in the control and elutriate dilution wells.

L. minor growth inhibition test was performed according to the OECD guideline 221 [44]. Plants for testing were bred in the laboratory in Steinberg culture medium composed of sterilized micro and macro element solutions, with pH adjusted to 5.5 ± 0.2. Cultures were maintained in axenic conditions in an acclimated chamber controlling illumination (100 μE/m^2/s obtained with a cool white fluorescent illumination) and temperature (24 ± 2 °C). A total of 3 colonies with three visible fronds were initially exposed per replicate (three replicates per elutriate dilution plus the control). The exposures were carried out in sterilized 150 mL Erlenmeyer's, filled with 100 mL of each elutriate dilution/control. The number of fronds and corresponding fresh and dry weight were measured per replicate, after 72 h of exposure. For this purpose all the fronds were collected from each Erlenmeyer and rinsed with distilled water. Afterwards, the fronds were counted and dried at 60 °C to a stable weight was achieved and the growth inhibition rate was calculated according to the protocol OECD 221 [44]. Validity criteria were checked.

D. magna immobilization test was performed according to the OECD guideline 202 [45]. Neonates for testing were obtained from cultures maintained in ASTM hard water medium [46] under a 16^L:8^D photoperiod and a temperature of 20 ± 2 °C, for several generations. All the assays were initiated with neonates (<24 h old), born between the 3rd and 5th broods, obtained from bulk group cultures. The 5 neonates were exposed to 50 mL of each elutriate dilution plus the ASTM control in glass vessels (4 replicates). After 24 and 48 h of exposure at the same conditions described for culture maintenance, the vessels were checked for immobilized individuals, which were counted and removed from the vessels. The pH (pH 330 from WTW), conductivity (LF 330 from WTW) and dissolved oxygen (Oxi 330 from WTW) were measured in the vessels in the beginning and at the end of the test.

2.4. Statistical analysis

The EC_x values with 95% confidence limits, for *D. magna* immobilization were calculated by Probit analysis [47] using the SPSS Statistics software, version 17.0. For *V. fischeri* the EC_{50} and their corresponding 95% confidence intervals were computed using the MicrotoxOmni® software version V1.18 [37]. For the growth inhibition assay with *R. subcapitata* and *L. minor* EC_x values and the 95% confidence limits were calculated using the nonlinear least squares regression procedure supplied by the software Statistica 12.0 (StatSoft, Inc., Tulsa, USA). One-way ANOVAs followed by Dunnett tests were also employed to find out potential significant differences in the endpoints assessed between the control and tested PG concentrations in soils (terrestrial assays) or elutriate dilutions (aquatic assays), using the software Sigmaplot version 11.0 [48–49]. Based on the ANOVA results ($p < 0.05$), the LOEC values (Lowest Observed Effect Concentration) were determined.

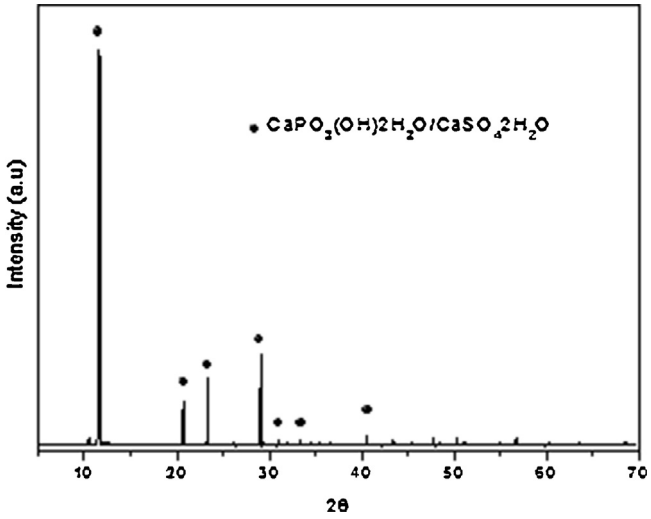

Fig. 1. XRD pattern of Tunisian phosphypsum.

