Earthworm assemblages in different intensity of agricultural uses and their relation to edaphic variables

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Abstract

The objective of this study was to relate earthworm assemblage structure with three different soil use intensities, and to indentify the physical, chemical, and microbiological variables that are associated to the observed differences in earthworm assemblage structure between soils. Three soil uses were evaluated: 1- Fifty year old naturalized grasslands, low use intensity; 2- ~~Cattle-grazing~~Recent agricultural fields, intermediate use intensity ~~converted to feedlot within the two years before the start of this work~~, and 3- Fifty year old intensive agricultural fields, high use intensity. Three different sites for each soil use were evaluated from winter 2008 through summer 2011. Nine earthworm species were identified across all sampling sites. The sites shared five species: the native *Microscolex dubius*, and the introduced *Aporrectodea caliginosa*, *A. rosea*, *Octalasion cyaneum*, and *O. lacteum*, but they differed in their relative abundances according to the system. The results show that earthworm community structure is linked to and modulated by soil properties. Both, species abundance and diversity showed significant differences depending on soil use intensity. A PCA analysis showed that species composition is closely related to the environmental variability. The ratio of native to exotic species was significantly lower in the intensive agricultural system when compared to the other two, lower disturbance Systems. *Microscolex dubius* was shown to be related to the naturalized grasslands and it was associated to Ca, pH, Mechanical Resistance, and to respiration. *Aporrectodea caliginosa* was related to high K levels, low enzymatic activity, slightly low pH, and low Ca, and appeared related to the highly disturbed environment. *Eukerria stagnalis* and *Aporrectodea rosea*, commonly found ~~un the~~in ~~cattle-grazing~~recent agricultural system, were related to high soil ~~humidity~~moisture condition, low pH, low Ca and low enzymatic activity. These results show that earthworm assemblages can be good descriptors of different soil use intensities. In particular, *Microscolex dubius*, *Aporrectodea caliginosa*, and *Aporrectodea rosea*, showed different temporal patterns and species associations, due to the changes in soil properties attributable to soil use intensity, defined as the amount and type of agricultural operations.

Comment [CEC1]: Related to the Cattle-grazing comment by reviewer 1

Comment [CEC2]: As requested by reviewer 1

Keywords: soil ecology, soil use intensity, earthworms, bioindicator, farming systemssoil biota

Comment [CEC3]: Reviewer 1

1. Introduction

The organisms living in the soil, collectively known as soil biota, play a crucial role in regulating processes like water infiltration and storage, decomposition and nutrient cycling, humus formation, nutrient transformation and transport; moreover, they stimulate the symbiotic activity in the soil, improve the organic matter storage, and prevent erosion (Coleman & Crossley, 1996; Lavelle et al., 2006).

Comment [CEC4]: Related to comment by reviewer 1

Several of the ecosystem services ~~of the~~provided by soil depend on the community of soil invertebrates (Lavelle et al., 2006), ~~being~~ earthworms are one of the most common components of the edaphic communities. Earthworms are considered ecosystem engineers because they improve decomposition processes and nutrient cycling (Lavelle et al, 1997; Six et al, 2004) and have a strong effect on the soils´ hydraulic properties (Lee, 1985; Edwards & Bohlen, 1996; Lavelle & Spain, 2001; Lavelle et al., 2006; Johnson-Maynard, Umiker, & Guy, 2007; Jouquet et al, 2008). As key detritivores, earthworms are essential for soil nutrient recycling, and maintenance of soil structure (Dennis et al, 2012)

The most important factors limiting earthworm populations are food supply, moisture, temperature, and the texture and soil chemical characteristics such as pH, organic matter and macronutrients content (Satchell, 1967; Lee, 1985; Curry, 2004). Earthworm populations are also affected by the direct and indirect effects related to the type and extension of the vegetation cover (Mather & Christensen, 1988; Falco & Momo, 1995). Due to the strong relation between earthworms and soils (Paoletti et al, 1998), modern agricultural practices can modify the physical and chemical soil environment thus modulating changes in abundance and composition of earthworm communities (Curry, Byrne & Schmidt, 2002). In this regard, Dale & Polasky (2007) indicate that in agricultural systems, changes in land cover are the direct result of management practices. Moreover, the use of pesticides and herbicides in intensive agricultural systems is known to affect earthworms at different levels, from gene expression and physiology, to the individual and population levels to community structure (Pelosi et al, 2014, Santadino, Coviella, & Momo, 2014). In a study encompassing five different land use intensities, Feijoo et al, (2011) found that high use intensity leads to a loss of native species. Furthermore, the use of heavy machinery prevents these native species from re-colonizing due to soil compaction. Therefore, when changes occur in agricultural practices, earthworm assemblages are able to respond to the ensuing changes in the

Comment [CEC5]: Related to comment on pesticide effects by reviewer 1.

soil's physical properties and environmental conditions~~Therefore, when changes in soil use occur due to different agricultural practices, earthworms' assemblages rapidly respond to them~~ (Lavelle et al, 1997; Johnson-Maynard, Umiker, & Guy, 2007).

Comment [CEC6]: Related to comment by reviewer 1

Since earthworm abundance and distribution are strongly influenced by the environmental conditions and the ecological status of the system (Paoletti, 1998, Falco & Momo, 2010, Pelosi, 2015), earthworm community structure can be successfully used as biological indicators of soil conditions (Paoletti, 1999, Momo, Falco, & Craig, 2003). Guéi & Tondoh (2012) found that earthworms can be used to monitor land-use types with different levels of soil quality.

Comment [CEC7]: Citation added

The use of bioindicators has the advantage of providing historical and functional information about soils. Earthworm community structure integrates this information on soil conditions both in space and time and provide signals of the soil ecological state.

