UNIVERSIDAD DE LA REPÚBLICA
FACULTAD DE AGRONOMÍA

POTENCIAL APORTE DE LAS LOMBRICES EN EL CONTROL BIOLÓGICO DE *Fusarium graminearum* EN AGROECOSISTEMAS URUGUAYOS

por

Gabriella JORGE ESCUDERO

TESIS presentada como uno de los requisitos para obtener el título de Doctora en Ciencias Agrarias

Montevideo
URUGUAY
Octubre 2018

Tesis aprobada por el tribunal integrado por Ing. Agr. (Dra.) Pilar Irisarri, Ing. Agr. (Dra.) Silvia Pereyra, y Dr. Cs. Biol. José Camilo Bedano, el 26 de octubre de 2018. Autora: Lic. Biol. (Mag.) Gabriella Jorge Escudero. Director Ing. Agr. (PhD) Carlos A. Pérez, Co-director PhD Jan E. Lagerlöf.

Dedico este trabajo a mi padre por predicar con el ejemplo.
Un ejemplo de vida.

AGRADECIMIENTOS

A Oswaldo Ernst, Eduardo Silva, Donato Marrero, Estanislao Chiazzaro, Alfonso Carriquiry, Adrián Ginestar, Silvana Machado y Fernando Ronca por permitir realizar los muestreos en los predios de los cuales estaban a cargo.

A Stella Zerbino por asesoramiento en metodología de muestreo.

A Enzo Grosso, por introducirme en el fascinante mundo de las lombrices.

A Juan Manuel Piaggio, Fabián Del Giorgio, Eduardo Llanos, Beatriz Irrisarri, Stefani Ramos, María Baeza, Romina Rostan, Araceli Peña, Sofía Ramos, Gustavo Acosta, Laura López, Leonardo Giménez, Martín Francia, Nazaret Ramírez Berruti y Noelia Benítez por colaboración en los muestreos y procesamiento de muestras.

A Samuel James, George G. Brown y Marie C. Bartz por asesoramiento en identificación morfológica.

A Elodie da Silva, Mailén Arleó y Claudio Borteiro por asesoramiento en técnicas moleculares y análisis de secuencias.

A Paula Persson por proveer la cepa de *Fusarium graminearum* en Suecia y a Silvia Pereyra por proveer el rastrojo de trigo con *F. graminearum* para los ensayos.

A Andrés Ferraz, Aurelio Enciso, y Sebastián García por colaborar en la colecta de lombrices para la experimentación.

A Aurelio Enciso y Luis Macedo por el diseño y construcción de recipientes para los experimentos.

A Hanna Friberg, Sara Söderlund, Helena Bötker, Jamlek Muturi John, Fredrick Ayuke, Nyashadzashe Marecha y Boitshepho Chube por asistencia en laboratorio en Suecia.

A Sebastián García, Mariana Pérez, Guillermo Stagno, Enrique Barboza, Renzo Callaba, Ángel Chocho por asistencia en laboratorio en Uruguay.

A Mario Michelazzo por asistencia con programas de SIG.

Al Laboratorio de Fertilidad del Depto. Suelos y Aguas por permitir utilizar sus instalaciones para el montaje del experimento.

A Laura Hernández por asistencia desde el Laboratorio de Fitopatología y al Departamento de Protección Vegetal por permitir el uso de la lupa.

A Anders Larsolle, por desarrollar un procedimiento especial con MATLAB para analizar el porcentaje de cobertura por rastrojo.

A Silvana Vero y Gabriela Garmendia por colaboración, capacitación y supervisión en el análisis de qPCR y al laboratorio de Bioquímica de Facultad de Química.

A María Noel Merentiel por asesoramiento en el manejo de índices de diversidad.

A Anahí Domínguez por asesoramiento en la instalación de ensayos de toxicidad.

A Soledad Martínez por el diseño de la figura del artículo de la revisión Control biológico de enfermedades y plagas promovido por lombrices.

A Claudia von Brömssen, Jorge Franco, Andrés Beretta y Mónica Cadenazzi por asistencia en los análisis estadísticos.

A mi hija, a mi compañero de vida y a mi madre por ser el sostén, a mi hermano Gerardo por las mejores fotos de lombrices que haya visto, y a mi tía Ethel por alentarme.

Al grupo vuelo, Blanca Elvira Borges y Andrea Ferrer por ayudarme a canalizar.

A los compañeros de los departamentos de Suelos y Aguas y de Sistemas Ambientales ¡por el aguante!

A Claudio Martínez y Diana Míguez, por su comprometida contribución como integrantes del comité de seguimiento del Doctorado y porque realmente me hicieron sentir acompañada.

A mis tutores, Carlos Pérez y Jan Lagerlöf, por su apoyo constante y por lograr que el olmo al fin diera peras.

A ANII, Uruguay, por financiar la Beca de Posgrados Nacionales.

A CSIC-UdelaR, Uruguay, por financiar dos becas para pasantía en el exterior y un proyecto de iniciación.

A Stiftelsen Olle Engkvist Byggmästare y Ekhagastiftelsen, Suecia, por financiamiento para el montaje de experimentos y análisis.

TABLA DE CONTENIDO

RESUMEN

Fusarium graminearum es el principal patógeno causante de la Fusariosis de la espiga (FE) en Uruguay, una de las principales enfermedades de los cereales a nivel mundial. Al sobrevivir saprofíticamente en los rastrojos, se ve favorecido en sistemas de producción en siembra directa, los cuales dejan altos porcentajes de residuos sobre el suelo. Su difícil control requiere de un manejo integrado que combine diversas medidas. En Uruguay se han estudiado alternativas de control biológico utilizando *Trichoderma* spp., pero no hay antecedentes a través de la fauna del suelo, en particular las lombrices. Éstas favorecen la productividad y sanidad vegetal tanto de forma indirecta (mediante una mejora de las propiedades del suelo) como directa (suprimiendo patógenos de plantas). Se ha demostrado que las lombrices reducen la biomasa de *Fusarium* en el suelo y en el rastrojo de trigo inoculado. En el presente estudio se exploró la posibilidad de integrar el uso de lombrices al control de *Fusarium* en agroecosistemas de trigo en Uruguay. Para esto se realizó una prospección de las comunidades de lombrices presentes en agroecosistemas donde se produce trigo en el sur y en el litoral oeste del país. Se encontró que éstas están compuestas por especies exóticas y nativas, dominando las primeras en el sur y las segundas en el litoral oeste. Entre las especies de lombrices encontradas se seleccionaron dos para evaluar en condiciones controladas su potencialidad para reducir el rastrojo de trigo inoculado con *F. graminearum*. La sobrevivencia y crecimiento de las lombrices no fue afectado por Fusarium. *Lumbricus* spp. redujeron hasta 50% de la cobertura por rastrojo mientras que *Glossoscolex rione* no se diferenció del control, probablemente debido a su hábito de vida con poca actividad en la superficie. Finalmente, ensayos de toxicidad mostraron que dos fungicidas utilizados para el control de FE, tuvieron un efecto negativo agudo y crónico sobre las lombrices, siendo la especie nativa *G. rione* más sensible que la especie de referencia (*Eisenia fetida*). Se concluye que existen lombrices en los agroecosistemas uruguayos capaces de contribuir al control de *Fusarium* y que para maximizar este servicio ecosistémico brindado por las lombrices se deberá considerar manejos del suelo que favorezcan su población.

Palabras clave: Fusariosis, lombrices nativas y exóticas, biocontrol, fitopatógeno, ensayos de toxicidad

POTENTIAL CONTRIBUTION OF EARTHWORMS TO THE BIOLOGICAL CONTROL OF *Fusarium graminearum* IN URUGUAYAN AGROECOSYSTEMS

SUMMARY

Fusarium graminearum is the main pathogen causing Fusarium Head Blight (FHB) in Uruguay, one of the most important cereal diseases worldwide. By surviving saprophytically in wheat straw, its survival is favored in no-till systems, which leave high percentages of plant residues on the ground. *Fusarium* is difficult to control, and requires an integrated management combining several control measures. In Uruguay, biological alternatives of control using *Trichoderma* spp. have been studied. However, there are no precedents of biological control through soil fauna, especially earthworms, which benefit plant growth and health both indirectly (through improved soil properties) and directly (by suppressing plant pathogens). Particularly, it has been shown that earthworms can reduce *Fusarium* biomass in the soil and in inoculated wheat straw. The present study explored the possibility of integrating the use of earthworms to control *Fusarium* in wheat agroecosystems in Uruguay. For this, earthworm communities in wheat systems in southern and western Uruguay were sampled. It was found that, while exotic earthworms dominated in the south, natives were dominant in the west. Among the earthworm species found, two were selected to evaluate their potential to reduce wheat straw inoculated with *F. graminearum* under controlled conditions. *Lumbricus friendi* (anecic) had a significant effect reducing soil straw cover, whereas *Glossoscolex rione* (endogeic) had no significant effect, probably due to their scarce activity on the surface. Finally, toxicity tests showed that two fungicides used to control FHB had an acute and chronic negative effect on earthworms, with native *G. rione* being more sensitive than the reference species (*Eisenia fetida*). It is concluded that there are earthworms in Uruguayan agroecosystems capable of contributing to the control of *Fusarium,* and to maximize this ecosystem service provided by the earthworms, soil managements that favor their population should be considered.

Keywords: Fusarium Head Blight, wheat, native and exotic earthworms, biocontrol of phytopathogen, toxicity tests

1: INTRODUCCIÓN

1.1. JUSTIFICACIÓN

Las exportaciones de productos de origen agropecuario representan el 74% de las exportaciones totales de Uruguay, dentro de las cuales los productos agrícolas son el primer producto seleccionado en exportaciones y el trigo se ubica en el tercer lugar luego de la soja y el arroz, con más de 600.000 toneladas exportadas en el año 2015 (DIEA, 2016). Esto hace que el trigo no sólo sea un componente fundamental en los sistemas de rotación de Uruguay, sino que además representa un rubro de exportación de importancia significativa.

Sin embargo, hay varias enfermedades que interfieren en el proceso productivo, siendo la fusariosis de la espiga (FE) una de las principales limitantes sanitarias que enfrenta la producción de trigo en Uruguay (Pereyra, 2013). La principal especie causante de dicha enfermedad en Uruguay es *Fusarium graminearum* (Umpiérrez et al., 2013). Debido a su capacidad de sobrevivir saprofíticamente en el rastrojo, este patógeno se ve favorecido por los sistemas en siembra directa, al aumentar el porcentaje de rastrojo dejado sobre la superficie y, por consiguiente, aumentar la disponibilidad de sustrato para su alimentación y esporulación (Steward, Pereyra y Díaz, 2004). Los beneficios ambientales ofrecidos por la siembra directa en términos de reducción de erosión de suelo, se podrían ver contrarrestados por las pérdidas causadas por los patógenos que sobreviven en el rastrojo, y los impactos ambientales negativos que pueden causar los aumentos en uso de agroquímicos, no sólo de herbicidas, sino también de fungicidas a causa de este problema. Los fungicidas utilizados mayormente para el control de la FE en Uruguay son metconazol, protioconazol y tebuconazol (Pereyra, 2013), los cuales presentan cada uno por separado toxicidad crónica moderada para lombrices (IUPAC, 2017). No obstante, no existen estudios del efecto de éstos cuando son aplicados a través de los formulados comerciales en los cuales se los combina entre ellos y con epoxiconazol, de toxicidad crónica alta (IUPAC, 2017). Pero además de los bemoles presentados por los fungicidas debido al riesgo ambiental que implica su uso, éstos han mostrado una eficiencia insuficiente en el control de la FE por lo que tampoco resuelven el

problema (Yuen & Schoneweis, 2007). Para el control de este fitopatógeno se necesita, por lo tanto, un manejo integrado que combine diversas medidas de control.

