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Earthworms to assess the innocuousness of spent biomixtures employed for glyphosate degradation

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ABSTRACT
In this study, the innocuousness of different biomixtures employed for glyphosate degradation was tested through *Eisenia fetida* earthworms. Eight biomixtures were prepared with local materials: alfalfa straw (AS), wheat stubble (WS), river waste (RW) and two different soils (A and B). Each biomixture was divided into two equal portions: one without glyphosate application (control substrate) and the other was sprayed with a commercial glyphosate formulation of 1,000 mg glyphosate a.i. kg^{-1} biomixture (applied substrate). The bioassay started when all sprayed biomixtures reached high percentages of glyphosate degradation (spent biomixtures). Three parameters were studied: survival, adults and juveniles biomass and reproduction. The results allowed the identification of three biomixtures (AWS, BWS and BWSRW) for good maintenance and development of *E. fetida*. In addition, at the end of the bioassay two of the viable biomixtures (AWS and BWS) showed the highest performance of juvenile earthworms compared to a reference soil. The Principal Component Analysis (PCA) indicated that the biomixtures containing high silt and clay percentages and minor density renders higher values of earthworm growth and reproduction. Therefore, these innocuous biomixtures can be used as organic amendments or recycled materials for new treatments on biobeds.

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Eisenia fetida; glyphosate; vermicomposting; spent biomixtures

Introduction

Agricultural production is mainly based on the combination of minimum and non-tillage practices, and the adoption of genetically modified glyphosate-resistant crops. Glyphosate (N-phosphonomethylglycine) has been used for more than three decades being the most non-selective herbicide applied in the field.[1,2] In Argentina, about 200 million liters of glyphosate were used in 2012 for agricultural sprays.[3] This situation has increased the concern about the possible adverse effects of this herbicide, especially its potential impact on soil, water contamination and ecosystem functioning.[4,5]

The biobeds, simple and cheap on-farm constructions, are biopurification systems (BPS) designed to collect and decontaminate wastewater with high concentration of pesticides. Biobeds are basically built by waterproofed excavations filled with a biologically active matrix (biomixture). The biomixtures are made up with soil, lignocellulosic materials and humidifying organic substrates mixed in different volumes ratios covered by a grass layer. They were developed in European countries and their use was extended to other countries by adapting them to local materials and conditions.[6,7] The biomixture is the most important component due to it allows pesticides degradation through the action of the microorganisms.[8,9]

Spent biomixtures (biomixtures that have been used for decontamination purposes) could potentially contain pesticide residues and must be considered as hazardous wastes which should be correctly treated. Particularly, spent biomixtures employed for glyphosate degradation contains low glyphosate concentrations but high AMPA (aminomethylphosphonic acid) concentrations, being this metabolite the main breakdown glyphosate degradation product.[10,11] Although little information is available concerning to AMPA toxicity,[5] it has been classified as a persistent pollutant in soils. This characteristic might result on higher toxicity risks compared to glyphosate on biomixtures. Possible ways of handling this material include dispersal, landfill disposal or incineration. Even some of these techniques could reach the complete depuration of biomixtures; they are rather expensive or cannot be considered as final processes since they include only pollutant matrix transference. Vermicomposting is the process by which worms are used to convert organic matter (usually wastes) into a humus-like matter known as vermicompost.[12] From an environmental and economic point of view, vermicomposting technology could be an efficient and viable method to treat spent biomixtures. Earthworms represent a higher proportion of terrestrial invertebrates biomass (approximately 80%) and play an important role in soil formation. They help to maintain its fertility.[13,14] Earthworms are substrate contamination bioindicators, providing early warning and serving as a field or laboratory toxicity test models.[15] *Eisenia fetida* is one of the standardized species for ecotoxicological tests, being a sensitive indicator of restored soils or substrates suitability.[16,17] Thus, they speed up organic