In this context, the objectives of this study were: 1) To assess earthworm community structure under three different soil use intensities: intensive agriculture, ~~cattle grazing~~recent agriculture, and naturalized grasslands. 2) To identify the physical, chemical, and microbiological variables related to the different soil use intensities affecting earthworm community structure. 3) To detect which earthworm species are typical of each set of soil conditions as affected by use intensity.

Comment [CEC8]: Related to the Cattle-grazing comment by reviewer 1

Comment [CEC9]: Rephrased for clarity

2. Materials and Methods

2.1 Sampling sites

This study was performed in the rolling pampas within the Argentine pampas, a wide plain with more than 52 million hectares (520~~.~~,000 km^2) of land suitable for cattle rising and cropping (Viglizzo et al, 2004). It is one of the largest and most productive agricultural regions in the world.

Agricultural systems based on crop–crop and crop–pasture rotations under grazing conditions have been very common in the region for over a century until the 1980s. The adoption of conservative tillage and no-till practices has significantly increased during the 1980s and 1990s. Although pesticides were extensively used since the 1960s, crops and pasture fertilization increased noticeably only during the 1990s (Viglizzo et al, 2003). The expansion of the land area used for annual crops on different environments means that the pampean ecosystem is currently under an intense disturbance regime (Navarrete et al, 2009).

The selected study sites are located in central Argentina, on ~~argiudol~~ Argiudoll~~soil~~s, (Mollisols, Typic~~al~~ ~~argiudols~~ Argiudolls (USDA, 2010)). The study sites were privately owned fields located in Navarro, Buenos Aires Province (34° 49’35” S, 59° 10´ 38” W), and Chivilcoy (35º 03’10” S; 59º 41’ 08” W) approximately 75 and 150 km west of Buenos Aires City, respectively (Fig. 1).

Comment [CEC10]: As suggested by reviewer 1

Weather regime in this region is temperate humid, with a ~~n average~~mean annual rainfall around 1000 mm. The mean annual temperature is 17 °C. Phytogeographycally, it is within the neotropical region, oriental district of the Pampean Province, and the dominant vegetation is a gramineous steppe (Cabrera & Willink, 1973).

2.2 Land use intensity in the selected sites

The systems analyzed differed only in their use intensity, as defined by the amount and type of agricultural operations, such as tillage, pesticide use, rotation, fertilization, and harvesting. Samplings were carried out on three different type of soil uses (agroecosystems) which represent three different levels of disturbance of the same ~~a~~Argiudoll ~~soil~~:

Comment [CEC11]: As requested by reviewer 1

High disturbance agroecosystem: Intensive agricultural systems (AG), sites with 50 years of continuous intensive agricultural practices~~.~~ ~~Under~~ under a regular corn-wheat-soybean rotation, currently under no-tillage, and chemical weed control ~~are~~ is used. During ~~the~~ cropping season, heavy machinery is used and insecticides, herbicides, and fertilizers ~~are~~ were applied several times a year.

Comment [CEC12]: Requested by reviewer 2

Intermediate disturbance agroecosystem: Recent agricultural system (RA). Cattle-grazing sites with 40 years under direct grazing, turned to a feedlot system ~~within the~~ two years before the start of this work. Originally managed under direct grazing, it moved to bale production (oat, maize, and sorghum) two years prior to the start of this study.

Comment [CEC13]: Related to the Cattle-grazing comment by reviewer 1

Comment [CEC14]: Requested by reviewer 2

Low disturbance agroecosystem Naturalized grasslands (NG), sites with no significant anthropic impact during the last 50 years.

Comment [CEC15]: Requested by reviewer 2

Nine ~~sampling~~ observation fields (three replicates for each one of the three agricultural systems) were evaluated. Replicates were separated from each other from at least several hundred meters to a few kilometers. At each site, five samples were taken every three months ~~covering~~ for a period of two years.

Comment [CEC16]: Requested by reviewer 2

~~Each~~ At each sampling date, five random samples were taken 25 meters apart from each other per each replicate (3) and treatment (3). Thus, a total of 45 samples were taken per sampling date. The size of each sample was of 25 x 25 x 25 cm.

The measured environmental variables were bulk density (BD), mechanical resistance (MR), ~~humidity~~ gravimetric moisture content (~~RH~~*w*), electrical conductivity (EC), organic mater (OM), pH, total N, total P, exchangeable Ca, exchangeable Mg, exchangeable K, and exchangeable Na. To characterize the sites, microbiological activity was assessed through soil respiration and free nitrogen-fixing bacteria activity (Nitrogenase Acetylene Reduction Activity, ARA). Methods used for chemical and physical analyses are shown in Table 1.

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Earthworm extraction from the soil samples was performed by handsorting. Earthworms were preserved with soil until identification in the laboratory, and later fixed and preserved in alcohol- formalin - glycerin following Righi (1979) and identified by external morphology using keys from Righi (1979) and Reynolds (Reynolds, 1996). Clitelated individuals were identified to species level and pre-clitelated ones to genus. At each site, earthworm taxonomic composition and population density were measured. Earthworm communities were characterized at each soil use intensity by population density, species richness both observed and estimated using the Chao index (Magurran, 2004), and diversity by the Shannon index (Zar, 1999).

The Chao index was calculated as:

$$\hat{S} = S_{obs} + (a^2 / 2b)$$

were:

$\hat{S}$: Species richness estimate

S_{obs}: Observed species richness

a: Number of species found in only one sample, and

b: Number of species found in only two samples

Comment [CEC17]: According to the Editor´s request to define the Chao index

The Shannon index (H´) was calculated as

$$H´: -\Sigma p_i * \log_2 p_i$$

were:

p_i: Number of individuals belonging to the species i / total number of individuals

Comment [CEC18]: According to the Editor´s request to define the Shannon index

Comment [CEC19]: Following Editor´s comment

2.3 Statistical analyses

Due to the non-normal distributions of the physical and chemical data, Kruskall-Wallis ANOVA tests were carried out to compare variables between treatments. The Shannon index comparisons were performed using ANOVA.