En este sentido resulta de interés explorar tecnologías de manejo complementarias que contribuyan al control de esta enfermedad. En Uruguay se han estudiado alternativas biológicas para disminuir el impacto de la FE mediante una reducción en la presión de inóculo presente en el rastrojo utilizando *Trichoderma* spp., componente de la microflora del suelo (Villar et al., 2014; et al., 2012). No obstante, no existen antecedentes nacionales de control biológico a través de la fauna del suelo, en particular las lombrices, lo cual ha mostrado ser promisorio en experiencias realizadas en Europa (Wolfarth et al., 2011), especialmente enfocados en el impacto de *Lumbricus terrestris* sobre el inóculo de *Fusarium* spp. en el rastrojo.

Existen escasos estudios nacionales sobre las comunidades de lombrices presentes en los sistemas agrícolas de Uruguay (Zerbino, 2012; 2010; Grosso & Brown 2007; Zerbino, 2005). En particular, la especie *L. terrestris* (Fam. Lumbricidae) utilizada en los experimentos de Wolfarth et al (2011) has sido reportada para nuestro país, pero también es posible que haya otras especies que puedan afectar negativamente al inóculo de *Fusarium* en el rastrojo, resultando en una disminución en la presión de inóculo de dicho patógeno. En ese caso, el manejo del suelo que favorezca el desarrollo de estas lombrices, podría contribuir a una reducción de la presión de inóculo presente en el rastrojo y por lo tanto a una reducción en el riesgo de ocurrencia de enfermedades epifítias. Por lo tanto, es importante conocer el efecto que tienen sobre las lombrices, los fungicidas utilizados para combatir la FE, entre otros.

En esta Tesis se propuso estudiar el efecto de las lombrices sobre el inóculo de *Fusarium graminearum* en el rastrojo. La interacción de la fauna edáfica con la microbiota y su potencial aplicación en el control de enfermedades, conforman una línea de investigación novedosa y promisoria a nivel mundial (Ayuke et al. 2017; Lagerlöf et al. 2015; Wolfarth et al. 2011; Oldenburg et al. 2008) y sin antecedentes para nuestro país. Esto contribuye al desarrollo de mecanismos sustentables de

control de esta enfermedad, con el propósito de reducir los riesgos para la salud humana, animal y ambiental, así como las pérdidas económicas de los productores.

1.2. HIPÓTESIS DE INVESTIGACIÓN

Se plantean tres hipótesis con respecto la potencial contribución que pueden hacer las lombrices en el control de FE en agroecosistemas que incluyen trigo en Uruguay:

1) Los suelos de los sistemas agrícolas que incluyen trigo en su rotación presentan lombrices nativas y exóticas.
2) Las especies encontradas pueden afectar al inóculo de *Fusarium graminearum* presente en el rastrojo.
3) Los fungicidas más frecuentemente utilizados en la agricultura para el control de *Fusarium* spp. afectan la biología de las lombrices en Uruguay.

1.3. OBJETIVOS

1.3.1. Objetivo general

Estudiar la potencialidad que tienen las lombrices presentes en agroecosistemas de trigo en Uruguay para contribuir al control biológico de la fusariosis de la espiga, reduciendo el inóculo de *Fusarium graminearum* en el rastrojo de trigo.

1.3.2. Objetivos específicos

1. Identificar las especies de lombrices presentes en distintos agroecosistemas de Uruguay.
2. Conocer la relación de los patrones de densidad, biomasa y composición de las comunidades con las propiedades físico-químicas del suelo, y de manejo del sistema, de los distintos sitos muestreados.
3. Cuantificar el efecto de dos especies de lombrices de hábito diferencial, sobre la presión de inóculo de *Fusarium graminearum* en rastrojo de trigo en condiciones controladas.
4. Cuantificar el efecto que tienen sobre las lombrices, los fungicidas utilizados para controlar *Fusarium*, para determinar si éstos interfieren en la actividad de las mismas.

1.4. ESQUEMA GENERAL DE LA TESIS

La estrategia de investigación utilizada con el fin de lograr los objetivos expuestos, incluyó muestreos a campo, trabajo en laboratorio de identificación de especies de lombrices (por métodos morfológicos y moleculares), así como ensayos en laboratorio con lombrices en mesocosmos, estudiando primero su efecto sobre el inóculo de *Fusarium* y luego cómo son afectadas por los fungicidas utilizados en estos agroecosistemas.

El **Capítulo 2** consta de una revisión bibliográfica sobre el rol de las lombrices en el control biológico de enfermedades y plagas de las plantas. Esta revisión fue aceptada para su publicación por la *Revista Brasileira de Agroecolgia*, y antecede a los resultados del trabajo de muestreo y experimentación. En este capítulo se describen los casos que han demostrado un efecto positivo significativo de las lombrices sobre la sanidad vegetal, indaga en los mecanismos por los cuales éstas pueden participar del control biológico de parástitos fitófagos o fitopatógenos, y discute las perspectivas de aplicación del control biológico mediante el uso de lombrices como parte de una agricultura sustentable y agroecológica.

En el **Capítulo 3** se presentan los resultados de los muestreos y posteriores análisis realizados para caracterizar las comunidades de lombrices presentes en sistemas de producción de trigo (**Objetivos 1 y 2**). Se muestrearon las rotaciones experimentales de EEMAC, en Paysandú, y chacras comerciales con sistemas de producción convencional y de transición a producción orgánica, en Montevideo. Además de medir la abundancia y biomasa de lombrices para cada sitio, se identificaron las especies de lombrices de las muestras obtenidas por taxonomía morfológica, complementada por métodos moleculares. A modo de contextualización se realizaron análisis de los suelos de los sitios muestreados. Con todos estos datos se elaboró un artículo sobre la composición de especies nativas y exóticas en los sitios estudiados, artículo que será enviado a publicar en la revista *Biological Invasions*. Este capítulo contiene además otro artículo donde se detalla el trabajo realizado de identificación

de especies con el marcador molecular COI, en la Facultad de Ciencias, bajo la supervisión de Dr. CF. Claudio Martínez Debat, el cual ha sido aprobado para su publicación por *Agrociencia Uruguay*.

En el **Capítulo 4** se incluye la información obtenida en los experimentos realizados para determinar el efecto de las lombrices sobre el inóculo de *Fusarium* (**Objetivo 3**), integrado por una serie de experimentos con lombrices exóticas y nativas realizados parte en Europa y parte en Uruguay. En el marco del programa de Doctorado realicé dos pasantías (2014 y 2015) en el Centro de Ecología de la Universidad de Agricultura de Suecia (SLU), Uppsala, Suecia, en el departamento donde trabaja mi co-tutor, PhD. Jan Lagerlöf. Durante las mismas trabajé junto a un equipo interdisciplinario en el armado de un experimento en el que se evaluó el efecto que tienen las especies *Lumbricus rubellus, L. terrestris* y *Aporrectodea longa*, lombrices comunes en ese país, sobre el inóculo de *Fusarium graminearum* en el rastrojo de trigo. Los datos obtenidos se plasmaron en un artículo que será enviado a publicar a la revista *Applied Soil Ecology*, que se incluye en la segunda sección del Capítulo 4. En un segundo artículo se presentan los resultados del experimento realizado en Uruguay con similar metodología utilizando lombrices nativas y exóticas seleccionadas a partir de los muestreos realizados anteriormente.

El **Capítulo 5** lo compone el artículo que reporta los resultados de los ensayos de toxicidad crónica y aguda, de los principales fungicidas utilizados en Uruguay para el control de la FE sobre una especie nativa de lombriz y sobre la especie estándar de las normas ISO, *Eisenia fetida*, como control (**Objetivo 4**). Estos ensayos fueron realizados en el laboratorio del LATU, bajo la dirección de la Dra. Diana Míguez, y los resultados serán eviados para su publicación a la revista *Environmental Toxicology an Chemistry* de SETAC (Society of Environmental Toxicology and Chemistry). Finalmente, en el **Capítulo 6** se realiza una discusión general en la cual se sintetiza el trabajo y sus proyecciones.

Si bien los capítulos 1, 2 y 6 han sido redactados completamente en español, los capítulos 3, 4 y 5, han sido redactados en inglés, ya que comprenden los artículos

preparados para revistas arbitradas internacionales. Por este motivo se respetan los formatos de citado y referenciado de cada revista en los artículos que ya cuentan con una revista asignada, de lo contrario se sigue el formato sugerido por la Guía para la presentación de trabajos finales de Posgrado (UPEP, 2017). Al inicio de cada uno de estos capítulos, se presenta un resumen en español. La sección de Bibliografía al final contiene las referencias de las citas de los capítulos 1 y 6, ya que las otras citas se referencian al final de cada artículo correspondiente.

2: REVISIÓN BIBLIOGRÁFICA

CONTROL BIOLÓGICO DE ENFERMEDADES Y PLAGAS PROMOVIDO POR LOMBRICES

Biological control of plant diseases and pests promoted by earthworms

G. Jorge Escudero[1,2], J.E. Lagerlöf[3], C.A. Pérez[4]

RESUMEN

A pesar de vaivenes en la historia al respecto de cómo se ha interpretado el efecto de las lombrices sobre el suelo y las plantas, actualmente se acepta como un hecho que estos anélidos tienen un efecto positivo indirecto sobre la productividad y sanidad vegetal, al mejorar las propiedades del suelo que sustenta la vida de las plantas. Lo que resulta más novedoso, quizás, es la acción directa que se ha descubierto que pueden tener las lombrices al suprimir ciertas plagas y enfermedades; o influyendo directamente sobre el sistema de defensa de las plantas. Estas líneas de investigación tienen varias décadas de desarrollo a nivel mundial, pero son muy poco conocidas en el mundo hispano-parlante, con escasa literatura en el tema escrita en español. La presente revisión pretende reunir los casos que han demostrado un efecto positivo significativo de las lombrices sobre la sanidad vegetal, indagar en los mecanismos por los cuales éstas pueden participar del control biológico de parástitos fitófagos o fitopatógenos, y discutir las perspectivas de aplicación del control biológico mediante el uso de lombrices como parte de una agricultura sustentable y agroecológica.

Palabras clave: biocontrol, oligochaeta, manejo fitosanitario alternativo

[1]Departamento de Suelos y Aguas, Facultad de Agronomía, Universidad de la República, Garzón 780, 12900 Montevideo, Uruguay.
[2]Departamento de Sistemas Ambientales, Facultad de Agronomía, Universidad de la República.
[3] Departamento de Ecología, Universidad Sueca de Agricultura (SLU), P.O Box 7044, SE-75007, Uppsala, Suecia.
[4]Departamento de Protección Vegetal, EEMAC, Facultad de Agronomía, Universidad de la República, Ruta 3 km 363, 60.000 Paysandú, Uruguay.

ABSTRACT

Despite some fluctuations in history regarding human interpretation of the effect of earthworms on soil and plants, it is now accepted as a fact that these worms have an indirect positive effect on productivity and plant health, by improving the properties of the soil where plants grow. What is less known, is that it has been discovered that earthworms can have a direct action on pests and diseases by suppressing them; or by directly influencing plants' defense system. These lines of research have several decades of development worldwide, but are very little known in the Spanish-speaking world, with little literature on the subject written in Spanish. The present review aims at bringing together the cases that have demonstrated a significant positive effect of earthworms on plant health, examining the mechanisms by which they can participate in the biological control of phytoparasites, herbivores or phytopathogens, and discussing the perspectives of application of biological control through the use of earthworms as part of sustainable and agroecological agriculture.