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pollutants biodegradation by the microorganisms present in a restored soil, optimizing this process and improving the quality of the substrate.[17,18] Earthworms are detritivorous, decomposing the residues through the action of digestive enzymes and aerobic and anaerobic microflora present in their intestinal tract.[19] There are previous studies that deal with the effects of glyphosate on grown and reproductivity of *E. fetida*. García-Torres et al.[20] reported that adverse effects upon adult fecundity and cocoon viability were observed from a glyphosate concentration of 5,000 mg kg^{-1}. In other work, glyphosate inhibited the growth of adults earthworms and the development of juveniles employing concentrations 2 or 3 times lower than the recommended application dose.[21]

The aim of this work is to assess the innocuousness of local biomixtures (prepared with different materials) employed for glyphosate degradation using *E. fetida* earthworm as a bioindicator. In order to evaluate the performance of the biological test on the spent biomixtures, some parameters were measured during the bioassay: earthworm survival, biomass and reproduction. In addition, a Principal Component Analysis (PCA) was performed in a reduced space to find the best conditions that provide a better habitat for earthworms (correlation between some biomixture parameters (%sand, %clay, Mg, density) on earthworms growth and reproduction).

Materials and methods

Preparation of biomixtures. Degradation assays

Eight biomixtures were prepared employing different substrates: two types of soil (soil A was obtained from a field in the north of Santa Fe province (Argentina) with more than 20 years of continuous soybean cultivation, while soil B was taken from the garden of a private residence in Santa Fe City (Argentina)), two types of lignocellulosic wastes (AS: alfalfa straw and WS: wheat stubble) and a humidifying material (RW: river waste). Stubble and straw were collected from the same field where soil A was obtained.

Physicochemical properties of the different soils, lignocellulosic materials and the river waste are presented in Tables 1 and 2, respectively. From the granulometric properties it is possible to determine two soil textural classes: silty clay loam (soil A) and loam (soil B). River waste substrate is a material used to mix together with soil and is intended for all kind of plants cultivation that requires non-acid soils. It is a commercial product purchased by Santa Isabel tree nursery (Santa Fe, Argentina).

Table 1. Physicochemical properties of employed soils.

Properties	Soil A	Soil B
Granulometry (%)		
Sand	6.4	52.3
Silt	66.6	33.9
Clay	27.0	13.8
Carbon dry basis (%)	1.97	2.40
Organic matter (%)	3.40	4.12
Phosphorus dry basis ($mg.kg^{-1}$)	0.023	0.029
Bulk density ($g.cm^{-3}$)	0.782	0.984
Porosity (%)	70.7	58.9
pH	5.96	6.88
Ash (%)	94.83	92.15
K ($mg.kg^{-1}$ soil)	462.71	472.99
Ca ($mg.kg^{-1}$ soil)	184.88	532.96
Mg ($mg.kg^{-1}$ soil)	84.36	51.89
Na ($mg.kg^{-1}$ soil)	10.39	27.74
N (%)	0.153	0.270

Table 2. Physicochemical properties of lignocellulosic materials and river waste.

Properties	*AS*	*WS*	*RW*
Organic matter (%)	79.5	82.2	18.2
Dry matter (%)	89.6	91.3	nd
Ash (%)	10.1	9.1	71.9
Raw fiber (%)	23.6	38.4	nd
P (%)	0.4	Not detected	0.001
N (%)	2.3	0.46	0.57

nd: no determined, AS: alfalfa straw; WS: wheat stubble; RW: river waste.

Each proposed biomixture was prepared by mixing the components in different volume ratios. The composition of the proposed biomixtures is presented in Table 3. Then, biomixtures were placed in glass boxes (20 × 30 × 50 cm) for composting. After 50 days of composting, each biomixture was divided into two equal portions: one without glyphosate application (control substrate) and the other was sprayed with a glyphosate solution prepared with the commercial formulation Eskoba® in order to obtain an initial concentration of active ingredient of 1,000 mg a.i. glyphosate kg dry $biomixture^{-1}$ (applied substrate). The degradation assays were carried out for 63 days taken samples to determine glyphosate concentration and its main metabolite AMPA. Glyphosate and AMPA extraction procedures were performed adapting different techniques.[4,22,23] Glyphosate and AMPA quantification was performed by HPLC/UV, for which the sample was previously derivatized with *p*-toluenesulfonyl chloride (Sigma Aldrich), according to Kawai et al.[24]