Comment [CEC20]: As requested by reviewer 2

The relationship between environmental variables and earthworm species abundances was analyzed by means of ~~a~~ principal component analysis (PCA) using abundances. Prior to analysis, the species abundances data were transformed using the Hellinger distances (Legendre & Gallagher 2001) ~~this results in an analysis that~~in order ti preserve the distances among samples. To calculate the Hellinger distances, the abundances are first divided by the sample total, and the result is square root transformed, then euclidian distances are computed. As a result, Hellinger distances are scalar measurements of the divergence in the distribution of samples. ~~This distances~~These distances are then used in PCA. ~~Prior to analysis, the species abundances data were transformed using the Hellinger method of Legendre & Gallagher (2001) such that the resulting PCA represents Hellinger distance between samples rather than Euclidean distance.~~ Physical and chemical variables were then fitted into the ordination space described by the first two principal components of the earthworm data by projecting biplot vectors. The statistical significance of the environmental variables is based on random permutations of the data and P-values were adjusted by a sequential multiple test procedure of Hommel (1988). The ordination analysis and vector fitting were produced using the R statistical language (R Core team, 2012) and the Vegan package (Oksanen et al, 2011).

Comment [CEC21]: Regarding the comment by the editor about Hellinger transformation

The relationship between the characteristics of the environment and earthworm presence was further analyzed at the genus level, assessing the sensitivity of the groups with the soil parameter values through a Mann-Whitney U-test. The program Statistica 7.0 (Stat Soft, Inc.) was used.

Comment [CEC22]: As requested by reviewer 2

3. Results

3.1 Physical and chemical soil parameters

Of all the physical - chemical and microbiological parameters evaluated, only four variables: exchangeable Sodium (Na) , Electrical conductivity (EC), Mechanical resistance (MR), and respiration) showed significant statistical differences between each of the three systems and only organic matter (OM) presented no differences (Table 1). From the four variables that separate the three systems, the naturalized grasslands showed the highest Na and EC values.

Microbiological activity and soil microfauna were assessed through soil respiration and nitrogen fixing bacteria activity, which separated the naturalized grasslands for their high value when compared to the other two agroecosystems.

3.2 Earthworm assemblage response to soil use intensity

Results show that each soil use presents a different species composition and abundance (Fig 2). The relative abundances of the earthworm species found in each system is shown in figure 3.

A total of 9 earthworm species were identified across all systems. Five species were common to all of them: the native *Microscolex dubius* (Fletcher, 1887) and the exotic *Aporrectodea caliginosa*, (Savigny, 1826) , *A. rosea* (Savigny, 1826); *Octolasion cyaneum* (Savigny, 1826), *O. lacteum* (Oerley, 1885), but differed in their abundances. The differences in abundance explain the significant differences found for the Shannon index values (ANOVA test $p<0.05$). The richness estimate ($\hat{S}$) and the observed richness (S_{obs}) only differed in the recent agricultural system (Table 2).

In the naturalized grasslands- the species identified as being the dominant (44% of all the individuals collected) was the epigeic native *M. dubius*, followed by the endogeic exotics *A. caliginosa*, *A. rosea*, *O. cyaneum*, and *O. lacteum*. The other endogeic exoyic species, *Aporrectodea trapezoides* (Dugés, 1828), and the native *M. phosphoreus* (Dugés,1837) were less frequent (Fig. 3a). Forty seven percent of all the individuals collected belonged to native species, and the ratio natives / exotics was 2:5.

Comment [CEC23]: Following editor´s comments

Comment [CEC24]: Authors added as requested by reviewer 1. Also naming of the especies was standardized from this point on

In the recent agricultural system the endogeic native *Eukerria stagnalis* (Kinberg, 1867) was dominant and the exotic *A. rosea* was also common. Other species that were present albeit with a low frequency were *A. caliginosa*, and *M. dubius*. *M. phosphoreus; O. cyaneum*, and *O. lacteum* appeared on either one or two sampling dates only. In this system, *E. stagnalis* represents 68% of all the individuals collected and *A. rosea* represents 22% (Fig. 3b).The ratio of native:exotic species was 3:4 .

In the intensive agricultural system, the most common species were the endogeic exotics *A. caliginosa*, *A. rosea*, and *A. trapezoides*. The other endogeic species *O. cyaneum*, and the epigeic native *M. dubius* were less frequent. *Octolasion tyrtaeum* (Savigny, 1826) was only detected in the first sampling date, and *O. lacteum* appears in two sampling dates with a single individual each. Here, the exotic species represent 95% of the individuals (Fig. 3c) , with *M. dubius* being the only native present in this system. The agricultural system had the lowest ratio of native.exotic species (1:6).

The differences in the chemical and physical soil parameters and species requirements determined the species´ co-occurrences found in each system. We observed these associations involving both native and introduced species, and combining different ecological categories. In this way the associations most frequently found in naturalized grasslands were: *A. rosea – M. dubius* (appearing together in 33% of the samples), *O. cyaneum – O. lacteum* (10%), and *A. rosea –O. cyaneum* (10%). In the recent agricultural sites *A. rosea –E. stagnalis* (67%) and in the agricultural system the most common associations were *A. caliginosa –A. rosea* (12.5%), and *A. rosea – M. dubius* (12,5%).

The relationship between the characteristics of the environment and earthworm presence assessing the sensitivity of the groups with the soil parameter values is shown in Table 3.