3. Results and discussion

3.1. Physico-chemical and mineralogical characterization of Sfax PG and PG amended soil samples

The powder X-ray diffraction pattern of PG is reported in Fig. 1. As shown, the main diffraction peaks correspond to brushite–gypsum ($CaPO_3(OH)\cdot 2H_2O/CaSO_4\cdot 2H_2O$)(JCPDS 009-0077/33-0311). Further, the physico-chemical characterization of the PG samples is summarized in Table 1. The results showed that CaO and sulphate (expressed as SO_3) were the major components (Table 1), corresponding to 30.7% and 43.8% (proportions expressed for the total of the elements detected), respectively. In addition, high concentrations of Cd and Zn were found (other metals were not conspicuous). Regarding the content of these metals the Sfax PG was similar to other Tunisian PG. Compared to PG from other parts of the world, both contained higher concentrations of these elements [27]. In fact, the composition of PG in terms of metals is variable and depends from their original content in the phosphate rock and the production process of PG [27]. As expected the pH

Table 1
Physico-chemical and mineralogical characterization of Sfax phosphogypsum (PG).

Parameters	
pH	4.26
Conductivity (mS cm^{-1})	2.76
TOC (%)	0.64
Soluble sulfates (g kg^{-1})	10.47
Total metal contents (mg kg^{-1})	
Cu	3.27
Ni	2.45
Zn	117.03
Fe	469.76
Cd	20.29
Co	0.39
Mn	3.03
Chemical composition of PG (major elements, %)	
CaO	30.7
P_2O_5	2.51
F	1.93
SO_3	43.8
Na_2O	0.06
SiO_2	1.38
Fe_2O_3	0.02
Al_2O_3	0.1
MgO	0.01

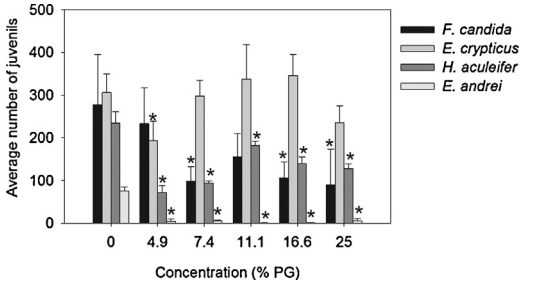

Fig. 2. Average number of juveniles (± standard error) of *F. candida*, *E. crypticus*, *H. aculeifer* and *E. andrei* exposed to different concentrations of PG-amended soil. Asterisks (*) indicate significant differences from the control ($p < 0.05$).

value of the PG was low (4.26) due to the use of sulfuric acid and, to a lesser extent, hydrochloric acid in the production process [6]. The conductivity of PG was high, caused by the presence of many salts and ionic forms. As expectable, the content of total organic carbon (TOC) was very low (Table 1).

The activity of radionuclides in PG was not analyzed in this study, but the USEPA classifies PG as a low-level radioactive material [26,50], especially due to the presence of uranium (U) (frequently associated to phosphate ore) and radium (^{226}Ra), resulting from uranium (U) decay series [6,15]. Accordingly, in a previous characterization [15] ^{238}U/^{232} Th ratios lower than one were recorded for the Sfax PG. Such ratios indicate disequilibrium between the concentrations of both elements, caused by the mobilization of U by phosphoric acid during the treatment, while the Th-radionuclide persists in the PG [15]. All recommendations supporting the use of PG as a soil fertilizer are based on concentrations of individual components (metals and radionuclides). Thus, neither cumulative effects of multiple soil amendments were evaluated, nor the potential synergistic effects of all of these elements were assessed. In fact, so far a limit value for ^{226}Ra in PG is available to regulate its use [26]. Further, the Brazilian ministry for Agriculture (MAPA) established maximum admissible values for toxic metals for fertilizers and soil amending products in general [51].

The highest percentage of PG added to the OECD soil prepared for soil and elutriate testing was 25% corresponding to a worst case scenario. Even at this concentration, the PG did not cause meaningful changes in soil pH, despite its acidity (Table 2). This observation is not in agreement with other authors who report a decrease in soil pH caused by PG amendments [52–53]. In opposition, it is in agreement with the use of PG to correct the low pH of acidic soils (see Introduction 1). However, these studies reported observations made in tests with natural soils, which buffering capacity might be lower than that in OECD artificial soil. Further no changes were observed in the percentage of OM due to the low TOC content of PG. Nevertheless, the addition of PG had caused a remarkable increase in the maximum water holding capacity (WHC) of the test soils.