The toxicity bioassay began 63 days after biomixtures were sprayed. At this time, six biomixtures reached nearly 100% of glyphosate degradation, except AAS and BWS which reached a degradation of 88.1% and 98.2%, respectively. Table 4 shows glyphosate and AMPA concentrations recorded at beginning of the bioassay. AMPA concentrations were between 50 and 100 mg kg^{-1}. In a previous work, more results about glyphosate and AMPA degradation of each biomixture were discussed.[10]

Chronic toxicity test

According to Karanasios et al.,[6] degradation does not mean detoxification. Toxicity is conditioned by biomixture composition. In this sense, the intrinsic characteristics of each biomixture and its

Table 3. Biomixtures composition.

Components		Biomixtures (% v/v)							
		AWSRW	AAS	AASRW	AWS	BWSRW	BAS	BASRW	BWS
Soil	A	25	50	25	50	—	—	—	—
	B	—	—	—	—	25	50	25	50
Lignocellulosic material	WS	50	—	—	50	50	—	—	50
	AS	—	50	50	—	—	50	50	—
Humidifying material	RW	25	—	25	—	25	—	25	—

AWSRW (soil A, wheat stubble, river waste), AAS (soil A, alfalfa straw), AASRW (soil A, alfalfa straw, river waste), AWS (soil A, wheat stubble), BWSRW (soil B, wheat stubble, river waste), BAS (soil B, alfalfa straw), BASRW (soil B, alfalfa straw, river waste) and BWS (soil B, wheat stubble).

Table 4. Glyphosate and AMPA concentrations at the beginning of the bioassay.

Spent biomixtures	Glyphosate concentration (mg kg^{-1})	AMPA concentration (mg kg^{-1})
AWSRW	<LQ	97 ± 10
AAS	119 ± 8	66 ± 7
AASRW	<LQ	68 ± 7
AWS	<LQ	99 ± 11
BWSRW	<LQ	71 ± 7
BAS	<LQ	55 ± 6
BASRW	<LQ	66 ± 7
BWS	18 ± 5	66 ± 7

<LQ: below limit of quantification, 10 mg kg^{-1}. AWSRW (soil A, wheat stubble, river waste), AAS (soil A, alfalfa straw), AASRW (soil A, alfalfa straw, river waste), AWS (soil A, wheat stubble), BWSRW (soil B, wheat stubble, river waste), BAS (soil B, alfalfa straw), BASRW (soil B, alfalfa straw, river waste) and BWS (soil B, wheat stubble).

Table 5. Adult survival at 56 days.

	Adult survival (%)	
Biomixtures	Control (c)	Applied (a)
AWSRW	95	0
AAS	95	0
AASRW	0	0
AWS	100	100
BWSRW	100	100
BAS	0	0
BASRW	0	0
BWS	100	100

AWSRW (soil A, wheat stubble, river waste), AAS (soil A, alfalfa straw), AASRW (soil A, alfalfa straw, river waste), AWS (soil A, wheat stubble), BWSRW (soil B, wheat stubble, river waste), BAS (soil B, alfalfa straw), BASRW (soil B, alfalfa straw, river waste) and BWS (soil B, wheat stubble).

composting time impacts in a symbiotic relationship between microorganisms and earthworms.[25] Therefore, a toxicity bioassay on biomixtures that has been used to degrade glyphosate is proposed in this work.