Aporrectodea, *Octolasion* and *Microscolex* genus were present in samplings with the same levels of Mg, K, and BD. *Octolasion* separated from *Aporrectodea* only for Ca levels, and its response to soil ~~humidity~~moisture, MR, and Respiration put it close to *Microscolex*. In turn, *Microscolex* differed from the other groups due to Na, pH, ARA, and high MR (MR 10 cm). On the other hand, *Eukerria* was related to places with low levels of Ca, K, pH, EC, ARA, BD, MR and high ~~humidity~~moisture content.

In order to know how the species´ composition explains the environmental variability, an indirect ordination PCA analysis was used, followed by a vector fitting (Fig. ~~3~~4). Interestingly, the analysis showed no relationship between species with fertility levels (N, P, and OM), but it did show a relationship with exchangeable Mg and Ca, ~~elements of low soil mobility~~.

The first two axes explain 58% of the variance. The environmental variables that were significantly related to the species ordination were: ~~RH~~moisture, K, ARA, Respiration, MR, Ca, and pH (adjusted P < 0.05).

As it can be seen in Fig. ~~3~~4, the ordination method shows that *M. dubius* appeared related to the levels of Ca, pH , MR and respiration. This species is well adapted to environments rich in Ca, neutral pH, high microbiological activity, and high mechanical resistance. The environment defined by *M. dubius* was related to the characteristics of the Naturalized Grassland system, and this species can be considered as indicative of the conditions prevailing in this system. In the same way *A. caliginosa* (Fig. 4) is related to high K levels, low enzymatic activity, low pH, and low Ca. These are characteristic of the Intensive agricultural system, being this cosmopolite, invasive species a good indicator of high perturbation sites. Finally, *E. stagnalis* and *A. rosea*, were related to the second ordination factor, and they describe an environment with high soil ~~humidity~~moisture, low pH, low Ca levels, and low ARA. These characteristics describe the Recent agricultural system. In this way, these species are clearly good descriptors of the three studied use intensity regimes of the same soil.

4. Discussion

These results show that the structure of the earthworm assemblage changes in relation to differences in soil use intensity in terms of its composition, abundance, and species associations. The data presented here show that, on the same soil and the same regimen of temperature and precipitations, the earthworm assemblage composition and abundance varied across the different systems ~~studied~~. ~~Therefore~~~~thus~~ ~~reflecting t~~These variations describe the physical and chemical differences of soil due to land use intensities and their associated management practices (Geissen et al, 2009).

Comment [CEC25]: Examples added as requested by reviewer 1

Comment [CEC26]: Following Editor´s comment #1

Comment [CEC27]: Citation added

Tillage, weed control, fertilization and soil cover are parameters that best characterize the different land use intensities (Curry, 2004; Viglizzo et al, 2004; Decaëns et al, 2008), modifying the physical (water and air movement) and chemical environment, thus changing habitat suitability.

In the AG system under highest use intensity, earthworm communities were affected directly by the changes caused by tillage practices or indirectly through changes in food supply. Several studies indicated that earthworm communities are more abundant and rich in species in undisturbed soils when compared to croplands (Emmerling 2001; Curry et al, 2008; Decaëns et al, 2008; Feijoo et al, 2011; Felten & Emmerling, 2011). In this study, however, this pattern was not observed. All three systems have the same richness value ~~and~~ but the abundances are consistently higher in the AG system with the highest use intensity. This system also showed the highest native species substitution by exotic ones (ratio 1:1.6). Total individuals belonging to invasive species varied from 16.6% in NG to 40~~,~~.4% in the AG system. These results agree with those of Lee (1985), Paoletti (1999), and Smith et al. (2008), who found that annual croplands have higher earthworm abundance than older fields. The dominance of introduced species is another characteristic of highly disturbed sites, as pointed out by Fragoso et al. (1999), Winsome (2006), and Chan and Barchia (2007).

Comment [CEC28]: Related to comment by reviewer 1 on invasive species

The results presented in this work indicate that earthworm assemblage response to the same soil subjected to different use intensities can be used as a biological indicator of agroecosystem ~~soil use intensity~~. This response can be explained in terms of quality and quantity of food (Bohlen et al, 1997), the long term use of inorganic fertilizers which have a positive effect on the total number of worms (Edwards & Bohlen, 1996; Curry, 2004), pH changes and the level of Ca in the soil (Lee, 1985; Paoletti, 1999; Smith et al, 2008).

In the intensive agricultural and ~~cattle grazing systems~~recent agricultural systems, microbiological activity was low (as assessed through respiration and ARA) when compared to the naturalized grasslands. This can be explained, as the result of a reduction in pH and Ca, as well as to the ecological categories of the earthworm species present (Scheu et al, 2002). Indeed, Scheu (2003) ~~indicates~~ indicated that the presence of endogean species significantly reduces bacterial biomass and the functioning of the microbial assemblage. In AG, 95% of the species present are exotic endogeans, while in the ~~CG~~ RA system 97% are endogean (70 % native, 30% exotic).

Comment [CEC29]: Related to the Cattle-grazing comment by reviewer 1

Comment [CEC30]: Related to the Cattle-grazing comment by reviewer 1

Soil use intensity was also indicated by the presence of a few species that were closely related to environmental variability. The intensification of the agricultural activities in the Pampas determined up to a 50% reduction in the calcium level (Casas, 2005). The ordination analysis related *M. dubius* with high Ca levels and thus, to less disturbed environments.

In this sense, Mele and Carter (~~Mele & Carter,~~ 1999) point out that the distribution and number of native species are negatively correlated with P, K, and Mg levels, these species being adapted to lower nutrient levels. In our study the only species that is related to higher K levels is *A. caliginosa*, which is the most abundant one in the agricultural system.

5. Conclusions

The data ~~gathered~~from this study indicate that the three agricultural systems ~~studied~~ are different in terms of the levels of exchangeable cations (Ca, K), pH, microbiological activity, and physical variables such as mechanical resistance and moisture. ~~Being the original, pristine soil the same~~While the soil type ~~a~~Argiudoll ~~for~~is the same for all ~~the~~ three systems ~~studied~~, changes in land use intensity ~~are the main~~ caused ~~for~~ the observed differences in soil chemical and physical properties. Earthworm species assemblage also reflected the changes in these variables and are therefore good descriptors of the studied systems.