The concentration of metals increased in elutriates gained from PG amended soils as the percentage added to the OECD soil increased (Table 3). Extremely high levels of phosphorus and calcium were also mobilized in all elutriates. The concentration of metals dissolved in elutriates varied only slightly in the different aquatic test media; except for the ASTM elutriate which showed lower concentrations for all metals analyzed. Among the metals measured in elutriates, the highest concentrations were recorded for zinc.

3.2. Ecotoxicological assays with terrestrial organisms

Tables 4 and 5 depict the toxicity parameters for the terrestrial species exposed to the OECD soil samples amended with different concentrations of PG, whereas Figs. 2 and 3 showed the variation

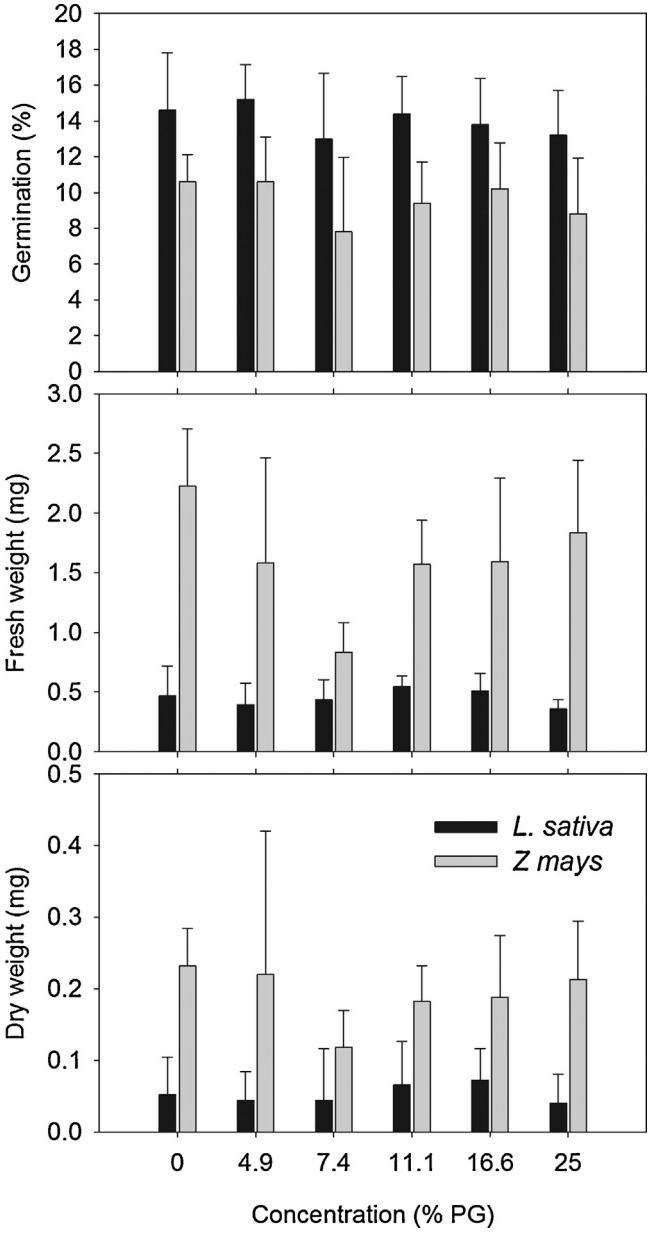

Fig. 3. Average germination (%), fresh weight (mg) and dry weight (mg) (± standard error) of *L. sativa* and *Z. mays* exposed to different concentrations of PG-amended soil.

of the average values of the endpoints analysed for each species. All the validity criteria established in the standard protocols were accomplished. Overall, the exposure of the four terrestrial invertebrate species (Tables 4 and 5; Fig. 2) to PG amended soils resulted in significant inhibitory effects on the reproduction ($p < 0.05$), with estimated LOEC values ranging from 4.9% PG (in the case of *E. andrei*, and *H. aculeifer*) to 7.4% PG (for *F. candida*). The lowest effect was found in the tests with *E. crypticus*, since no significant effect was found at all concentrations except the lowest one. Since there is no dose-effect relationship this difference has to be ignored, meaning that the LOEC is >25% PG. In addition, the concentration-effect relationship is not easily to interpret in the tests with *H. aculeifer* (despite there is always a significant difference to the control).