In order to test biomixtures innocuousness, the experiment began when glyphosate degradation was almost 100%. Each biomixture consisted in three replicates with unsprayed substrate (control substrate) and three with treated substrate (applied substrate) after 63 days of glyphosate degradation. Notice that three replicates is the minimum number of replicates recommended by OECD.[16] Each replicate was placed in a transparent polypropylene box (24 × 16 × 9 cm) with small holes, containing 300 g of composting substrate and eight *E. fetida* adult individuals (average weight 300 ± 25 mg) grown in our ecotoxicology laboratory. Biomixtures moisture was maintained at 30–35% with distilled water, the room temperature was 25 ± 2°C and the illumination was constant; according to the protocol OECD[16] with slightly modifications. The worms were fed weekly following the methodology detailed in Masin and Rodríguez.[26]

Three parameters were studied: survival (live adult organisms/total), adults and juveniles biomass (wet weight in g), and reproduction (cocoons number per worm and juveniles number per cocoon). These parameters were recorded at 3, 56 and 116 days. The bioassay ended at 116 days. The performance of adult and juvenile earthworms was compared to a reference soil (named as Ref. soil) taken from a forestland in San Vicente village, Santa Fe, Argentina.

Results and discussion

The innocuous test (or bioassay) began with eight different biomixtures, each one divided into two portions: control and applied substrates. The adult survival values obtained are shown in Table 5.

At 56 days, adult survival was 100% on biomixtures AWS, BWS and BWSRW for both (control and applied substrates). In AWSRW and AAS, only there were survivors in control substrates with the same percentage (95%). Finally, in BASRW, BAS and AASRW there were not adult survivors at 3 days on both substrates being the individuals covered by the putrefaction white fungus (Fig. 1). Therefore, AS was the substrate present on biomixtures unviable for earthworms while the WS was the lignocellulosic material employed in the biomixtures that proved to be innocuous, considering control and applied substrates. This could be due to the great influence of lignocellulosic raw materials nature (WS or AS) on the biodegradation of organic matter during composting.

Mendoza Hernández[27] and Al-Maliki and Scullion[28] argued that organic waste and/or its immature compost may accumulate organic compounds that are unpalatable or may be toxic to organisms. Thus, the evidenced effects on the biomixtures AWSRW and AAS (applied), BASRW, BAS and AASRW (controls and applied) could be related to this phenomenon. These substrates could require more time to reach a complete organic matter degradation improving its quality.

It can be seen in Figure 2 the estimated means for two parameters: adult weight change in a period of 56 days (Fig. 2a) and reproduction at 116 days (Fig. 2b), where reproduction = fecundity × viability. This analysis of variance compares the means of seven groups (AWSc, AWSa, BWSc, BWSa, BWSRWc, BWSRWa, where the letters c and a indicate control and applied, respectively, and Ref. soil is reference soil) to test

Figure 1. Dead earthworms at 3 days of exposure on control biomixtures: (A) BASRW (soil B, alfalfa straw, river waste), (B) BAS (soil B, alfalfa straw) and (C) AASRW (soil A, alfalfa straw, river waste).

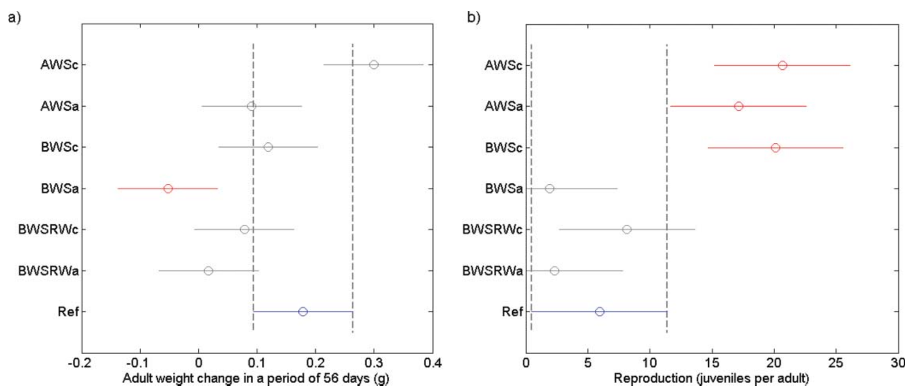

Figure 2. Multiple comparisons of group means with 95% confidence intervals for the true mean difference. The comparison interval of the Ref. group mean is highlighted (in blue). Two characteristics are analyzed: (a) adult weight change in a period of 56 days and (b) reproduction at 116 days. Where: AWSc (soil A, wheat strubble, control); AWSa (soil A, wheat strubble, applied); BWSc (soil B, wheat strubble, control); BWSa (soil B, wheat strubble, applied); BWSRWc (soil B, wheat strubble, river waste, control); BWSRWa (soil B, wheat strubble, river waste, applied); Ref. soil (reference soil).

the hypothesis that they are all equal, against the general alternative that they are not all equal.