The high diversity and highest number of earthworms found in the AG system under no tillage are consistent with the results by Pelosi, Bertrand & Roger-Estrade (2009), who found that soil tillage and surface mulch are important parameters for the study of the effects of agricultural practices on earthworm communities.

M. dubius was associated to sites with high levels of calcium, microbiological activity and high mechanical resistance and describes the naturalized grassland. *E. stagnalis* is primarily associated with high ~~humidity~~moisture condition ~~as seen in the cattle grazing system in which it is the dominant species.~~, and *A. caliginosa* is associated to highly disturbed environments, with high K levels, low ~~CE~~ EC and ~~NA~~Na, and low microbiological activity, all typical of the intensive agricultural system.

Comment [CEC31]: Following Editor´s comment #1. See rebuttal letter for explanation

Comment [CEC32]: Related to the Cattle-grazing comment by reviewer 1

Comment [CEC33]: Following comment by reviewer 2 about repeated Concepts on Es y Ac species

E. *stagnalis* is indicative of high ~~humidity~~moisture condition, increased soil acidity, and a reduction in the levels of calcium and potassium, which are conditions prevalent in the intermediate use intensity system.

A. caliginosa is the species best adapted to the most disturbed environment. This implies that the population recovers quickly after a disturbance (Curry, 2004, Felten & Emmerling, 2011; Decaëns, 2011), as it is known not to be significantly affected by changes in litter quality (Curry & Schmidt, 2007).

It is interesting to note that the earthworm species most related to the different systems, are not linked to the variables most usually measured: OM, N, and P. Therefore, monitoring these species would provide indirect ~~estimations of those scarcely~~indicator of ~~measured~~ nutrient variables, such as Mg, Ca or K (Fig. 4), thus complementing the information provided by other more common soil analyses in agroecosystems.

The patterns in the distribution and abundance of earthworm species observed in this work followed the differences in the physical and chemical variables measured on the different systems ~~studied~~. An important difference between the studied systems is related to the agrochemicals used in the AG system. Earthworms are strongly affected by herbicide and insecticide use (Pelosi et al, 2014, Santadino, Coviella, & Momo, 2014), and their effect is also reflected in the differences in earthworm composition. All these differences are, in turn, a reflection of the different management practices applied to the same argiudol soil.

The richness, composition and abundance as well as the species associations found, reflected the physical, chemical, and biological changes brought about as a result of the different intensities of the agricultural practices used on each ~~tested~~ system. The high abundance of the native *M. dubius* was associated to the less anthropi~~zed~~c activity ~~system~~. As a result, a strong population density reduction of this species can be interpreted as indicative of a high disturbance regime. On the other hand, *A. caliginosa* increased its density as disturbance increased. *A. caliginosa* presence ~~in high densities,~~ is clearly associated to high disturbance environments as it was indeed found in several other published works (Johnston, et al, 2014; Lüscher et al, 2014).

An increase in soil use intensity, leads to changes in the physical and chemical properties of the same original soil. Earthworm assemblages are then affected by two main mechanisms. Firstly, by the agrochemical and mechanical perturbations

Comment [CEC34]: Following comment by reviewer 1

Comment [CEC35]: ADD TO BIBLIO BEFORE submitting introduced by intensive agricultural practices. Secondly, by the effects that changes in the chemical variables have on earthworm assemblages, (eg. Ca content). On the other hand, as ecosystem engineers earthworms affect edaphic variables, for example microbiological activity. Therefore, the association between presence and abundance of the different earthworm species, can be used as a biological indicator of the physical and chemical conditions of the soil they inhabit. These results show that the structure of the earthworm assemblages can be reliably used for monitoring different soil use intensities.

Comment [CEC36]: On the comment about an attempt to provide a set of indicators based on earthworms by reviewer 1.

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Comment [CEC37]: References section completely revised for consistency.

Comment [CEC38]: Added citation

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Comment [CEC39]: Added citation

Comment [CEC40]: Added citations

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Comment [CEC41]: Added citation

Comment [CEC42]: Added citations

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Comment [CEC43]: Added citation