With the exception of the test with *H. aculeifer* the same tendency was found when comparing EC_{20} values (Table 5) and the LOECs, indicating in both cases a higher toxicity of PG amended soils for *E. andrei* and *F. candida* than for the other two invertebrate

Table 2
Average values ± standard deviation of physico-chemical properties of PG-amended soils used for terrestrial and aquatic tests. OM: organic matter; WHC: maximum water holding capacity.

	Percentage of PG in OECD soil (%)	pH	OM (%)	WHC (%)
Terrestrial assays	0.0	5.64 ± 0.00	5.48 ± 0.19	34.75 ± 1.93
	4.9	5.48 ± 0.02	5.11 ± 0.10	36.94 ± 1.24
	7.4	5.26 ± 0.05	5.56 ± 0.56	46.18 ± 3.21
	11.0	5.19 ± 0.03	5.70 ± 0.10	47.94 ± 1.43
	17.0	5.29 ± 0 .07	5.80 ± 0.19	45.15 ± 1.66
	25.0	5.6 ± 0.03	5.25 ± 0.15	44.4 ± 0.42
Aquatic assays	0.0	5.94 ± 0.11	6.84 ± 1.39	35.0 ± 3.44
	6.25	5.53 ± 0.043	5.35 ± 0.26	37.14 ± 1.13
	12.5	5.55 ± 0.06	5.70 ± 0.15	36.53 ± 2.63
	25.0	5.58 ± 0.025	5.84 ± 0.086	42.64 ± 1.05
	100.0	5.23 ± 0.18	5.83 ± 0.082	75.28 ± 5.63

Table 3
Total concentrations of phosphorus, calcium and metals in elutriates obtained with different test media from OECD soil amended with different percentages of PG.

	P (mg/L)	Ca (mg/L)	Ni (μg/L)	Cu (μg/L)	Zn (μg/L)	Cd (μg/L)	U (μg/L)
Steinberg medium elutriates							
0%	18.0	61.0	1.0	1.9	58.0	0.2	0.5
6.25%	36.0	650.0	4.9	4.3	159.0	1.8	0.6
12.5%	37.0	656.0	3.8	3.1	155.0	2.7	0.4
25.0%	38.0	637.0	6.0	3.6	165.0	4.3	0.7
Deionized water elutriates							
0%	1.0	8.0	<1.0	1.5	11.0	<0.1	0.2
6.25%	8.9	584.0	4.3	3.5	125.0	1.6	0.7
12.5%	11.0	564.0	4.8	3.3	138.0	2.9	0.7
25.0%	12.0	562.0	6.1	3.7	148.0	4.1	0.8
Woods MBL medium elutriates							
0%	0.7	46.0	1.6	3.7	67.0	0.4	0.8
6.25%	8.5	596.0	4.5	5.2	122.0	1.9	0.5
12.50%	10.0	588.0	4.9	4.7	125.0	3.0	0.5
25.0%	12.0	592.0	7.5	5.6	138.0	4.2	1.1
ASTM medium elutriates							
0%	0.4	25.0	<1.0	1.3	12	<0.1	0.17
6.25%	7.3	545.0	2.6	2.4	84	1.2	0.35
12.5%	8.1	537.0	2.3	3.2	82	1.8	0.27
25.0%	9.1	546.0	3.0	2.3	88	2.4	0.35

species: *E. andrei* ≅ *F. candida* > *H. aculeifer* > *E. crypticus*. Notwithstanding, the earthworm *E. andrei* was clearly the most sensitive organism to PG amended soil, with a significant reduction in the number of juveniles produced even in the lowest concentration tested (93.6% of inhibition when exposed to 4.90% PG).

Although several metals were recorded in the composition of Sfax PG, for which mixture effects cannot be discarded, the corresponding concentrations were all below the HC_{50} values (hazard concentration for 50% of the invertebrate, plant and microbial processes) proposed by Jänsch et al. [54], except for Cd (HC_{50} = 6.78 mg kg^{-1}). However, and considering that only a maximum of 25% of PG was mixed into the OECD soils, such soil value for Cd was not surpassed. Thus, the toxic effects observed in invertebrates cannot be exclusively caused by cadmium.