Keywords: biocontrol, oligochaeta, alternative phytosanitary management

"Suelo sano: planta sana, gente sana"

Ana Primavesi

Frase dicha en presentación oral en
"Taller Latinoamericano sobre Control Orgánico de Plagas y Enfermedades",
MAPO, Facultad de Ciencias Agropecuarias-UNC, Córdoba, Argentina, 2003

2.1. LAS LOMBRICES: IMPORTANTE COMPONENTE DEL SUELO, CON INFLUENCIA POSITIVA SOBRE EL CRECIMIENTO Y LA SANIDAD VEGETAL

Debido a su tamaño, las lombrices, oligoquetos terrestres (Annelida, Clitellata), son las principales contribuyentes a la biomasa invertebrada de la fauna edáfica en praderas y agroecosistemas, a pesar de no dominar numéricamente la fauna del suelo. En diversos sistemas de rotación de cultivos y pasturas de zonas templadas se ha contabilizado que las lombrices representan entre un 25% y 50% aprox. de la abundancia de la fauna edáfica (ZERBINO et al., 2008; ZERBINO, 2010). Según el tipo de suelo, el uso y el manejo del mismo, se pueden encontrar entre 1 y 1000 individuos por m^2, lo cual en biomasa representa entre 0,1 y 100 g m^{-2} (EDWARDS y BOHLEN, 1996; LAGERLÖF et al., 2012; LEE, 1985; SCHIEDECK et al., 2009a; ZERBINO, 2005).

La presencia de lombrices se asocia comúnmente con suelos de buena calidad. Actualmente es aceptado en la comunidad científica además que las lombrices son sin duda el componente biótico más importante de los suelos en términos de procesos de formación del suelo, la estructura y la fertilidad del mismo (JOHNSON y SCHAETZL, 2015; LAVELLE y SPAIN, 2001; EDWARDS, 2004). Diversos estudios han probado cómo los oligoquetos favorecen las propiedades físicas del suelo (al mejorar la estructura y la formación de agregados, aumentar la porosidad y disminuir la densidad aparente), así como sus propiedades hídricas (en la regulación del agua, aumentando la infiltración y disminuyendo el escurrimiento) (BLOUIN et al., 2013; SHIPITALO y LE BAYON, 2004). Otros estudios muestran como este grupo de macrofauna afecta las propiedades químicas del suelo (pudiendo modificar el pH por sus secreciones y acelerar la mineralización del N ingiriendo suelo y residuos orgánicos) (EDWARDS y BOHLEN, 1996).

Los procesos biológicos de altas tasas de ingestión de suelo y residuos orgánicos, así como de ingestión selectiva de partículas finas, interactúan con la bioturbación, es decir las modificaciones físicas realizadas por las lombrices al ambiente, como lo son

la construcción de galerías y acumulación de pellets fecales. De esta interacción resulta una modificación en los procesos de infiltración, formación de agregados y pedogénesis (LAVELLE, 1997). Por estos motivos es que se ha incluido a las lombrices dentro de la categoría de ingenieros ecosistémicos, definido por Jones, Lawton y Shachak (1994) como aquellos "organismos que directa o indirectamente regulan los recursos para otras especies, al causar cambios en el estado físico de materiales bióticos o abióticos, modificando, manteniendo o creando nuevos hábitats" para otros organismos. De esta manera, además, modifican las propiedades biológicas del suelo, influyendo en la biomasa y estructura de la comunidad microbiana al crear micro-hábitats y fragmentando los residuos, lo cual fomenta el desarrollo de microorganismos. A su vez, a través de su movilidad también los puede diseminar (DOUBE et al., 1994; BROWN, 1995; BROWN et al., 2004). Más recientemente, por los efectos que estos anélidos tienen en el ecosistema suelo, se destaca su rol catalizando servicios ecosistémicos, en particular los de soporte como ser la formación de suelo y el ciclado de los nutrientes (BLOUIN et al., 2013). Servicios ecosistémicos son aquellas funciones o procesos ecológicos que directa o indirectamente contribuyen al bienestar humano o tienen un potencial para hacerlo en el futuro (CAMACHO VALDEZ y RUIZ LUNA, 2012).

El efecto positivo que tienen sobre el suelo, también se traslada a la vegetación que sobre él crece. Dos revisiones, una realizada en base a 28 estudios en suelos tropicales y otra con 67 estudios a nivel mundial, concluyen que tres de cada cuatro estudios reportan un efecto positivo de la presencia de lombrices sobre la biomasa vegetal (BROWN et al., 1999; SCHEU, 2003). Más recientemente, un meta-análisis realizado con datos provenientes de 58 estudios a nivel mundial, concluye que este efecto positivo se traduce en 25% de aumento en el rendimiento de cultivos y 23% de aumento en la biomasa vegetal aérea, incrementándose cuando los residuos vegetales son devueltos al suelo, y disminuyendo cuando la disponibilidad de N en el suelo es alta (VAN GROENIGEN et al., 2014). Jana et al. (2010) plantean que las lombrices tienden a contrarrestar los efectos de suelos pobres sobre el crecimiento y desarrollo de las plantas. No obstante, Blouin et al. (2006) corroboraron que las lombrices de la

especie *Millsonia anomala* tenían efecto positivo sobre las plantas de arroz, independientemente de si el N representaba o no una limitante en el sistema.

El concepto de las lombrices como benefactoras del suelo y, por ende, de las plantas que este sustenta, era manejado hace ya más de 4000 años por los egipcios, y reconocido por el filósofo Aristóteles. Sin embargo, durante la época positivista, éstas cayeron de su pedestal, pasando a ser consideradas pestes que debían ser eliminadas de los jardines. Los intentos singulares primero de Rev. Gilbert White en 1777 y luego de Charles Darwin en 1881, por revindicar en sus escritos el valor de las lombrices en el suelo, no tuvo eco sino cientos de años después, cuando a finales del siglo XX, la investigación en la fauna del suelo cobró fuerza (BROWN et al., 2004). La revalorización del rol de las lombrices en el suelo va de la mano de un cambio de los paradigmas dominantes que han impulsado el desarrollo agrícola. Nulo era el rol que cumplían mientras se entendía al suelo como un mero chasis para las plantas, cuyo requerimiento para su desarrollo podía ser suplido completamente por insumos externos y sintéticos.

Siguió en una categoría subvalorada aun cuando el suelo comienza a comprenderse como un sistema complejo de propiedades físicas químicas y biológicas que interactúan, ya que estas últimas se asociaban principalmente a la actividad microbiana. Actualmente, productores brasileros de diversos sistemas agrícolas (convencional, agroecológico y en transición hacia sistemas agroecológicos), enfatizan la importancia de las lombrices para la tierra, ya sea por su capacidad de transformar las características físicas de ésta, en beneficio de un mejor establecimiento y crecimiento de las plantas, o por su capacidad de disponibilizar nutrientes requeridos por las mismas (SCHIEDECK et al., 2009b). Esto evidencia que, a nivel de productores, si bien esta revalorización se puede enmarcar en una evolución hacia una comprensión más holística del agroecosistema, aún se ve mayormente acotada a las propiedades físicas y químicas.

Ampliando esta visión holística del suelo como un componente vivo, es que comienza a aceptarse un tímido rol de las lombrices interactuando junto con otros integrantes de la macro y mesofauna, llegando así a beneficiar las plantas de una manera indirecta, a través de las propiedades del suelo (BROWN et al., 2004, CUNHA et al., 2016), no sólo a nivel de producción de biomasa sino también desde el punto de vista sanitario. Un paso más se da cuando se comienza a encontrar evidencia de que la macrofauna, y en particular las lombrices, pueden tener un efecto directo sobre los patógenos o parásitos de plantas, reduciendo los niveles de daño por estos causados (BROWN et al., 2004; FRIBERG et al., 2005; SCHRADER et al., 2013; WURST, 2010). Paulatinamente, en esta evolución de paradigmas y comprensión del funcionamiento del suelo, se comienza a aceptar que la parte aérea y la subterránea de los ecosistemas terrestres pueden estar interconectadas (DE LA PEÑA, 2009; WARDLE et al., 2004; WURST, 2010). Esta revisión se centrará en los efectos de las lombrices en el suelo, excluyéndose aquellos estudios que prueban los efectos supresores de enfermedades por parte de vermicompuestos, que ya han sido extensamente revisados (EDWARDS et al., 2004; MEGHVANSI et al., 2011; SIMSEK-ERSAHIN, 2011).

2.2. VARIAS DÉCADAS DE INVESTIGACIÓN SOBRE LA ACCIÓN DE LOMBRICES EN EL CONTROL BIOLÓGICO DE ENFERMEDADES Y PLAGAS

Ya en los años 60 algunos investigadores habían comenzado a observar que la actividad de las lombrices enterrando la hojarasca alrededor de árboles frutales podía proteger a los mismos de enfermedades, dado que eliminaba el ambiente donde se alojaban los patógenos causales de estas enfermedades durante el invierno (HIRST y STEDMAN, 1962; RAW, 1962). Dichas observaciones fueron el puntapié inicial para estudios posteriores que muestran la interacción de fauna del suelo, y en particular las lombrices, con fitopatógenos (BROWN et al., 2004; FRIBERG et al., 2005; WURST, 2010).

Las tablas 1 y 2 resumen las investigaciones que se han realizado desde fines del siglo pasado hasta la actualidad sobre el potencial que tienen las lombrices para controlar ciertos patógenos o parásitos, incluyendo experimentos en condiciones controladas, en invernáculos y a campo. Aquí se listan aquellos estudios en que las lombrices tuvieron un efecto significativo sobre la reducción del patógeno o parásito, y/o sobre la incidencia de la enfermedad o daño.

En este sentido, las lombrices han mostrado ser supresoras de enfermedades fúngicas de hortalizas, arbustivas, frutales, pasturas y cereales (Tabla 1). Principalmente se trata de hongos que habitan el suelo y atacan la raíz, o que sobreviven saprofíticamente en el rastrojo u hojarasca, desde donde esporulan e infectan a nivel foliar o de inflorescencia.
Por otro lado, se ha obtenido en varios casos una disminución en el número de parásitos en presencia de lombrices (YEATES, 1980; 1981; BOYER et al., 1999; BOYER et al., 2013; ILIEVA-MAKULEC y MAKULEC, 2007; SENAPATI, 1992); pero, además se ha observado que las lombrices pueden mitigar el daño, sin necesariamente reducir la población de parásitos, actuando a nivel de la resistencia de la planta frente a la plaga (BLOUIN et al., 2005; LAFONT et al., 2007; WURST et al., 2008) (Tabla 2). Los mecanismos de acción son abordados en la siguiente sección.

Una cantidad menor de estudios reportan casos en que las lombrices no tuvieron un efecto significativo sobre la sanidad vegetal (AYUKE et al., 2017; CLAPPERTON et al., 2001; JORGE-ESCUDERO et al., *s/p*; NEWINGTON et al., 2004; STEPHEN et al., 1994c; WOLFARTH et al., 2011b) o incluso que las lombrices han aumentado las poblaciones de nematodos fitófagos (POVEDA et al., 2005; TAO et al., 2009). De acuerdo a Tao et al. (2009), el mulch utilizado presentó un efecto negativo sobre el crecimiento de las raíces jóvenes, limitando la población de nematodos, que se alimenta de ellas. En este caso, la actividad de las lombrices promoviendo el crecimiento vegetal, y contrarrestando el efecto negativo del mulch, aumentó la

disponibilidad de este sustrato para los nematodos, resultando en un aumento en su población.