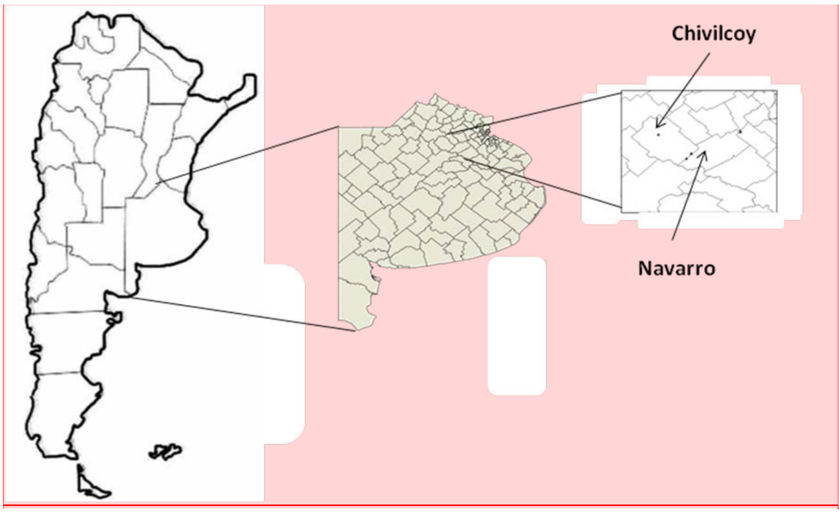

Figure 1: Map showing the location of the sampling sites

Comment [CEC44]: Location map added as suggested by reviewer 1

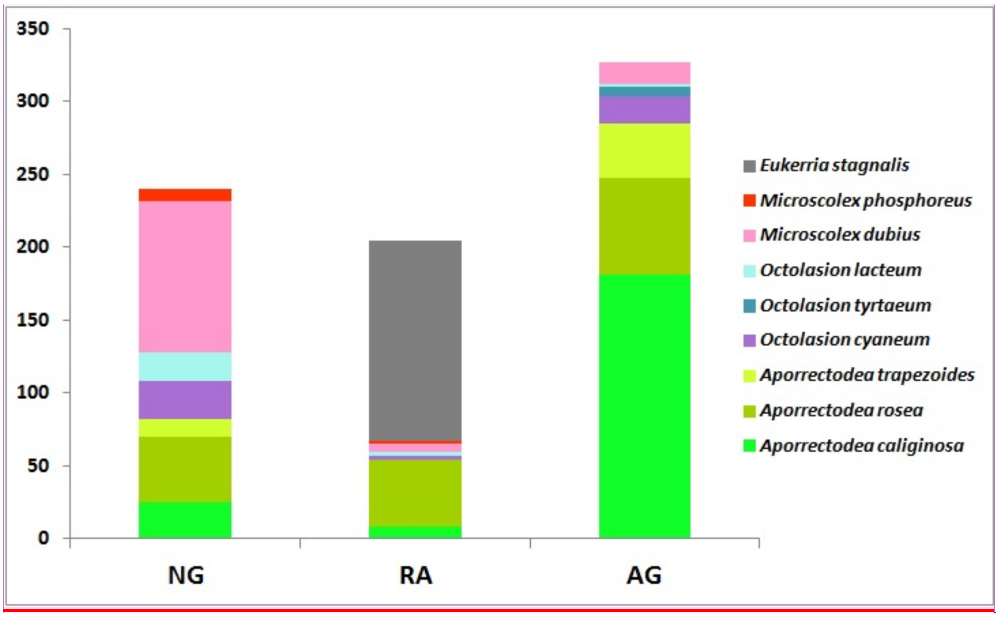

Figure 21: Abundance (N) of each earthworm species throughout the total sampling period for each system. NG: Naturalized grasslands; RA: Recent agriculture; AG Intensive agriculture

Comment [BM45]: Label Axes!