As far as calcium is considered, it has long been regarded only as an essential element for the metabolism and innate immune system of earthworms [55–57], while its toxicity has been neglected, probably because high calcium concentrations occur only in very particular situations. In fact several vital cell functions are governed by calcium signals. However, it was also demonstrated that a high overload of calcium into cells may indirectly elicit serious cell damages or even cell death by apoptosis, necrosis or autophagy. This could happen by activating reactions that depend on the presence of Ca^{2+}, namely through: (i) the activation of hydrolytic enzymes,

Table 4
One-way ANOVA summary and estimated Lowest Observed Effect Concentration (LOEC) values (Dunnett's test; $p \leq 0.05$) regarding the response of terrestrial species (*E. andrei*, *F. candida*, *E. crypticus*, *H. aculeifer*, *L. sativa* and *Z. mays*) exposed to several concentrations of PG-amended soil. df: degrees of freedom, MS_{res}: mean square variation, F_{ratio}: F statistic, *p*-value: probability.

Species	Endpoint	df	MS_{res}	F_{ratio}	p_{value}	LOEC (%)
E. andrei	Number of juveniles	5	...	...	0.003	4.9
F. candida	Number of juveniles	5	3.50E+04	6.316	<0.001	7.4
E. crypticus	Number of juveniles	5	1.80E+04	6.828	<0.001	>25.0
H. aculeifer	Number of juveniles	5	1.76E+04	71.955	<0.001	4.9
L. sativa	Germination (%)	5	3.63	0.488	0.782	>25.0
	Fresh weight (mg)	5	2.45E−02	0.927	0.481	>25.0
	Dry weight (mg)	5	8.62E−04	1.049	0.412	>25.0
Z. mays	Germination (%)	5	6.27	0.792	0.566	>25.0
	Fresh weight (mg)	5	8.26E−01	2.293	0.077	>25.0
	Dry weight (mg)	5	8.41E−03	0.814	0.552	>25.0

Table 5
EC_x values, plus corresponding 95% confidence intervals within brackets, estimated for the response of the terrestrial species (*E. andrei*, *F. candida*, *E. crypticus*, *H. aculeifer*, *L. sativa* and *Z. mays*) exposed to several concentrations of PG-amended soil.

Species	Endpoint	EC_{10} (%)	EC_{20} (%)	EC_{50} (%)
E. andrei	Number of juveniles	<4.9% (a.t.t.)	<4.9% (a.t.t.)	<4.9% (a.t.t.)
F. candida	Number of juveniles	1.24 (0.00–4.33)	2.77 (0.00–7.57)	10.88 (2.39–19.36)
E. crypticus	Number of juveniles	24.61 (23.94–25.28)	24.98 (-,-)	> 25%
H. aculeifer	Number of juveniles	24.41 (5.04–43.78)	27.37 (0.00–77.82)	33.31 (0.00–224.0)
L. sativa	Germination (%)	>25% (b.t.t.)	>25% (b.t.t.)	>25% (b.t.t.)
	Fresh weight (mg)	>25% (b.t.t.)	>25% (b.t.t.)	>25% (b.t.t.)
	Dry weight (mg)	>25% (b.t.t.)	>25% (b.t.t.)	>25% (b.t.t.)
Z. mays	Germination (%)	>25% (b.t.t.)	>25% (b.t.t.)	>25% (b.t.t.)
	Fresh weight (mg)	>25% (b.t.t.)	>25% (b.t.t.)	>25% (b.t.t.)
	Dry weight (mg)	>25% (b.t.t.)	>25% (b.t.t.)	>25% (b.t.t.)

b.t.t. – Stands for below toxicity threshold, which means that an inhibition lower than 10, 20 or 50% has occurred thus the corresponding EC_x value was not calculated. a.t.t. – Stands for above toxicity threshold, which means that extremely high inhibitory effects occurred in all the concentrations tested, or only in the non-diluted elutriate, thus preventing the calculation of EC_x values.

such as endonucleases that degrade DNA and, (ii) the breakdown of mitochondrial membrane potential after excessive mitochondrial sequestering of calcium, with the subsequent generation of ROS, impairment of cell energetic metabolism and generation of active signals responsible for inducing cell apoptosis [58–59]. Thus, based on the high levels of dissolved calcium recorded in the soil elutriates (including the one prepared with water), we suspect that extremely high Ca^{2+} concentrations have been added via PG to the OECD soil, where it was highly bioavailable for soil invertebrates, causing cytotoxic effects that have compromised their reproduction. Furthermore, low concentrations of metals in the soil pore water (as shown by elutriates obtained with deionized water) may have facilitated the influx of Ca^{2+} into cells, since calcium modulates the sequestration and elimination of different metals in the chloragogeneous tissue [60]. Clear evidences of changes in the metabolism of calcium in *E. andrei* exposed to uranium mining wastes were demonstrated by Lourenço et al. [61].