En los casos en que las lombrices no tuvieron efecto sobre la sanidad, se manejaron varias hipótesis. Clapperton et al. (2001) lo adjudicaron a la baja densidad de lombrices utilizada, ya que sus resultados mostraron inconsistencia con estudios anteriores con las mismas especies. Ayuke et al. (2017) estudiaron el efecto de *Aporrectodea caliginosa* y *Ap. longa* sobre la incidencia de la mancha negra de la hoja (*Alternaria brassicae*) en plantas de colza (*Brassica napus*), en ensayos de laboratorio y a la intemperie. El hecho de no haber obtenido un efecto significativo de estas lombrices sobre la enfermedad, lo atribuyeron a que, además de trabajar con especies de plantas diferentes a las ya reportadas en la literatura, el patógeno no actuó a nivel radicular como en los casos reportados, sino a nivel foliar. Siendo el suelo y la rizósfera el sitio de acción directa de las lombrices utilizadas, sólo podía esperarse que tuvieran algún tipo de efecto indirecto mediado por la planta, lo cual no pudo comprobarse en este caso particular. Tampoco pudo comprobarse que las lombrices de la especie *Octolasion tyrtaeum* compensaran el daño foliar causado por *Spodoptera litoralis* en *Sinapis arvensis*, en un complejo sistema de planta – lombriz – parásito radicular – herbívoro foliar – áfido - parasitoide-polinizador. Las lombrices favorecieron indirectamente a los áfidos a través de su efecto positivo en el crecimiento de las plantas, el cual fue independiente de la presencia de los otros componentes del sistema estudiado (POVEDA et al., 2005). Ya antes un estudio mostraba que la tasa de consumo de herbívoros foliares no había sido afectada por la presencia de una comunidad compuesta por seis especies de lombrices de los géneros *Allolobophora, Aporrectodea y Octolasion* (NEWINGTON et al., 2004).

Evidentemente, según las especies o comunidades que componen el sistema planta-patógeno-lombriz, se obtienen interacciones y respuestas diferentes (DE LA PEÑA, 2009). Wurst et al. (2003) pudo demostrar que la magnitud del efecto de las lombrices sobre la incidencia de los áfidos, varió entre especies de plantas. Wolfarth et al. (2011b) comprobó que si bien una especie de lombriz, *L. terrestris*, controlaba

el inóculo de *Fusarium* alojado en el rastrojo de trigo remanente en la superficie del suelo, otra especie, *Ap. caliginosa*, no tenía un efecto sobre el mismo. Esto fue explicado por la ecología de cada especie y su ámbito de acción: la primera sube a la superficie y entra en contacto directo con el rastrojo y el hongo, mientras que la segunda se mantiene dentro del suelo. Las mismas especies de lombrices que tienen efecto en ciertas condiciones ambientales, también pueden dejar de tenerlo si estas cambian. Jorge-Escudero et al., (*s/p*) observaron que las lombrices, *L. rubellus* y *Ap. longa*, no afectaron el inóculo de *Fusarium* en el rastrojo de trigo cuando se les ofreció una fuente de alimentación alternativa (estiércol incorporado al suelo). Este efecto del sustrato alternativo coincide con lo encontrado por Stephens et al. (1994c), quienes observaron que *Ap. trapezoides y Ap. rosea* no disminuyeron la enfermedad radicular provocada por *Rhizoctonia solani* cuando el suelo estuvo cubierto con *mulch* orgánico. En resumen, se puede decir que los efectos de control biológico por parte de las lombrices son contexto-dependientes (WURST, 2010).

2.3. MECANISMOS DE ACCIÓN DE CONTROL BIOLÓGICO DE FITÓFAGOS O FITOPATÓGENOS

2.3.1. Características de los oligoquetos que habilitan o condicionan su efecto supresor

Para lograr una mejor comprensión de los mecanismos por los cuales las lombrices pueden favorecer la sanidad vegetal, es necesario conocer la ecología de este grupo de oligoquetos terrestres.

Las lombrices son invertebrados anélidos, vermiformes, cuyo contenido corporal está compuesto por agua entre 75% y 90%. Por este motivo su actividad estará condicionada por la humedad del suelo. Frente a una reducción en el contenido de agua del suelo las lombrices migrarán hacia zonas más profundas, o cesarán su actividad y entrarán en diapausa arrollándose sobre sí mismas para asegurar una mejor conservación de la humedad corporal (EDWARDS y BOHLEN, 1996). Dado que respiran por la piel, secretan un mucus de base proteica por glándulas de la

epidermis para mantener la cutícula siempre humectada y permitir el intercambio gaseoso (EDWARDS y BOHLEN, 1996). Este mucus, junto con las secreciones intestinales estabiliza agregados del suelo y las paredes de las galerías realizadas por estos animales (EDWARDS, 2004). La sustancia mucilaginosa además contiene tanto compuestos altamente biodisponibles, que promueven la actividad microbiana, como agentes antimicrobianos que la inhibe. El efecto positivo, neutro o negativo de las lombrices sobre los microorganismos dependerá de las especies en interacción (BROWN, 1995; WANG et al., 2011). Los oligoquetos terrestres prefieren en general un ambiente relativamente neutro a levemente ácido, aunque algunas especies tienen un rango más amplio de tolerancia y otras están adaptadas a vivir en ambientes ácidos con pH menor a 5. Se ha sugerido que la actividad de las lombrices tiende a aumentar el pH en un suelo a través de las secreciones de las glándulas calcíferas, secreciones intestinales y excreción de amonio (EDWARDS y BOHLEN, 1996). Se alimentan de microorganismos que se desarrollan en el suelo, en la rizósfera o en restos vegetales en descomposición. Para su nutrición dependen de un rango de microorganismos en el cual priman los hongos, en segundo lugar, vendrían los protozoarios, mientras que las bacterias y actinobacterias tendrían una menor importancia en la dieta (EDWARDS y FLETCHER, 1988).

Se han clasificado las lombrices en tres grupos ecológicos según su hábito de vida, y estrategia de alimentación denominados epígeas, anécicas y endógeas (BOUCHÉ, 1977; LAVELLE, 1988). Las epígeas, habitan en la superficie del suelo donde se concentran restos vegetales o estiércoles animales de los cuales se alimentan participando del proceso de descomposición; entre ellas están las lombrices utilizadas para el vermicompostaje o lombricompostaje. Por otro lado, están las anécicas, que generan galerías verticales subiendo a buscar restos vegetales a la superficie, los cuales entierran generando una incorporación de materia orgánica al suelo; la más conocida quizás por su gran tamaño y característica peregrina es *L. terrestris.* Finalmente, las endógeas se mantienen sin subir a la superficie en el horizonte superior del suelo, generando galerías más irregulares que la anterior y están generalmente asociadas a la rizósfera donde se encuentra una diversidad de

microorganismos que servirá de alimento. Estas estrategias determinarán el rango de acción de las mismas, condicionando los mecanismos por los cuales las lombrices controlan los parásitos y patógenos de plantas, los cuales pueden ser directos, es decir de acción directa sobre el fitopatógeno o fitófago, o indirectos a través de efectos ejercidos sobre el suelo, la comunidad microbiana o sobre la planta (Figura 1).

Tabla 1. Enfermedades fúngicas controladas por lombrices

Cultivo	Nombre común de la enfermedad	Nombre científico del patógeno	Especie de lombriz que mitiga el efecto del patógeno	Mecanismo de control propuesto	Referencias
Berenjena: *Solanum melongena*	Verticilosis	*Verticillium dahliae*	*Lumbricus terrestris*	Supresión mediada a través de la actividad microbiológica	Elmer (2009)
Col: *Brassica oleracea var. capitata*	Hernia	*Plasmodiophora brassicae*	*Pheretima hilgendorfi*	Cambios en las propiedades físicas, químicas y biológicas; posible ingestión del hongo.	Nakamura et al. (1995; 1996)
Espárrago: *Asparagus officinalis*	Declina-miento del espárrago	*Fusarium oxysporum f. sp. asparagi; F. proliferatum*	*L. terrestris*	Supresión mediada a través de la actividad microbiológica	Elmer (2009)
Grosella: *Ribes rubrum*	Antracnosis	*Drepanopeziza ribis*	*L. terrestris*	Incorporación al suelo de la hojarasca, la cual es la única fuente de inóculo de este patógeno.	Kennel (1990)
Manzano: *Malus domestica*	Sarna del manzano	*Venturia inaequalis*	*L. terrestris*	Incorporación al suelo de la hojarasca donde el patógeno se mantiene durante el invierno en forma de pseudotecios.	Kennel (1990); Niklas y Kennel (1981)
Trébol y Ryegrass	Damping off	*Rhizoctonia solani*	*Aporrectodea trapezoides* *Ap. rosea*	Secreción de CaCO3 influye en la solubilidad y disponibilidad de nutrientes para los vegetales	Stephens y Davoren (1997)
Tomate: *Solanum lycopersicum*	Marchitez vascular	*F. oxysporum f. sp. lycopersici* Race 1	*L. terrestris*	Supresión mediada a través de la actividad microbiológica	Elmer (2009)

Tabla 1 (cont.). Enfermedades fúngicas controladas por lombrices

Cultivo	Nombre común de la enfermedad	Nombre científico del patógeno	Especie de lombriz que mitiga el efecto del patógeno	Mecanismo de control propuesto	Referencias
Trigo: *Triticum* aestivum	Damping off	*Rhizoctonia solani*	*Ap. trapezoides* *Ap. rosea*	Ingestión de las hifas; reducción de la susceptibilidad del trigo por aumento de N y Zn en el suelo similar al producido por el laboreo; aceleración de la descomposición.	Stephens et al. (1993); Stephens et al. (1994b); Stephens et al. (1994c)
Trigo: *Triticum* aestivum	Fusariosis de la Espiga	*Fusarium culmorum; F. graminearum*	*L. terrestris*	Incorporación al suelo del rastrojo infestado; ingestión del hongo; efecto promotor de actividad microbiana a través de secreción de mucus, el cual contiene compuestos altamente biodisponibles.	Oldenburg et al. (2008); Schrader et al. (2013); Wolfarth et al. (2011a)
Trigo: *Triticum* aestivum	Pudrición radical o mal del pie (take all)	*Gaeumannomyces graminis var. tritici (Ggt)*	*Ap. trapezoides* *Ap. rosea* *Ap. caliginosa*	Perturbación mecánica del suelo; ingestión de las hifas; promoción y dispersión de microorganismos antagonistas del hongo.	Doube et al. (1994); Hume et al. (2015); Puga - Freitas et al. (2016); Stephens et al. (1994[a]); Stephens y Davoren (1995)
Trigo: *Triticum* aestivum	Mancha Ocular del Trigo	*Tapesia yallundae; Oculimacula yallundae*	*L. terrestris*	Incorporación al suelo del rastrojo infestado; ingestión del hongo; aumento de la porosidad del suelo, lo cual limita condiciones favorables para la diseminación del hongo como ser el anegamiento; efecto indirecto sobre la resistencia de la planta al patógeno al mejorar el estado nutricional de la misma.	Bertrand et al. (2014; 2015)

Tabla 2. Parásitos controlados por lombrices

Cultivo	Nombre común del parásito	Nombre científico del parásito	Especie de lombriz que mitiga el efecto del parásito	Mecanismo propuesto	Referencias
Arroz: *Oryza sativa*	Nemátodos fitófagos	*Hiischmanniella, Heliocotylenchus.*	*Lampito mauritii*	Aumento de la población de nematodos bacteriófagos y fungívoros por aportes de nitrógeno por sus excreciones, mucus, tejido muerto y por sus efectos físicos en el suelo; reducción de la población de nematodos fitófagos posiblemente por ingestión voluntaria.	Senapati (1992)
Arroz: *Oryza sativa*	Nemátodo fitófago	*Heterodera sacchari*	*Millsonia anomala*	La presencia de lombrices no disminuyó la cantidad de parásitos, pero aumentó la tolerancia de las plantas a los mismos de una forma sistémica, observándose una sobre- expresión del gen de *lox*, implicado en el inicio de una vía de respuesta al estrés.	Blouin et al. (2005)
Arroz: *Oryza sativa*	Nemátodo fitófago	*Pratylenchus zeae*	*Pontoscolex corethrurus*	Ingestión y digestión; excreción de fluidos con enzimas que afectan la fertilidad, viabilidad y germinación de los cistos; activación de microorganismos antagonistas.	Boyer et al. (2013)
Arroz: *Oryza sativa*	Nemátodo fitófago	*Heterodera sacchari*	*P. corethrurus*	Disminución de la población luego de que los cistos pasan por el tracto digestivo de la lombriz.	Boyer et al. (2013)
Banana: *Musa acuminata*	Nemátodo sedentario parásito	*Radopholus similis*	*P. corethrurus*	Si bien la presencia de lombrices no redujo el número de nematodos, al aumentar el desarrollo radicular, la densidad de los mismos disminuyó y la severidad del daño radicular también.	Lafont et al. (2007)