Comment [CEC46]: Because of insertion of Fig 1 as suggested by reviewer 1

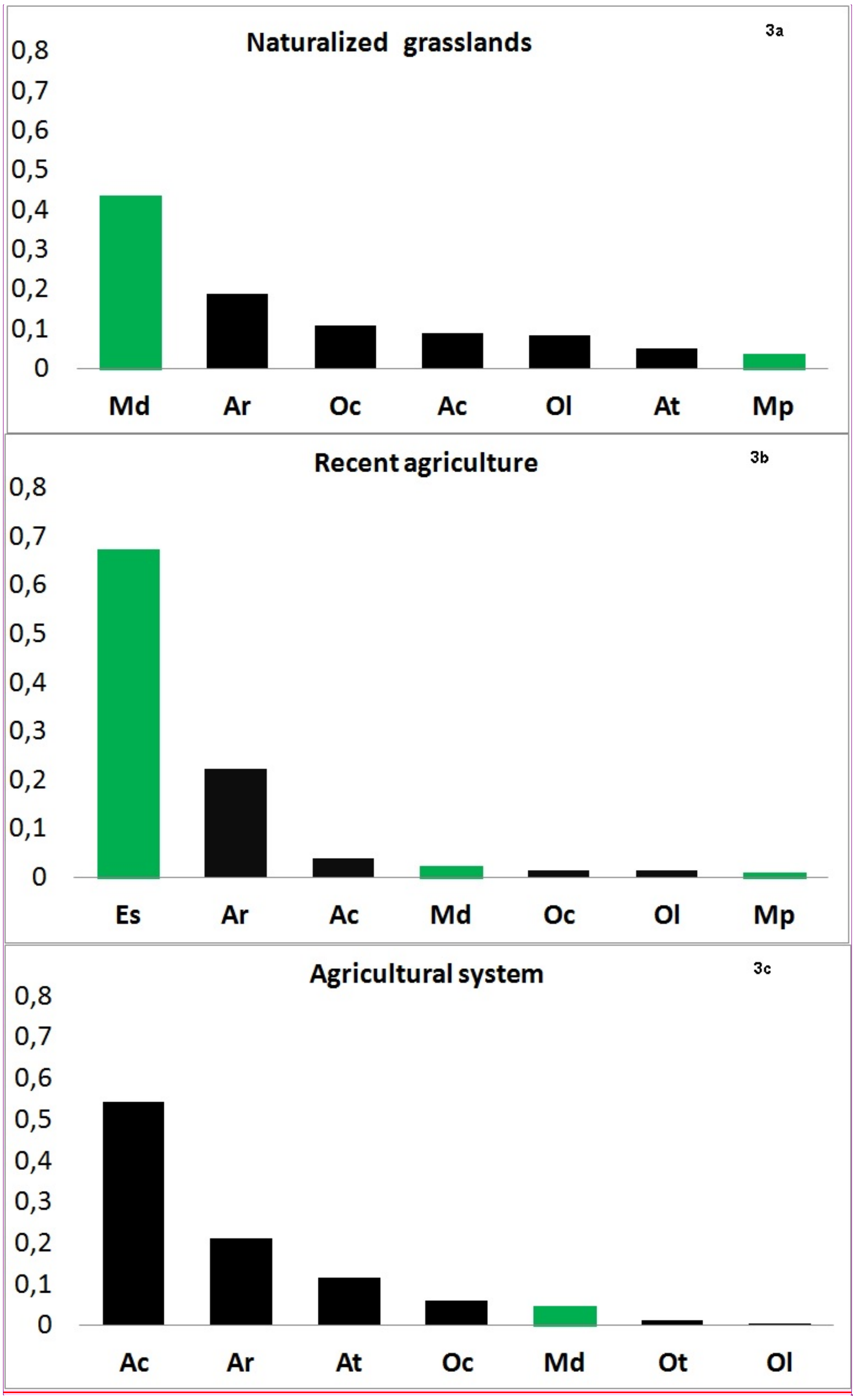

Figure 3: Relative abundance (ni/N) for each earthworm species identified in the three systems. 3a Naturalized grassland (NG), 3b Recent agricultural (RA), 3c Agricultural system (AG). Ac :*A. caliginosa*, Ar: *A. rosea*; At: *A. trapezoides*; Ot: *O. tyrtaeum*; Md: *M. dubius*; Oc: *O. cyaneum*; Ol: *O. lactuem*; Es: *E. stagnalis; Mp: M. phosphoreus.* Native species shown in green, exotics in black.

Comment [BM47]: Change axis, in decimal point! Label the axes!

Comment [CEC48]: Related to the Cattle-grazing comment by reviewer 1

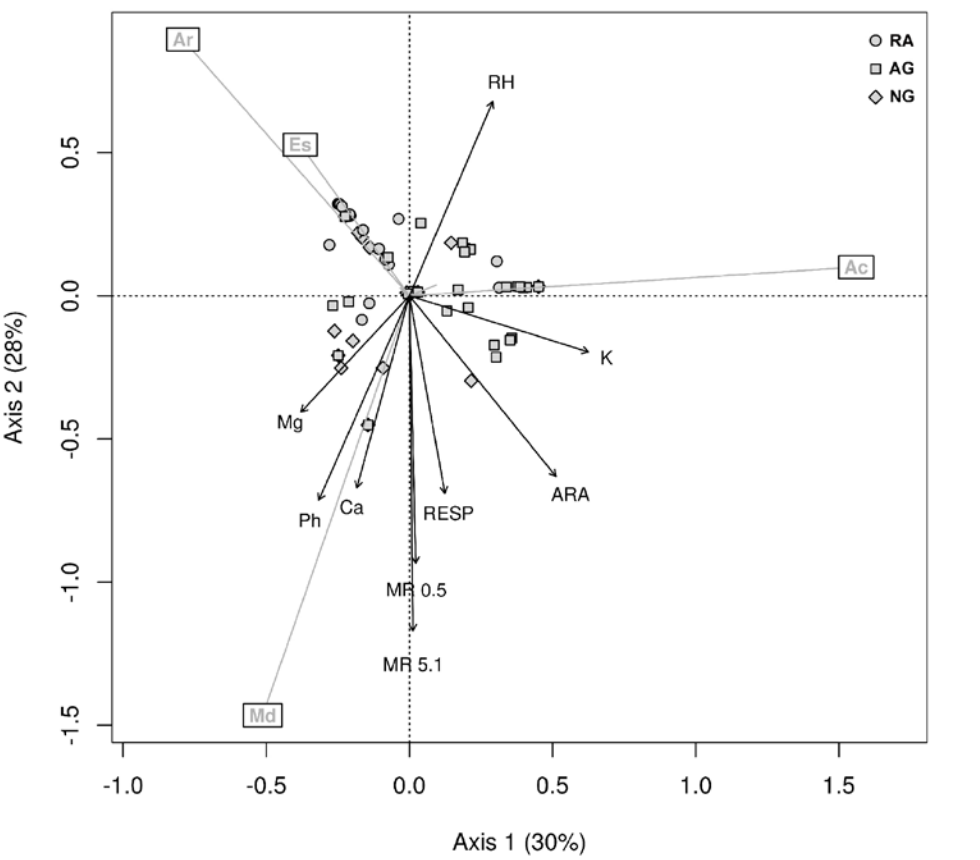

Figure 4~~3~~: PCA biplot of Hellinger transformed earthworm species, only the four most abundant ones are shown. The arrows are significant environmental variables fitted into the ordination space. The percentage of explained variance is shown in each axis. Ac: *A. caliginosa;* Ar: *A. rosea;* Es: *E. stagnalis*; Md: *M. dubius.*

Comment [BM49]: Change RH to moisture!

Table 1: Physicochemical and microbiological parameters measured (n=150 per system)

Parameters	Method	System					
		NG		~~C~~GRA		AG	
Available P (ppm)	Kurtz and Bray	11± 8.5	b	15 ± 12	a	14 ± 12	a
OM (%)	Walkey-Black	4 ± 1.5	a	4 ± 1.5	a	4 ± 1.4	a
EC (dS*m^{-1})	Conductivity meter	1,5 ± 1.3	a	0.8 ± 0.5	b	0.7 ± 0.5	c
pH	1:xx soil to water ratio???	7.5 ± 1.0	a	6 ± 0.6	b	6 ± 0.5	b
Bulk density (g*cm^{-3})	Cylinder method	1.2 ± 0.2	a	1.1 ± 0,1	b	1.2 ± 0.1	a
~~RH~~ *Moisture content* (g water*g soil^{-1})	~~Calculation~~gravim=etric	0.2 ± 0.1	a	0.3 ± 0.1	b	0.2 ± 0.1	a
Exch. Ca (cmol*kg soil^{-1})	titration with EDTA	6.7 ± 1.3	a	5 ± 0.5	b	6 ±0.7	a
Exch. Mg (cmol*kg soil^{-1})	titration with EDTA	1.8 ± 0.4	a	1.5 ± 0.7	b	1.6 ± 0.5	b
Exch. Na (cmol*kg soil^{-1})	flame photometry	1.3 ± 0.5	a	0.8 ± 0.2	b	0.7 ± 0.2	c
Exch. K (cmol*kg soil^{-1})	flame photometry	1.6 ± 0.5	a	1.3 ± 0.3	b	1.6 ± 0.5	a
Total N (%)	Kjeldahl	0.28 ± 0.1	a	0.32 ± 0.1	b	0.29 ± 0.05	b
Nitrogenase activity (nanolitres of ethylene* g dry soil*incubation hour $^{-1}$)	ARA	0.3 ± 0.3	a	0.2 ± 0.2	b	0.2 ± 0.3	b
Respiration (mg CO_2*g dry soil day^{-1})	alkaline incubation	0.09 ± 0.06	a	0.07 ± 0.05	b	0.05 ± 0.05	c
MR 0-5 cm (kg*cm^{-2})	Cone penetrometer	10 ± 6	a	2.5 ± 3	b	5.5 ± 4	c
MR 5~~=~~-10 cm (kg*cm^{-2})	Cone penetrometer	13 ± 7	a	5 ± 5	b	8 ± 5	c