The disjoint comparison intervals indicate that the group of means is significantly different. While on the contrary, two groups of means are not significantly different because their intervals overlap. Then, AWSa, BWSc and BWSRWa are viable because its adult weight change intervals overlap with the Ref. soil interval, but the AWSa interval has a greater overlap. Clearly, BWSa is not innocuous (Fig. 2a). In addition, Figure 2b shows three groups that have means significantly different than Ref. soil. In summary, AWSa has a reproduction significantly greater than Ref. soil (Fig. 2b) and the growth is similar to it. Therefore, AWSa results the most viable biomixture. The characteristic of viable biomixtures and Ref. soil are shown in Table 6.

Average performance per individual on innocuous biomixtures related to the Ref. soil is shown in Figure 3. According to Figure 3a, it can be noticed that the adult biomass increased at 56 days in the followings substrates: control BWS, control and applied AWS and control and applied BWSRW. That is probably due to the mutualism relationship between earthworms and microorganisms during the exposure, similar to the nutrient enrichment processes where *E. fetida* would modify the substrate structure with mucus production.[29,30] This mucus stimulates the appearance of a more active and specialized microflora for residue degradation.[19]

Table 6. Characteristics of viable biomixtures and reference soil.

Composition (%)	AWS	BWS	BWSRW	Ref. soil
Sand	3	27	23	1.98
Silt	31	17.7	15	63.6
Clay	12.6	7.2	6	34.42
Organic matter	4.1	4.4	7.8	4.5
Ca	8.6	27.9	23.3	14.51
Mg	3.9	2.7	2.3	3.63
Nitrogen	0.085	0.156	0.230	0.266
Bulk density (g.cm^{-3})	0.838	0.938	0.749	0.930
Phosphorus dry basis	1.1×10^{-6}	1.5×10^{-6}	1.4×10^{-4}	2.9×10^{-6}
pH	6.27	6.30	7.23	6.10

AWS (soil A, wheat stubble), BWS (soil B, wheat stubble), BWSRW (soil B, wheat stubble, river waste).

Casabé et al.[31] and Santos et al.[32] reported that soil organisms, including earthworms, tolerate glyphosate contaminated substrates showing no alterations in behavior, even at higher conditions than those applied in the crops. Others studies[33,34] related to glyphosate and earthworms stated that the lack of glyphosate direct effects on growth and reproduction of these soil organisms could be due to the herbicide capacity to stimulate and increase the availability of soil nutrients (for example, nitrates and phosphates) which are derived from their degradation. These nutrients could stimulate the microorganisms that are a food source for earthworms. Regarding earthworms fecundity, biomixtures control BWS and applied AWS showed the highest values (Fig. 3b) where the number of cocoons in the applied AWS biomixture exceeded the respective control in detriment to biomass (Fig. 3a). Santamaría and Ferrera-Cerrato[35] reported coincident results, indicating an inverse relationship between weight and reproduction, where high *E. fetida* reproduction rate corresponds to a weight loss per individual at the end of the test. Figure 3d shows that juveniles biomass after 116 days was high in all viable biomixtures (control and applied). The favorable evolution of *E. fetida* on these biomixtures, particularly on AWS, is related to the capacity to degrade or stabilize the organic residues under aerobic conditions and at the same time promote their degradation by microbial action. Autochthonous microorganisms have the capacity to remove pollutants from soil, but their mobility is limited. Earthworms increase the contact between contaminant and the soil microorganism. If the availability of the contaminant is low (e.g., glyphosate and AMPA according to the soil type) being its degradability high, the application of earthworms will speed up the contaminant removal from soil.[17]