Tabla 2 (cont.). Parásitos controlados por lombrices

Cultivo	Nombre común del parásito	Nombre científico del parásito	Especie de lombriz que mitiga el efecto del parásito	Mecanismo propuesto	Referencias
Hierba: *Plantago lanceolata*	Áfidos	*Myzus persicae*	*Aporrectodea caliginosa*	La reproducción de los áfidos se redujo en presencia de las lombrices en suelo sin autoclavar. Las lombrices pueden afectar a los herbívoros influyendo en las concentraciones de metabolitos secundarios, como ser fitosteroles y glicósidos iridoides y por tanto en los mecanismos de defensa.	Wurst et al. (2003; 2004)
Maíz: *Zea mays*	Nemátodos fitófagos	*Pratylenchus vulnus*	*Amynthas corticis*	Creación de macroporos y compactación del suelo de las paredes laterales de las galerías, lo cual impide el movimiento de los nematodos; ingestión pasiva de nematodos junto con el suelo y digestión por parte de enzimas digestivas.	Boyer et al. (1999)
Maíz: *Zea mays*	Plaga del taladro	*Sesamia calamistis*	*Am. corticis*	La asociación de las lombrices y *Lotus uliginosus* generaron condiciones que favorecieron los depredadores de los estadios larvales del taladro, que habitan en el suelo.	Boyer et al. (1999)
Manzano: *Malus domestica*	Minadores	*Phyllonorycter blancardella*	*Lumbricus terrestris*	Incorporación al suelo de la hojarasca donde el parásito se mantiene durante el invierno.	Kennel (1990); Laing et al. (1986)
Mostaza blanca: *Sinapis alba*	Nemátodos parásito	*Meloidogyne incognita; Pratylenchus penetrans*	*Ap. caliginosa*	La presencia de lombrices contrarrestó las reducciones de biomasa aérea y de N en la planta causadas por nematodos. Afectan la concentración de metabolitos secundarios, que disuaden herbívoros generalistas, al disponibilizar N en el suelo. Pero también, independiente de la disponibilidad de N, afecta de manera diferencial a los diferentes metabolitos.	Lohmann et al. (2009)

Tabla 2 (cont.). Parásitos controlados por lombrices

Cultivo	Nombre común del parásito	Nombre científico del parásito	Especie de lombriz que mitiga el efecto del parásito	Mecanismo propuesto	Referencias
Pastura: *Festuca rubra*	Nemátodos fitófagos	*Paratylenchus; Tylenchorhynchus.*	*Ap. caliginosa*	Disminuyen los nematodos fitófagos, y aumentaron los bacteriófagos y fungívoros, por una priorización de la ruta de descomposición, manteniendo la población total de nematodos con un número similar al control sin lombrices.	Ilieva-Makulec y Makulec (2007)
Pasturas mixtas: *Agrostis capillaris, Anthoxanthum odoratum, F. rubra, Holcus lanatus, Poa pratensis, Lotus corniculatus.*	Nemátodos fitófagos	*Paratylenchus; Pratylenchus; Filenchus; Meloidogynidae.*	*L. rubellus; Octolasion sp.*	Si bien las lombrices no redujeron el número de nematodos fitófagos, al promover un aumento de la biomasa radicular, aumentaron la resistencia de los pastos a los mismos.	Wurst et al. (2008)
Peral: *Pyrus communis*	Psila del peral, mieleta, sila	*Psylla piri*	*L. terrestris*	Incorporación al suelo de la hojarasca donde el parásito se mantiene durante el invierno.	Kennel (1990)

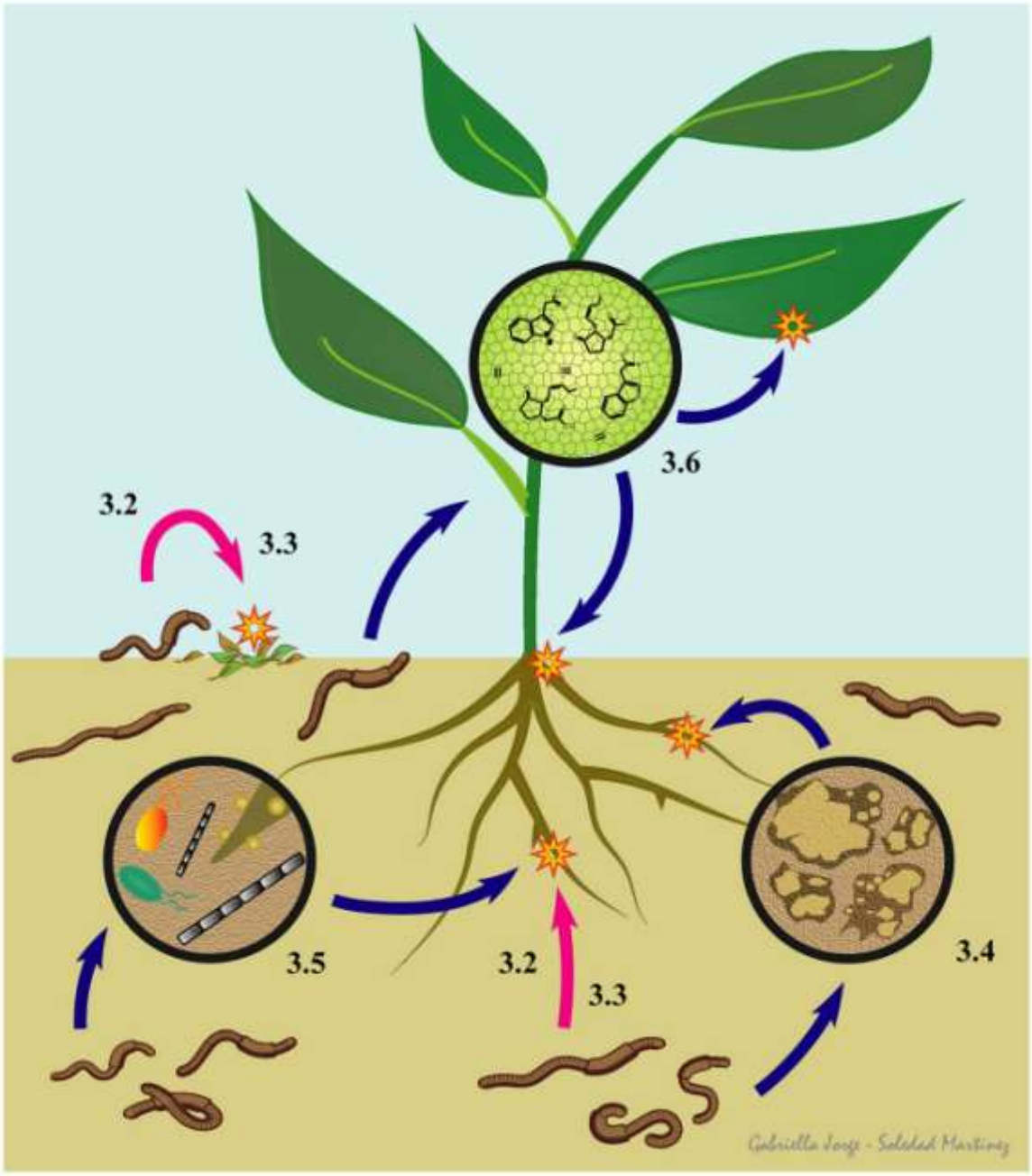

Figura 1. Esquema de los mecanismos de acción de las lombrices en el control biológico de fitófagos, parásitos o fitopatógenos, explicados en las secciones 2.3.2 a 2.3.6. Las estrellitas representan los fitopatógenos, fitófagos o parásitos. Las flechas color rosa representan acción directa de las lombrices sobre patógenos radiculares o sobre los que sobreviven saprofíticamente en la horjarasca o rastrojo (3.2 y 3.3); las flechas azules representan efectos indirectos mediados por el suelo (3.4), los microorganismos (3.5) y las plantas (3.6). Idea original: Gabriella Jorge. Diseño Gráfico: Soledad Martínez

2.3.2. Efectos directos: ingestión y digestión

El consumo preferencial de ciertos microorganismos por parte de las lombrices (WOLFARTH et al., 2011a) que termina favoreciendo a los antagonistas también se ha estudiado para otros grupos de fauna edáfica como ser nemátodos fungívoros y colémbolos, y se ha demostrado que en varios casos los hongos patógenos son más atractivos que los saprófitos o antagonistas de los patógenos, tales como *Trichoderma* spp. (HASNA et al., 2008; LAGERLÖF et al., 2011). Una de las razones posiblemente sea que los hongos patógenos contienen menores cantidades de sustancias no digeribles o tóxicas para la fauna edáfica (RUESS et al., 2000). Las lombrices también tienen preferencias por ciertas especies fúngicas. Bonkowski et al. (2000) encontró que la mayoría de las lombrices probadas preferían hongos de etapas tempranas de la sucesión frente a los de las etapas tardías y micorrizas. Dentro del primer grupo se encuentran la mayoría de los patógenos de plantas. Además, luego de pasar por el tracto digestivo de las lombrices, ciertas esporas fúngicas tienen una tasa menor de germinación, como ser *Trichoderma sp.* y *Mucor hiemalis*, o incluso pierden totalmente la viabilidad, por ejemplo, *Fusarium lateritium* y *Agrocybe temulenta* (MOODY et al., 1996).

También se ha comprobado que las lombrices ingieren nematodos, aunque aún no hay acuerdo con respecto a si es voluntaria o simplemente acompaña la ingestión de suelo realizado por la lombriz; no obstante, luego del pasaje por el tracto digestivo se da una disminución de la población de éstos (SENAPATI, 1992; BOYER et al., 1999; BOYER et al., 2013)

2.3.3. Efectos directos: mecánico y químico

Como mecanismo directo alternativo, se ha sugerido a nivel teórico que la actividad de las lombrices en la rizósfera puede generar disrupción mecánica de hifas. No obstante, esto no se ha comprobado empíricamente aún. Al incorporar el rastrojo o la hojarasca donde sobreviven parásitos y patógenos saprofíticamente, la lombriz actúa directamente sobre éstos, ya que reduce o destruye su hábitat limitando su

reproducción (JORGE-ESCUDERO et al., *s/p*; KENNEL 1990; OLDENBURG et al., 2008; WOLFARTH et al., 2011a).

Desde el punto de vista químico, las lombrices producen sustancias propias de su sistema inmune que pueden afectar directamente a los microorganismos. Se han encontrado varios péptidos antimicrobianos (AMPs, por su sigla en inglés) en el líquido celómico y tejido de lombrices, y hasta en vermicompuestos, que tienen actividad antimicrobiana frente a hongos, bacterias Gram positivas y Gram negativas (CHO et al., 1998); así como polisacáridos compuestos por cinco partes moleculares que confirmaron tener un amplio espectro antimicrobiano contra bacterias y hongos fitopatógenos (WANG et al., 2007). La excreción de fluidos con enzimas también puede afectar la fertilidad, viabilidad y germinación de los cistos de nematodos (BOYER et al., 2013).

2.3.4. Efectos indirectos: mediados por el suelo

Se ha sugerido que la creación de macroporos y compactación del suelo en las paredes de las galerías por parte de las lombrices puede impedir el movimiento de los nematodos (BOYER et al., 1999). Por otro lado, el aumento de la porosidad del suelo evita condiciones de anegamiento que puedan resultar favorables para la diseminación de hongos fitopatógenos allí presentes (BERTRAND et al., 2014; et al., 2015).