Therefore, although calcium seems to be the most concerning element of PG, which may pose serious risks to soil biota, further studies are required to assess the toxicity of high concentrations of calcium to soil invertebrates and to obtain risk limits for this element, while also clarifying the relationship between PG toxicity and high levels of calcium.

In contrast to the toxicity of PG to soil invertebrates, no toxicity was found for both plant species (*Z. mays* and *L. sativa*) exposed to PG-amended soils (Tables 4 and 5; Fig. 3). Regarding the parameters (germination, fresh weight and dry weight) measured for each plant species, no significant differences ($p > 0.05$) were observed between the plants from controls and those exposed to soils amended with the different percentages of PG (Fig. 3).

No information from literature is available regarding the toxicity of PG on maize. However, *L. sativa* has shown to be sensitive to metal-contaminated soils [62] mainly due to its high capacity to bioaccumulate metals. Nevertheless, in PG-amended OECD soil no significant phytotoxic effects were observed for both species tested. May be the potential effects of metals like Cd on plants have been counteracted by the simultaneous addition of essential nutrients such as calcium and phosphorus [63]. Enamorado et al. [23] suggested that Ca^{2+} added by PG can have an antagonistic effect preventing the uptake of at least some metals (especially cationic forms) by plants. As shown by Wang et al. [64] the electrostatic displacement of toxic metal ions from binding sites in cellular membrane exterior surface (CMS_C) seems to be the most important mechanism in this context (e.g., Cu^{2+} and Ni^{2+} in the presence of Cd^{2+}). Fan et al. [65] and Li et al. [66] observed the same ameliorating effect of calcium and phosphate amendments on copper and lead phytotoxicity. Calcium is able to prevent the inhibition of growth and the cytotoxicity of Cd^{2+} in hairy root cells of *Wedelia trilobata* by decreasing the rate of chromosomal aberration and by enhancing the mitotic index of the cells [63]. Siddiqui et al. [67] found that the protective effect of calcium in *Vicia faba* was related to an improved activity of antioxidant enzymes and with its capacity for increasing the concentration of photosynthetic pigments by acting as a secondary messenger for cytokinin action. Thus, the biosynthesis of chlorophyll is promoted. This protective effect of Ca co-occurs with the provision of essential nutrients such as Zn, Cu, and Fe by PG, which promotes the growth of plants. However, the antagonist effect of calcium does not act similarly for all the metals and plant species, suggesting that high concentrations of these elements can overcome the efficiency of ameliorating mechanisms. Therefore, although the low phytotoxic effects of PG amendments were, at least in part, expected it stills necessary to evaluate the effects of cumulative amendments. In recent studies, accumulation of metals (e.g., Cd, Fe) and radionuclides in plants was found after PG applications [23]. For example, Abril et al. [68] found that Cd concentrations in tomato fruits from an area with a history of three decades of PG application, in SW Spain, were one order of magnitude higher than those found in tomatoes from the market. Thus, although plants seem to be less sensitive to PG amendments, it is necessary to evaluate whether after cumulative applications of PG the negative effects of calcium will not override the beneficial ones.

3.3. Ecotoxicological assays with aquatic organisms

Tables 6 and 7 summarize the ecotoxicological parameters estimated for the aquatic species exposed to elutriates obtained from PG-amended soils. Similarly to the terrestrial assays, only the results of final tests are presented. All validity criteria have been fulfilled in these tests. The bioluminescence inhibition of the bacteria *V. fischeri* increased with the concentration of PG added to soils (Table 7). High toxic effects were observed for elutriates obtained from 12.5% and 25% PG-amended soils, whereas no toxicity was found for elutriates from 0.00% and 6.25% PG-amended soils. Nevertheless, the maximum bioluminescence inhibition (32.2%) was observed only for the elutriate obtained from the OECD soil amended with 25% of PG.