Little information is available for AMPA toxicity on invertebrates present in soil. In a recent work, Domínguez et al.[5] studied the effect of AMPA (concentrations between 0.1 to 2.5 mg kg^{-1}) on mortality and reproduction of *Eisenia andrei* species using an artificial soil. In both, acute and chronic assays, no significant mortality was recorded; but the results suggest that earthworms coming from parents grown in contaminated soils may have reduced growth. It is important to highlight that AWS biomixture had a high performance in all parameters evaluated even it had the higher AMPA concentration at the beginning of the bioassay (99 ± 11 mg kg^{-1}).

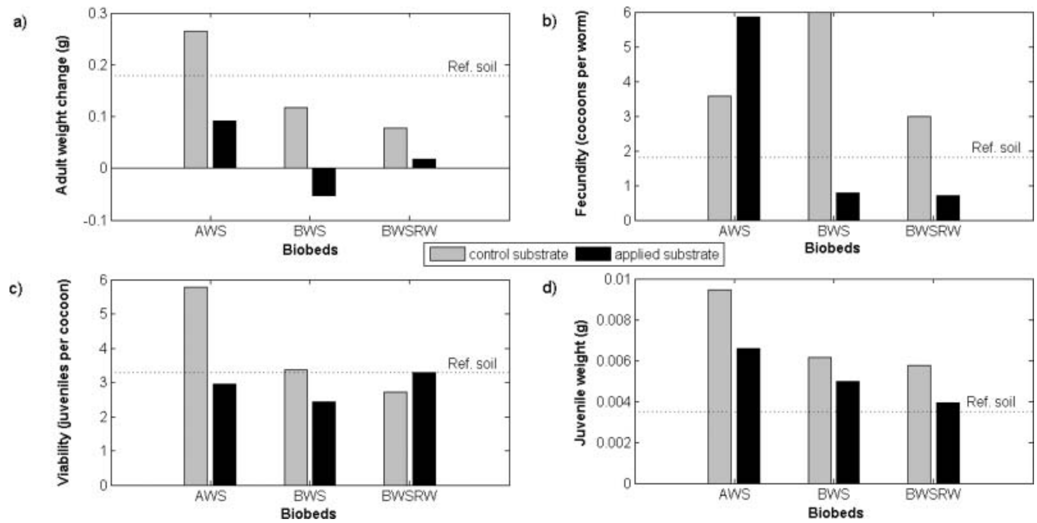

Figure 3. Average performance per individual on innocuous biomixtures related to the Ref. soil. (a) Adult weight change in a period of 56 days; (b) Fecundity at 56 days of exposure; (c) Viability at 116 days; (d) Juvenile weight at 116 days.

Studied parameters on biomixtures (adult weight change, fecundity, viability and juvenile weight) were also compared in Figure 3 with those obtained from the Ref. soil. At the end of the bioassay (116 days), the performance of juvenile earthworms was higher on biomixtures than on the Ref. soil (Fig. 3d).

The bioassay can be considered as a combination of composting and vermicomposting processes. The main factors that affected vermicomposting (characterized by growth G and reproduction R) were: nutrients (Mg), aeration (D) and water holding capacity. Water holding capacity and nutrients availability were characterized by the percentage of Clay (Cl) and Sand (Sa). Therefore, nutrients availability and water capacity would be higher when the percentage of silt and clay increases in the biomixtures. In the other hand, moisture content for vermicomposting process is essential; low bulk density values