Varios estudios aluden a que ocurren efectos en cascada, doblemente mediados por el suelo y la planta. La mejora de la estructura del suelo provocada por el rol de ingeniero ecosistémico de las lombrices, y la solubilización de nutrientes por su participación como catalizadoras de la descomposición de la materia orgánica, podrían reducir la patología o el daño indirectamente al fortalecer el estado nutricional y su sistema de defensa, y por consiguiente la salud de la planta (BERTRAND et al., 2014; et al., 2015; BROWN et al., 2004; FRIBERG et al., 2005; DE LA PEÑA, 2009). Un mayor desarrollo radicular, conseguido por la

solubilización de nutrientes y el micro-laboreo del suelo por parte de las lombrices, diluye el daño radicular frente a una misma población de nematodos (LAFONT et al., 2007) y aumenta la resistencia de las plantas a los mismos (WURST et al., 2008).

El aumento de nutrientes disponibles para las plantas en presencia de lombrices ha sido tradicionalmente atribuido a otro efecto en cascada mediado por los microorganismos (ver sección 2.3.5). No obstante, las lombrices pueden tener un efecto sobre las propiedades químicas del suelo independiente de los microorganismos descomponedores del suelo. Stephens y Davoren (1997) sugirieron que la secreción de carbonato de calcio de las glándulas clacíferas influye en la solubilidad y disponibilidad de nutrientes para los vegetales. La actividad de las lombrices tiene un fuerte impacto en el ciclo del nitrógeno, ya que, al ingerir grandes cantidades de suelo y residuos orgánicos, acelera la mineralización del nitrógeno (EDWARDS y BOHLEN, 1996). Wurst et al. (2006) comprobó que las lombrices aumentaron la absorción de N por parte de las plantas, aun cuando la biomasa microbiana se mantuvo incambiada.

2.3.5. Efectos indirectos: mediados por los organismos benéficos

Como se mencionó en las secciones 2.1 y 2.3.1, las lombrices promueven la actividad microbiana. Se ha estudiado que el número de microorganismos puede aumentar 1000 veces con el pasaje a lo largo del tracto digestivo de la lombriz (EDWARDS y FLETCHER, 1988). Clapperton et al. (2001) pudieron confirmar que la población, composición y actividad de la comunidad microbiana del suelo es afectada por la presencia de lombrices en el mismo. Muchos de estos microoganismos pueden resultar antagonistas de los patógenos o ser promotores del crecimiento vegetal. Elmer (2009) sugiere que la supresión de la verticilosis en la berenjena, el declinamiento del espárrago y la marchitez vascular del tomate fue mediada a través de un aumento en la actividad microbiológica benéfica. En este sentido, la siembra de suspensiones de suelo de la rizósfera en medios selectivos

mostró mayores densidades de pseudomonas fluorescentes y de actinobacterias filamentosas en presencia de lombrices.

Gracias a su movilidad, las lombrices pueden distribuir en el suelo a estos microorganismos benéficos y ser considerado incluso como un aliado para la inoculación de los mismos, frente a una aplicación de una suspensión en superficie, estas lo distribuyen en el perfil hacia mayores profundidades, asegurando que el inóculo del microorganismo llegue hasta la rizósfera (BROWN et al., 2004; DOUBE et al., 1994; EDWARDS y BOHLEN, 1996; HUME et al., 2015; JAYASINGHE y PARKINSON, 2009; SINGER et al., 1999; STEPHENS et al., 1994a; STEPHENS y DAVOREN, 1995). Recientemente se ha demostrado que las lombrices distribuyen en el suelo convenientemente virus utilizados para control biológico de larvas de lepidópteros (INFANTE-RODRÍGUEZ et al., 2016). En este sentido, debe probarse que los microrganismos seleccionados no tengan un efecto negativo sobre las lombrices, como lo hicieron Lagerlöf et al. (2015) cuando probaron que *Ap. caliginosa* y *Ap. longa* no fueron afectadas por altas dosis de una cepa de la bacteria promotora de crecimiento, *Bacillus amyloliquefaciens.* A su vez, Puga-Freitas et al. (2012b) concluyeron que las lombrices pueden favorecer particularmente el desarrollo de las bacterias promotoras de crecimiento vegetal.

Algunos trabajos han mostrado que las lombrices también podrían diseminar fitopatógenos (EDWARDS y FLETCHER, 1988; MONTECCHIO et al., 2014; TOYOTA y KIMURA, 1994), o nematodos fitófagos (ELLENBY, 1945). No obstante, Doube et al. (1994) concluyen que "los efectos beneficiosos de las interacciones entre las lombrices de tierra y los microorganismos pesan más que los efectos nocivos, especialmente si se pueden idear e implementar prácticas de manejo que favorezcan a los microorganismos benéficos". Más de 20 años después, aún siguen siendo escasos los trabajos que documentan efectos negativos a causa de la diseminación de patógenos por parte de lombrices, pudiendo deberse a una menor importancia de este fenómeno, sin descartar la posibilidad de un sesgo por parte de los investigadores en la selección de los casos para su estudio y publicación.

Las lombrices también pueden estimular macroorganismos benéficos de la fauna del suelo (micro y meso fauna). Varios autores han observado un aumento de la población de nematodos bacteriófagos y fungívoros en presencia de lombrices, muy probablemente por los aportes de nitrógeno en sus excreciones, mucus y tejido muerto, y por priorizar la ruta de descomposición (SENAPATI, 1992; ILIEVA-MAKULEK y MAKULEK, 2007). Asimismo, se ha visto que generan condiciones que favorecen depredadores naturales que atacan a los estadios larvales del taladro *Sesamia calamistis* (BOYER et al., 1999).

2.3.6. Efectos indirectos: mediados por las plantas

Ya se discutió en la sección 2.1 como las lombrices favorecen el rendimiento y la biomasa vegetal, lo cual puede ocurrir como efecto cascada por varios mecanismos indirectos mediados por los efectos que tienen las lombrices sobre el suelo (ver sección 2.3.4) o sobre organismos benéficos (ver sección 2.3.5). Al generar un ambiente que reduce las limitantes de nutrientes, agua y aire para el desarrollo vegetal, las plantas se encuentran menos vulnerables frente a las amenazas. Recientemente, además se ha descubierto que las lombrices pueden incidir sobre el sistema de defensa de la planta de un modo sistémico por varias vías, ya sea por contacto físico o químico. Por ejemplo, pequeños daños que las lombrices, *Millsonia anomala*, pueden hacer a las raíces de las plantas de arroz por su actividad en la rizósfera, pueden desencadenar una sobreexpresión del gen *lox*, implicado en el inicio de una vía de respuesta al estrés. La expresión de este gen desencadena reacciones fisiológicas que preparan a la planta para tolerar mejor el ataque de nematodos (BLOUIN et al., 2005). Jana et al. (2010) también han documentado que las lombrices de la especie *Ap. caliginosa* afectan la expresión de genes implicados en la proliferación celular (gen HBT) y en la respuesta al estrés (PLDα) en *Arabidopsis thaliana*.

Los oligoquetos terrestres pueden afectar a los herbívoros influyendo en las concentraciones de metabolitos secundarios y por tanto activando los mecanismos de defensa (WURST et al., 2003; et al., 2004). Esto ocurre al influir positivamente en la disponibilidad de nitrógeno en el suelo, requerido en ciertas concentraciones para la formación de estos metabolitos (en ese caso mediado por el suelo). Además, hay evidencia de que puede existir al menos algún otro mecanismo, aun no dilucidado, independiente de la disponibilidad de nitrógeno (LOHMANN et al., 2009; JANA et al., 2010).

Actualmente se está investigando cómo moléculas-señal similares a las fitohormonas, encontradas en los *pellets* fecales de las lombrices, pueden estimular el crecimiento y el sistema de defensa de la planta, afectando las vías de señalización hormonal, comparable al efecto de las rizobacterias promotoras de crecimiento. Estas moléculas pueden ser compuestos similares a las auxinas, etileno, estimuladores de defensa de las plantas, o una combinación de estos. Este fenómeno se ha observado para otros integrantes de la fauna del suelo, como ser protozoarios y colémbolos. Aún no está claro si estas moléculas son producidas por las lombrices mismas, o por las bacterias promotoras de crecimiento vegetal, cuya proliferación fuera estimulada por las lombrices (JANA et al., 2010; CANELLAS et al., 2011; PUGA-FREITAS et al., 2012a).

2.4. APLICACIÓN Y PERSPECTIVAS

Las lombrices forman parte del llamado control biológico de conservación o conservacionista (*conservation biological control*) definido por Eilenberg et al. (2001), debido a sus diversos efectos sobre fitopatógenos, fitófagos y parásitos. La generación de conocimientos de ecología y cría de las especies locales podría resultar en su inclusión dentro de alguna de las otras tres categorías por ellos definidas: (1) el control biológico clásico, en el cual se realiza la introducción de un enemigo natural generalmente exótico para un establecimiento permanente y el consiguiente control de la plaga; (2) el control biológico de inoculación, en el que se introduce una

pequeña población de un organismo vivo, y el control se dará de manera no permanente una vez que se haya reproducido y multiplicado esa población inicial; o (3) el control por inundación, cuando el control se da exclusivamente por la liberación masiva de los organismos controladores en sí mismo.

Friberg et al. (2005) concluyeron que el manejo del suelo y el ambiente, de forma tal que favorezca la fauna benéfica, representa una posibilidad realista para una agricultura y horticultura sustentable. Sin duda, en la producción agroecológica, las lombrices se tornan un importante recurso a tomar en cuenta, que no debería ser descuidado. Además de ser el principal aliado para los productores que no quieren o no pueden fertilizar sus suelos (VAN GROENIGEN et al., 2014), a partir de esta revisión se evidencia el aporte de las lombrices a la sanidad de las plantas por múltiples vías.

El mecanismo, por el cual ocurre el control biológico, y su eficiencia, son caso-específicos, dependiendo de la ecología tanto del patógeno o parásito, como de las especies de lombrices y especies vegetales intervinientes. Por este motivo, resulta necesaria la investigación en esta área referida a especies y condiciones locales para cada agroecosistema. En resumen, tanto los efectos directos como los indirectos son sustanciales en la supresión resultante de la enfermedad o plaga. Un aspecto interesante es que en muchos casos aquí descritos no se combate la amenaza, sino que se actúa a nivel de la resiliencia de la planta. El manejo sustentable del suelo lo mantiene sano, conservando sus elementos bióticos sanos, como lo es la fauna y la vegetación, y en mejores condiciones de resistir o sobrellevar distintos tipos de estrés biótico como lo son las plagas y las enfermedades.

En suma, las lombrices cumplen una gran diversidad de funciones en el suelo, la mayoría de las cuales están sub-estudiadas, más aún en el caso del control biológico de enfermedades y plagas. Dada la necesidad de encontrar sistemas de producción sustentables a nivel mundial y local, por un lado, y la capacidad que tienen las lombrices de brindar servicios ecosistémicos, por otro, la investigación en esta línea

debería tomar mayor protagonismo, por el potencial que implica. La comprensión de los aportes que pueden realizar las diferentes lombrices locales al sistema suelo-planta-organismos asociados, así como el conocimiento de su biología y ecología permitirán diseñar manejos del suelo que favorezcan sus poblaciones de forma de aumentar su contribución a los agroecosistemas.

BIBLIOGRAFÍA

AYUKE, F.O.; LAGERLÖF, J.; JORGE, G.; SÖDERLUND, S.; JOHN MUTURI, J.; SAROSH, B.R.; MEIJER, J. Effects of biocontrol bacteria and earthworms on the severity of *Alternaria brassica*e disease and the growth of oilseed rape plants (*Brassica napus*). **Applied Soil Ecology**, v 117, p. 63 – 69, 2017.

BERTRAND, M.; BLOUIN, M.; BAROT, S.; CHARLIER, A.; MARCHAND, D.; ROGER-ESTRADE, J. Biocontrol of eyespot winter wheat fungal disease by an anecic earthworm *Lumbricus terrestris*, L.) In: 13th ESA CONGRESS, 13, 25-29 August 2014, Debrecen, Hungary. **Annals of event**, 2014. p. 51 -52

BERTRAND, M.; BLOUIN, M.; BAROT, S.; CHARLIER, A.; MARCHAND, D.; ROGER-ESTRADE, J. Biocontrol of eyespot disease on two winter wheat cultivars by an anecic earthworm (*Lumbricus terrestris*). **Applied Soil Ecology**, v 96, p. 33-41, 2015.