The microalgae *R. subcapitata* was slightly more sensitive since significant inhibitory effects on growth were recorded for algae exposed to elutriates from OECD soils amended with 6.5, 12.5 and 25% of PG (Tables 6 and 7; Fig. 4). Provided that, in general, low inhibitory effects were observed for *R. subcapitata*, the LOEC values obtained tended to overestimate the effects since although significant the inhibition recorded in the growth rate of *R. subcapitata* was always below 20%, preventing EC_{20} and EC_{50} estimations.

As regards to the floating aquatic plant *L. minor* (Tables 6 and 7; Fig. 4), no significant differences (p>0.05) were found between control and the various dilutions of the different elutriates tested.

Table 6
One-way ANOVA summary and estimated Lowest Observed Effect Concentration (LOEC) values (Dunnett's test; $p \leq 0.05$) regarding the response of the aquatic species *R. subcapitata* and *L. minor* exposed to several concentrations of the aqueous extracts of PG-amended soil. df: degrees of freedom, MS_{res}: mean square variation, F_{ratio}: F statistic, *p*-value: probability.

Species/endpoint	% PG in soil	df	MS_{res}	F_{ratio}	p_{value}	LOEC (%)
R. subcapitata/growth inhibition	0.0	5	1.66E-3	1.296	0.307	>100
	6.25	5	2.73E-2	10.085	<0.001	51.2
	12.5	5	8.05E-2	51.772	<0.001	≤41
	25.0	5	6.60E-2	27.444	<0.001	51.2
L. minor/growth inhibition	0.0	6	4.14E-4	1.121	0.399	>100
	6.25	6	1.72E-3	1.049	0.436	>100
	12.5	6	2.44E-3	1.752	0.181	>100
	25.0	6	1.84E-3	0.97	0.48	>100

Table 7
EC_x values, plus 95% confidence intervals within brackets, estimated for the response of the aquatic species (*R. subcapitata*, *L. minor* and *D. magna*) exposed to several dilutions of the aqueous extracts of PG-amended soil.

Species/endpoint	% PG in soil	EC_{10} (%)	EC_{20} (%)	EC_{50} (%)
V. fischeri/luminescence inhibition	0.0	b.t.t.	b.t.t.	b.t.t.
	6.25	b.t.t.	b.t.t.	b.t.t.
	12.5	b.t.t.	2.06 (0.28–14.92)	14.4 (2.07–100.5)
	25.0	b.t.t.	4.39 (1.21–1.59)	b.t.t.
R. subcapitata/growth inhibition	0.0	b.t.t.	b.t.t.	b.t.t.
	6.25	b.t.t.	b.t.t.	b.t.t.
	12.5	57.7 (42.7–72.8)	b.t.t.	b.t.t.
	25.0	69.8 (51.6-–88.0)	b.t.t.	b.t.t.
L. minor/growth inhibition	0.0	b.t.t.	b.t.t.	b.t.t.
	6.25	b.t.t.	b.t.t.	b.t.t.
	12.5	b.t.t.	b.t.t.	b.t.t.
	25.0	b.t.t.	b.t.t.	b.t.t.
D. magna/immobilization	0.0	b.t.t.	b.t.t.	b.t.t.
	6.25	b.t.t.	b.t.t.	a.t.t.
	12.5	b.t.t.	b.t.t.	a.t.t.
	25.0	b.t.t.	b.t.t.	a.t.t.

b.t.t. – Stands for below toxicity threshold, which means that an inhibition lower than 10, 20 or 50% has occurred thus the corresponding EC_x value was not calculated. a.t.t. – Stands for above toxicity threshold, which means that extremely high inhibitory effects occurred in all the dilutions tested, or only in the non-diluted elutriate, thus preventing the calculation of EC_x values.