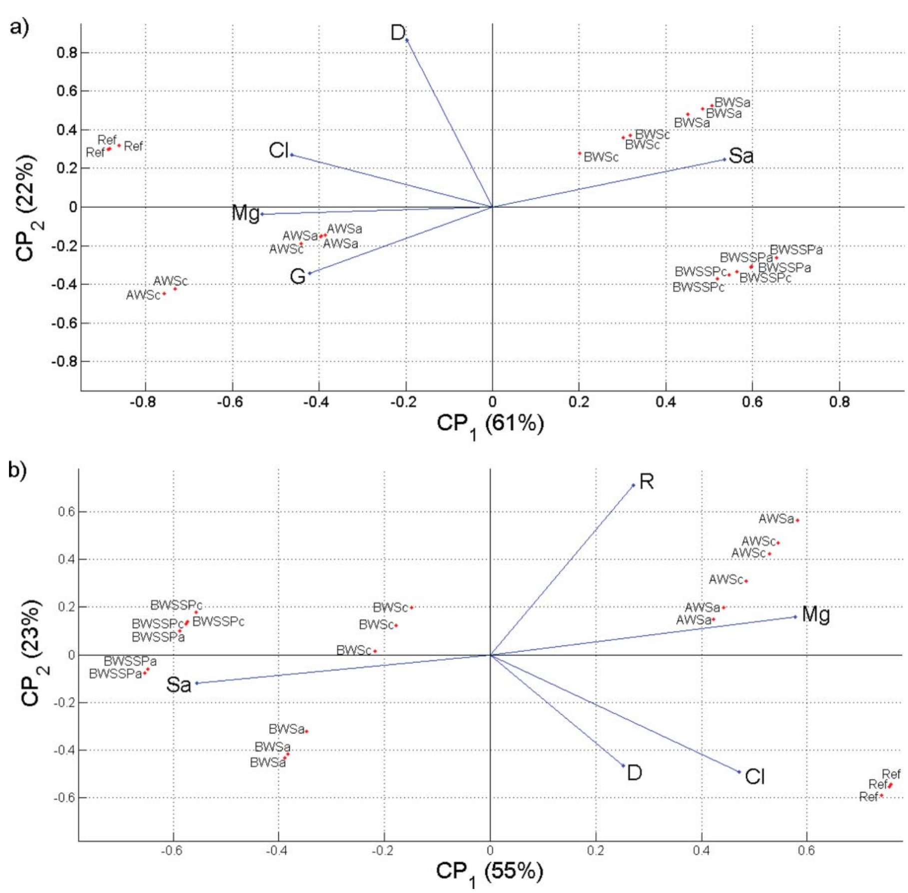

Figure 4. Biplot based on 1st principal components (PC_1) and 2nd principal components (PC_2) of data matrix (21 × 5). (a) Correlation structure of Sa, Cl, Mg, and D with G. (b) Correlation structure of Sa, Cl, Mg and D with R.

would provide more aeration to biomixtures (D). Thus, vermicomposting process would be faster.

A PCA (Principal Component Analyses) was performed to analyze the correlation between these variables (Sa, Cl, Mg, D, G and R) in a reduced space.[36] Figures 4a and 4b show biplots only considering the first two principal components, where the treatments are also identified. The projections dispersion in these reduced spaces represents approximately the 80% of the data variability. These biplots allow to visualize the magnitude and sign of each variable contribution (Sa, Cl, Mg, D, G or R) to these first two principal components (CP_1 and CP_2) and how each treatment is represented in terms of these components (or latent variables). These figures also show the relationships between Sa, Cl, Mg, D and G or R.

Figures 4a and 4b show positive correlations of Mg and Clay respect to G and R, and negative correlations of Sand (Sa) and Density (D) respect to G and R.

Biomixture AWS has better growth (G) and reproduction (R), because nutrient concentration was higher in this biomixture. In addition, other factors as aeration (determine by minor density) and nutrients availability imply higher values of G and R.

Conclusions

According to the evaluated bioassay parameters, the spent biomixtures AWS, BWS and BWSRW resulted safe. In consequence, these innocuous biomixtures can be used as organic amendments or recycled material for new treatments on biobeds. Also, PCA indicates that biomixtures containing high silt and clay percentages and minor density renders higher values of earthworm growth and reproduction. Vermicomposting technology was an efficient and viable method to decontaminate spent biomixtures, especially when AMPA, one of the main glyphosate breakdown products is present at high concentrations. Also, this simple bioassay could be used as a standard method on spent biomixtures to easily determine its toxicity.

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