BLOUIN, M.; ZUILY-FODIL, Y.; PHAM-THI, A.-T.; LAFFRAY, D.; REVERSAT, G.; PANDO, A.; et al. Belowground organism activities affect plant aboveground phenotype, including plant tolerance to parasites. **Ecology Letters**, n. 8, p. 202-208, 2005.

BLOUIN, M.; BAROT, S.; LAVELLE P. Earthworms (*Millsonia anomala*, Megascolecidae) do not increase rice growth through enhanced nitrogen mineralization. **Soil Biology & Biochemistry**, v. 38, p. 2063–2068, 2006.

BLOUIN, M.; HODSON, M.E.; DELGADO, E.A.; BAKER, G.; BRUSSAARD, L.; BUTTF, K.R.; DAI, J.; DENDOOVEN, L.; PERES, G.; TONDOH, J.E.; CLUZEAU, D.; BRUN, J.-J. A review of earthworm impact on soil function and ecosystem services. **European Journal of Soil Science**, v. 62, n. 4, p. 161–182, 2013.

BONKOWSKI, S.; GRIFFITHS, B.S.; RITZ, K. Food preference of earthworms for soil fungi. **Pedobiologia**. v. 44, p. 666–676, 2000.

BOUCHÉ, M.B. Strategies lombriciennes. in: Lohm, U., Persson, T. (Eds.), **Soil organisms as components of ecosystems.** Proc. 6th Int. Coll. Soil Zool. Ecol. Bull. 25, Stockholm, pp. 157-163. 1977.

BOYER, J.; MICHELLON, R.; CHABANNE, A.; REVERSAT, G.; TIBERE, R. Effects of trefoil cover crop and earthworm inoculation on maize crop and soil organisms in Reunion Island**. Biology & Fertility of Soils,** v. 28, n.4, p. 364–370, 1999.

BOYER J.; REVERSAT, G.; LAVELLE, P.; CHABANNE, A. Interactions between earthworms and plant-parasitic nematodes. **European Journal of Soil Biology**, v. 59, p. 43-47, 2013.

BROWN, G.G.; PASHANASI, B.; VILLENAVE, C.; PATRON, J.C.; SENAPATI, B.K.; GIRI, S.; BAROIS, I.; LAVELLE, P.; BLANCHART, E.; BLAKEMORE, R.J.; SPAIN, A.V.; BOYER, J. Effects of Earthworms on Plant Production in the Tropic. In: LAVELLE, P.; BRUSSAARD, L.; HENDRIX, P. (Org.) **Earthworm Management in Tropical Agroecosystems.** Wallingford-New York: CAB. International, 1999. p. 87-147.

BROWN, G.G.; EDWARDS, C.A.; BRUSSAARD, L. How earthworms affect plant growth: Burrowing into the mechanisms. In: Edwards C.A. (Org.). **Earthworm ecology** (2nd ed). London: Boca Raton: CRC Press, 2004. p. 19–49.

BROWN, G.G. How do earthworms affect microfloral and faunal community diversity? **Plant & Soil**, v. 170, n. 1, p. 209–231, 1995.

CAMACHO VALDEZ, V.; RUIZ LUNA, A. Marco conceptual y clasificación de los servicios ecosistémicos. **Revista Bio Ciencias**, v. 1, n. 4, p. 3-15, 2012.

CANELLAS, L.P.; DANTAS, D.J.; AGUIAR N.O.; PERES, L.E.P.; ZSÖGÖN, A.; OLIVARES, F.L.; DOBBSS, L.B.; FACANHA, A.R.; NEBBIOSO, A.; PICCOLO A. Probing the hormonal activity of fractionated molecular humic components in tomato auxin mutants. **Annals of Applied Biology**, p. 1-10, 2011.

CHO, J.H.; PARK, C.B.; YOON, Y.G.; KIM, S.C. Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization. **Biochimica et Biophysica Acta**, v. 1408, p. 67-76, 1998.

CLAPPERTON, M.J.; LEE, N.O.; BINET, F.; CONNER, R.L., Earthworms indirectly reduce the effects of take-all (*Gaeumannomyces graminis* var. tritici) on soft white spring wheat (*Triticum aestivum* cv. Fielder). **Soil Biology & Biochemistry**, v. 33, n. 11, p. 1531-1538, 2001.

CUNHA, L; BROWN, G.G.; STANTON, D. W. G.; DA SILVA, E.; HANSEL, F. A.; JORGE ESCUDERO, G.; MCKEY, D.; VIDALTORRADO, P.; MACEDO,

R.S.; VELASQUEZ, E.; JAMES, S.W.; LAVELLE, P.; KILLE, P. Soil animals and pedogenesis: The role of earthworms in anthropogenic soils. **Soil Science** (E), v.: 181, p.: 110 - 125, 2016.

DE LA PEÑA, E. Efectos de la biota edáfica en las interacciones planta-insecto a nivel foliar. **Ecosistemas**, v. 18, n. 2, p. 64-78, 2009.

DOUBE, B.M.; STEPHENS, P.M.; DAVOREN, C.W.; RYDER, M.H. Interactions between earthworms, beneficial soil microorganisms and root pathogens. **Applied Soil Ecology**, v. 1, p. 3-10, 1994.

EDWARDS, C.A.; FLETCHER, K.E. Interactions between Earthworms and Microorganisms in Organic-matter Breakdown. **Agriculture, Ecosystems & Environment**, v. 24, p. 235-247, 1988.

EDWARDS, C.A.; BOHLEN, P.J. **The biology and ecology of earthworms**. London: Chapman and Hall, 1996. 426p.

EDWARDS C.A. **Earthworm ecology** (2nd ed). London: Boca Raton: CRC Press, 2004.424 p.

EDWARDS, C.A.; DOMÍNGUEZ, J.; ARANCON, N. Q. The influence of vermicomposts on plant growth and pest incidence. In: SOIL ZOOLOGY FOR SUSTAINABLE DEVELOPMENT IN THE 2IST CENTURY, 2004, Cairo. **Resumen de trabajos presentados,** Cairo: [s.n.] Shakir, S.H. Hanna; Mikhaïl, W.Z.A. (Org.), 2004. p.397-420.

EILENBERG, J.; HAJEK, A.; LOMER, C. Suggestions for unifying the terminology in biological control. **BioControl**, v. 46, p. 387-400, 2001.

ELLENBY, C. Influence of earthworms on larval emergence in the potato-root eelworm, *Heterodra rostochiensis* Wollenweber, **Annals of Applied Biology**, v. 31, p. 332-339, 1945.

ELMER, W.H. Influence of earthworm activity on soil microbes and soilborne diseases of vegetables. **Plant Disease**, v. 93, n. 2, p. 175–179, 2009.

FRIBERG, H.; LAGERLÖF, J.; RÄMERT, B. Influence of soil fauna on fungal plant pathogens in agricultural and horticultural systems. **Biocontrol Science & Technology,** v. 15, n. 7, p. 641-658, 2005.

HASNA, M.K.; LAGERLÖF, J.; RÄMERT, B. Effects of fungivorous nematodes on corky-root disease of tomato grown in compost amended soil. **Acta Agriculture Scandinavica, Section B., Plant & Soil Science**, v. 58, p. 145-153, 2008.

HIRST, J.M.; STEDMAN, O.J. The epidemiology of apple scab (*Venturia inequalis* (Cke) Wint). **Annals of Applied Biology,** v. 50, p. 551-567, 1962.

HUME, E.A.; HORROCKS, A.J.; FRASER, P.M.; CURTIN, D.; MEENKEN, E.D.; CHNG, S.; BEARE, M.H. Alleviation of takeall in wheat by the earthworm *Aporrectodea caliginosa* (Savigny). **Applied Soil Ecology**, v. 90, p. 18–25, 2015.

ILIEVA-MAKULEC, K.; MAKULEC, G. Does the activity of the earthworm *Aporrectodea caliginosa* modify the plant diversity effect on soil nematodes? **European Journal of Soil Biology,** v. 43, p. 157–164, 2007.

INFANTE-RODRÍGUEZ, D.A.; BERBER, J.J.; MERCADO, G.; VALENZUELA-GONZÁLEZ, J.; MUÑOZ, D.; WILLIAMS, W. Earthworm mediated dispersal of baculovirus occlusion bodies: Experimental evidence from a model system. **Biological Control**, v. 100, p. 18–24, 2016.

JANA, U.; BAROT, S.; BLOUIN, M.; LAVELLE, P.; LAFFRAY, D.; REPELLIN, A. Earthworms influence the production of above- and belowground biomass and the expression of genes involved in cell proliferation and stress responses in *Arabidopsis thaliana*. **Soil Biology & Biochemistry,** v. 42, p. 244–252, 2010.

JAYASINGHE, B.A.T.D.; PARKINSON, D. Earthworms as the vectors of actinomycetes antagonistic to litter decomposer fungi. **Applied Soil Ecology,** v. 43, p.1–10, 2009.

JOHNSON, D.L.; SCHAETZL, R.J. Differing views of soil and pedogenesis by two masters: Darwin and Dokuchaev. **Geoderma.** p. 237–238, 2015.

JONES, C.G.; LAWTON, J.H.; SHACHAK, M. Organisms as ecosystem engineers. **Oikos,** v. 69, p. 373-386, 1994.

JORGE-ESCUDERO, G.; PÉREZ, C.A.; FRIBERG, H.; SÖDERLUND, S.; VERO, S.; GARMENDIA, G.; LAGERLÖF, J. Contribution of anecic and epigeic earthworms to biological control of Fusarium graminearum in wheat straw**. *S/p.***

KENNEL, W. The role of the earthworm *Lumbricus terrestris* in integrated fruit production, **Acta Horticulturae**, v. 285, p. 149-156, 1990.

LAFONT, A.; RISEDE, J.M.; LORANYER-MERCIRIS, G.; CLERMONT-DAUPHIN, C.; DOREL, M.; RHINO, B.; LAVELLE, P. Effects of the earthworm Pontoscolex corethrurus on banana plants infected or not with the plant-parasitic nematode Radopholus similis. **Pedobiologia**, v. 51, p. 311–318, 2007.

LAGERLÖF, J.; AYUKE, F.; BEJAI, S.; JORGE, G.; LAGERQVIST, E.; MEIJER, J.; MUTURI JOHN, J. Potential side effects of biocontrol and plant-growth

promoting *Bacillus amyloliquefaciens* bacteria on earthworms. **Applied Soil Ecology**, v. 96, p. 159 – 164, 2015.
LAGERLÖF, J.; INSUNZA, V.; LUNDEGÅRDH, B.; RÄMERT B. Interaction between a fungal plant disease, fungivorous nematodes and compost suppressiveness. **Acta Agriculture Scandinavica Section B – Soil & Plant Science**, v. 61, p. 372-377, 2011.

LAGERLÖF, J.; PÅLSSON, O.; ARVIDSSON, J. Earthworms influenced by reduced tillage, conventional tillage and energy forest in Swedish agricultural field experiments. **Acta Agriculture Scandinavica Section B – Soil & Plant Science,** v. 62, p. 235-244, 2012.

LAING, J.E.; HERATY, J.M.; CORRIGAN, J.E. Leaf burial by the earthworm, *Lumbricus terrestris* (Oligochaeta: Lumbricidae), as a major factor in the population dynamics of *Phyllonorycter blancardella* (Lepidoptera: *Gracillariidae*) and its parasites. **Environmental Entomology**, v. 15, p. 321-326, 1986.

LAVELLE, P. Earthworm activities and the soil system. **Biology & Fertility of Soils**, v. 6, p. 237-251, 1988.