Further it was not possible to estimate either the EC_x or LOEC values, evidencing the tolerance of *L. minor* to the elements provided by PG addition to soil elutriates, including the extremely high concentrations of calcium and phosphorus (Table 2). The tolerance of this species is not a surprise, because although, *L. minor* has been considered as a standard species for ecotoxicological assays, several recent studies demonstrated the lack of sensitivity of this species to complex mixtures of contaminants, including metals, being even recommended for the treatment of effluents [69]. The response of this aquatic plant species is also in line with the results obtained for the terrestrial plants. Similarly to what was described for invertebrates, calcium is also a crucial element for plants as it stabilizes membranes, activates or blocks ions channels to regulate ion-flux and is required for the activity of several enzymes, as those involved in mitochondrial processes of energy conversion [70]. In opposition, at high concentrations, Ca^{2+} can also become cytotoxic, by giving rise to insoluble calcium phosphate that precipitates in the cytosol, compromising ATP metabolism with subsequent cell death [70]. However, it has been demonstrated that under other stressful conditions (e.g., cold, drought, osmotic stress, metals exposure) calcium availability may contribute for improving plant's tolerance since different calcium-dependent protein kinases (CDPK) have key roles in the responses of plants to abiotic stress [71–72]. Further Ca^{2+} may prevent the uptake of metals like Cd^{2+}, and can also increase the endogenous glutathione (GSH) content thus improving the capacity of the cells to deal with oxidative stress [73].

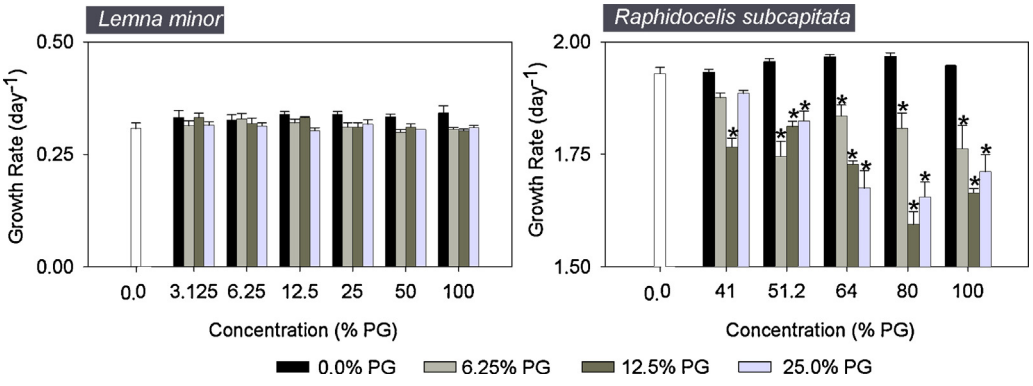

Fig. 4. Growth rate (day^{-1}) for the microalgae *R. subcapitata* and for the macrophyte *L .minor* exposed to the treatment dilutions of PG-soil aqueous extracts. Asterisks (*) indicate significant differences from the control treatment ($p < 0.05$).

Such complex interactions involving calcium-signatures and calcium mediated responses to stress may have contributed for the highest resistance, observed in this study, for plants and algae to OECD-soil amended with PG, and corresponding elutriates, respectively.

The cladoceran *D. magna* was also affected when exposed to elutriates obtained from the all OECD soils amended with PG (Table 7). A similar response pattern was found with 100% of immobilization only in non-diluted elutriates (100%), contrasting with the total absence of immobilized neonates in the remaining dilutions. This pattern hindered the calculation of EC values for this species. However, it was evident that elutriates had a clear acute toxic effect on *D. magna*, even when only 6.25% of PG was added to the OECD soil. Thus similarly to terrestrial species, this aquatic invertebrate was the most sensitive species to PG soil amendments. Considering that ASTM elutriates were those with the lowest concentration of metals provided by PG amendment of OECD soil, calcium appears again as a strong candidate to be responsible for the acute effects on this *cladoceran* species. Comparing the response of the four aquatic species exposed to the PG-soil aqueous extracts, the overall decreasing order of sensitivity was *D. magna* > *V. fischeri* > *R. subcapitata* > *L. minor*.

4. Conclusions

In summary, this study clear supports new concerns related with the application of PG as a fertilizer in the agricultural soils. The ecotoxicological effects on invertebrates were particularly remarkable and calcium seemed to have an important role in the effects observed. The addition of high levels of calcium to the soils is likely sufficient to disrupt the calcium metabolism with consequences in the reproduction and even in the survival of invertebrates (*D. magna*). It was also observed that PG may contribute to a certain level with metals, calcium and phosphorus to soils that may be easily mobilized to the aqueous phase, affecting aquatic resources and its species. In particular invertebrates like cladocerans which have a crucial role in the equilibrium of aquatic food chains. The results obtained, indicate that serious efforts should be made to set clear limits for PG application in soils, not only based on ^{226}Ra or metal contents.

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