NG: naturalized grassland; ~~C~~GRA: ~~cattle grazing~~recent agriculture, AG; agricultural system. Different letters within a row indicate significant differences between systems, P < 0.05 Kruskall-Wallis ANOVA tests. Means ± SE shown. P: Phosphorus; OM: Organic Matter; EC: Electrical conductivity; ~~RH: Relative humidity~~; Ca: Calcium; Mg: Magnesium; Na: Sodium; K: Potassium; MR: Mechanical resistance; ARA: Acetylene Reduction Activity.

Comment [CEC50]: Related to the Cattle-grazing comment by reviewer 1

Comment [CEC51]: All decimal points changed according to Editor´s comment

Comment [BM52]: Please check

Comment [CEC53]: Related to comment by reviewer 1

Comment [CEC54]: Requested by reviewer 2

Comment [CEC55]: Requested by reviewer 1

Table 2: Observed and estimated species richness, mean density (± SE), and Shannon diversity index. S_{obs} = Observed richness. Different letters in the Shannon index column indicate significant differences (one-way ANOVA $p < 0.05$)

	Richness observed (S_{obs})	Richness estimate (Chao)	Density (individual /m^2)	Shannon index
Naturalized grassland	7	7 ± 0	46 ± 19	0.53 a
Recent agriculture	7	8.5 ± 1.,5	40 ± 55	0.37 b
Agricultural system	7	7.25 ± 0,.4	76 ± 56	0.57 a

Comment [CEC56]: Related to the Cattle-grazing comment by reviewer 1

Table 3: Mean (range) values of each measured variable as they relate to earthworm genus presence (Non-clitelated specimens included)

Parameter	*Aporrectodea*	*Octolasion*	*Microscolex*	*Eukerria*
OM (%)	4.4 (3.7-5.3) a	4.8 (4.4-6.1) a	4.9 (3.2-5.9) a	4.7 (4-5.9) a
N (%)	0.29 (0.26-0.33) a	0.29 (0.26-.33) a	0.29 (0.27-0.34) a	0.29 (0.25-0.34) a
Available P (ppm)	8.7 (4.4-17.6) a	7.7 (3.6-15.2) a	9.3 (4.8-15) a	6.8 (4.4-14.1) a
Exch. Ca (cmol*kg soil^{-1})	6.0 (5.5-6.4) a	6.6 (6.1-9) b	6.1 (5.8-7) c	5 (4.6-5.4) d
Exch. Mg (cmol*kg soil^{-1})	1.7 (1.1-2 a	1.7 (1.1-1.9) a	1.6 (1.5-1.9) a	1.1 (1-1.6) b
Exch. Na (cmol*kg soil^{-1})	0.8 (0.7-1) a	0.74 (0.4-0.9) a	0.9 (0.7-1.1) b	0.8 (0.7-1.1) a
Exch. K (cmol*kg soil^{-1})	1.3 (1.1-1.7) a	1.5 (1.2-1.8) a	1.3 (1.1-1.8) a	1.1 (1-1.4) b
pH	6.2 (5.8-7) a	6.3 (6-6.8) a	6.8 (6.2-7.2) b	6 (5.6-6.5) c
~~Ec~~ EC (dS*m^{-1})	0.6 (0.3-0.9) a	0.6 (0.3-0.9) a	0.7 (0.3-1.2) a	0.4 (0.2-0.7) b
Nitrogenase activity (nanolitres of ethylene * g dry soil*incubation hour^{-1})	0.15 (0.07-0.3) a	0.26 (0.11-0.35) a	0.27 (0.14-0.37) b	0.15 (0.12-0.18) a
Respiration (mg CO_2*g dry soil day^{-1})	0.04 (0.03-0.09) a	0.04 (0.03-0.09) ab	0.07 (0.04-0.1) b	0.05 (0.02-0.07) a
~~RH~~ Moisture content (%)	0.3 (0.2-0.3) a	0.25 (0.17-0.29) ab	0.2 (0.2-0.3) b	0.3 (0.3-0.4) c
Bulk density (g*cm^{-3})	1.2 (1.1-1.3) a	1.21 (1.1-1.3) a	1.2 (1.1-1.3) a	1.1 (1-1.2) b
MR 0-5 (kg/cm^2)	4.6 (2.25-8.2) a	4.9 (3-8) ab	7.8 (4.3-12.5) b	0.78 (0-3) c
MR 5=10 (kg/cm^2)	6.5 (3.5-10.8) a	7.6 (4-11.5) a	10 (7-17) b	2.6 (0.8-5.5) c

Comment [BM57]: Check! 0.3 is listed a in the first column but c in the fourth column

Variables measured at the sampling points were each earthworm genus was recorded. Different letters within each row indicate significant differences between earthworm genus, $P < 0.05$, Mann–Whitney U-test pairwise comparisons. Abbreviations as in Table 1.