LAVELLE, P. Faunal activities and soil processes: Adaptive strategies that determine ecosystem function. **Advances in Ecological Research**, v. 27, p. 93–132, 1997.

LAVELLE, P.; SPAIN A. V. **Soil ecology**. Boston-Massachusetts: Kluwer Academic, 2001. 654pp.

LEE, K. **Earthworms: Their Ecology and Relationships with Soils and Land Use.** New York: Academic Press, 1985. 411p.

LOHMANN, M.; SCHEU, S.; MULLER, C. Decomposers and root feeders interactively affect plant defence in *Sinapis alba*. **Oecologia**, v. 160, p. 289–298, 2009.

MEGHVANSI, M.K.; SINGH, L.; SRIVASTAVA, R.B.; VARMA, A. Assessing the Role of Earthworms in Biocontrol of Soil-Borne Plant Fungal Diseases. In: Ayten Karaca (Org.) **Biology of Earthworms**. Noida, UP: Springer, 2011. p. 173-190.

MONTECCHIO, L.; SCATTOLIN, L.; SQUARTINI, A.; BUTT, K.R. Potential spread of forest soil-borne fungi through earthworm consumption and casting. **iForest** t 8: 295-301 [online 2014-08-26] URL: http://www.sisef.it/iforest/contents/?id=ifor1217-008, 2014.

MOODY, S.; PIEARCE, T.G.; DIGHTON, J. Fate of some fungal spores associated with wheat straw decomposition on passage through the guts of *Lumbricus terrestris*

and *Aporrectodea longa*. **Soil Bioliology & Biochemistry**, v. 28, n.4/5, p. 533-537. 1996.

NAKAMURA, Y.; ITAKURA, J.; MATSUZAKI, I. Influence of the earthworm *Pheretima hilgendorfi* (Megascolecidae) on *Plasmodiophora brassicae* clubroot galls of cabbage seedlings in pot. **Edaphologia,** v. 54, p. 39-41, 1995.

NAKAMURA, Y. Interactions between earthworms and microorganisms in biological control of plant root pathogens. **Farming Japan**, v. 30, n. 6, p. 37-43, 1996.

NEWINGTON, J.E.; SETÄLÄ, H.; BEZEMER, T.M.; JONES, T.H. Potential Effects of Earthworms on Leaf-Chewer Performance. **Functional Ecology**, v. 18, n. 5, p. 746-751, 2004.

NIKLAS, J.; KENNEL, W. The role of the earthworm, *Lumbricus terrestris* (L.) in removing sources of phytopathogenic fungi in orchards, **Gartenbauwissenschaft**, v. 46, p. 138-142, 1981.

OLDENBURG, E.; KRAMER, S.; SCHRADER, S.; WEINERT J. Impact of the earthworm *Lumbricus terrestris* on the degradation of *Fusarium*-infected and deoxynivalenol-contaminated wheat straw. **Soil Biology & Biochemistry**, v. 40, p. 3049-3053, 2008.

POVEDA, K.; STEFFAN-DEWENTER, I.; SCHEU, S.; TSCHARNTKE, T. Effects of decomposers and herbivores on plant performance and aboveground plant–insect interactions. **Oikos,** v. 108, p. 503–510, 2005.

PUGA-FREITAS, R.; BAROT, S.; TACONNAT, L.; RENOU, J.P.; BLOUIN, M. Signal molecules mediate the impact of the earthworm *Aporrectodea caliginosa* on growth, development and defence of the plant *Arabidopsis thaliana*. **Plos One**, v. 7, n. 12, p. 1-10, 2012a.

PUGA-FREITAS, R.; BELKACEM. L.; BAROT, S.; BERTRAND, M.; ROGER-ESTRADE, J.; BLOUIN, M. Transcriptional profiling of wheat in response to take-all disease and mechanisms involved in earthworm's biocontrol effect. **European Journal of Plant Pathology**, v. 144, p. 155–165, 2016.

PUGA-FREITAS, R.; ABBAD, S.; GIGON, A.; GARNIER-ZARLI, E.; BLOUIN, M. Control of Cultivable IAA-producing bacteria by the plant *Arabidopsis thaliana* and the earthworm *Aporrectodea caliginosa*. **Applied & Environmental Soil Science**, p. 1-4, 2012b.

RAW, F. Studies of earthworm populations in orchards. **Annals of Applied Biology**, v. 50, p. 389-404, 1962.

RUESS, L.; ZAPATA, J.E.G.; DIGHTON, J. Food preference of a fungal-feeding *Aphelenchoides* species. **Nematology**, v. 2, p. 223-230, 2000.

SCHEU, S. Effects of earthworms on plant growth: patterns and perspectives. **Pedobiologia,** v. 47, p. 846–856, 2003.

SCHIEDECK, G.; SCHIAVON, G.de A.; SCHWENGBER, J.E. Densidade e biomassa de minhocas em pomar de pessegueiro sob diferentes manejos do solo. **Revista Brasileira de Agroecologia**, v. 4, n. 2, p. 2725-2728, 2009a.

SCHIEDECK, G.; SCHIAVON, G. de A.; MAYER, F A.; LIMA, A.C.R. Percepção de agricultores sobre o papel das minhocas nos agroecossistemas. **Revista Brasileira de Agroecologia**, v. 4, n. 2, p. 856-859, 2009b.

SCHRADER, S.; WOLFARTH, F.; OLDENBURG, O. Biological control of soil-borne phytopathogenic fungi and their mycotoxins by soil fauna. A review. **Bulletin UASMV serie Agriculture,** v. 70, n. 29, p. 291-298, 2013.

SENAPATI, B.K. Biotic interactions between soil nematodes and earthworms. **Soil Biology & Biochemistry,** v. 24, p. 1441–1444, 1992.

SHIPITALO, J.M.; LE BAYON, R.C. Quantifying the Effects of Earthworms on Soil Aggregation and Porosity. In: Edwards C. A. (Org.), **Earthworm ecology** (2nd ed). London: Boca Raton: CRC Press, 2004. p. 19–49.

SIMSEK-ERSAHIN, Y. The Use of Vermicompost Products to Control Plant Diseases and Pests. In: Ayten Karaca (Org.) **Biology of Earthworms**. Noida, UP: Springer, 2011. p. 191-214.

SINGER, A.C.; CROWLEY, D.E.; MENGE, J.A. Use of an anecic earthworm, *Pheretima hawayana*, as a means for delivery of fungal biocontrol agents. **Pedobiologia**, v. 43, p. 771-775, 1999.

STEPHENS, P.M.; DAVOREN C.W.; DOUBE B.M.; RYDER M.H.; BENGER A.M.; NEATE S.M. Reduced severity of *Rhizoctonia solani* disease on wheat seedlings associated with the presence of the earthworm *Aporrectodea trapezoides* Lumbricidae). **Soil Biology & Biochemistry,** v. 25, p. 1477-1484, 1993.

STEPHENS, P.M.; DAVOREN, C.W.; RYDER, M.H.; DOUBE, B.M. Influence of the earthworms *Aporrectodea rosea* and *Aporrectodea trapezoides* on *Rhizoctonia* disease of wheat seedlings and the interaction with a surface mulch of cereal/pea straw. **Soil Biology & Biochemistry**, v. 26, p. 1285-1287, 1994a.

STEPHENS, P.M.; DAVOREN, C.W.; RYDER, M.H.; DOUBE, B.M.; CORRELL, R.L. Field evidence for reduced severity of *Rhizoctonia* bare patch disease of wheat, due to the presence of the earthworms *Aporrectodea rosea* and *Aporrectodea trapezoides.* **Soil Biology & Biochemistry**, v. 26, p. 1495-1500, 1994b.

STEPHENS, P.M.; DAVOREN, C.W.; RYDER, M.H.; DOUBE, B.M. Ability of the lumbricid earthworms *Aporrectodea rosea* and *A. trapezoides* to reduce the severity of take-all under greenhouse and field conditions. **Soil Biology & Biochemistry,** v. 26, p. 1291-1297, 1994c.

STEPHENS, P.M.; DAVOREN, C.W. Effect of the lumbricid earthworm *Aporrectodea trapezoides* on wheat grain yield in the field, presence or absence of *Rhizoctonia solani* and *Gaumannomyces graminis var tritici.* **Soil Biology & Biochemistry**, v. 33, n.11, p. 1531–1538, 1995.

STEPHENS, P.M.; DAVOREN, C.W. Influence of the earthworm *Aporrectodea trapezoides* and *A. rosea* on the disease severity of *Rhizoctonia solani* on subterranean clover and ryegrass. **Soil Biology & Biochemistry**, v. 29, p. 511-516, 1997.

TAO, J.; CHEN, X.; LIU, M.; HU, F.; GRIFFITHS, B.; LI, H. Earthworms change the abundance and community structure of nematodes and protozoa in a maize residue amended rice–wheat rotation agro-ecosystem. **Soil Biology & Biochemistry**, v. 41, p. 898–904, 2009.

TOYOTA, K.; KIMURA, M. Earthworms disseminate a soil-borne plant pathogen, *Fusarium oxysporum* f. sp. *Raphani.* **Biology & Fertility of Soils,** v. 18, p. 32-36, 1994.

VAN GROENIGEN, J.W.; LUBBERS, I.M.; VOS H.M.J.; BROWN, G.G.; DE DEYN, G.B.; VAN GROENIGEN, K.J. Earthworms increase plant production: a meta-analysis. **Scientific Reports-Nature**, v. 4, p. 1-7, 2014.

WANG, C.; SUN, Z.; LIU, Y.; ZHANG, X.; XU, G. A novel antimicrobial vermipeptide family from earthworm *Eisenia fetida*. **European Journal of Soil Biology,** v. 43, p. 127-134, 2007.

WANG, C.; ZHENJUN, S.; ZHENG, D.; LIU, X. Function of mucilaginous secretions in the antibacterial immunity system of *Eisenia fetida*. **Pedobiologia,** v. 54, p.57–62, 2011.

WARDLE, D.A.; YEATES, G.W.; WILLIAMSON, W.M.; BONNER, K.I.; BARKER, G.M. Linking Aboveground and Belowground Communities: The Indirect Influence of Aphid Species Identity and Diversity on a Three Trophic Level Soil Food Web. **Oikos**, v. 107, n. 2, p. 283-294, 2004.

WOLFARTH, F.; SCHRADER, S.; OLDENBURG, E.; WEINERT, J.; BRUTTONE, J. Earthworms promote the reduction of *Fusarium* biomass and deoxynivalenol content in wheat straw under field conditions. **Soil Biology & Biochemistry**, v. 43, p. 1858-1865, 2011a.

WOLFARTH, F.; SCHRADER, S.; OLDENBURG, E.; WEINERT, J. Contribution of the endogeic earthworm species *Aporrectodea caliginosa* to the degradation of deoxynivalenol and *Fusarium* biomass in wheat straw. **Mycotoxin Research**, v. 27, p. 215–220, 2011b.

WURST, S.; LANGEL, R.; REINEKING, A.; BONKOWSKI, M.; SCHEU, S. Effects of earthworms and organic litter distribution on plant performance and aphid reproduction. **Oecologia,** v. 137, p. 90–96, 2003.

WURST, S.; DUGASSA-GOBENA, D.; LANGEL, R.; BONKOWSKI, M.; SCHEU, S. Combined effects of earthworms and vesicular-arbuscular mycorrhizas on plant and aphid performance. **New Phytologist**, v. 163, p. 169–176, 2004.

WURST, S.; LANGEL, R.; RODGER, S.; SCHEU, S. Effects of belowground biota on primary and secondary metabolites in *Brassica oleracea*. **Chemoecology,** v. 16, p. 69–73, 2006.

WURST, S.; ALLEMA, B.; DUYTS, H.; VAN DER PUTTEN, W.H. Earthworms counterbalance the negative effect of microorganisms on plant diversity and enhance the tolerance of grasses to nematodes. **Oikos,** v. 117, p. 711–718, 2008.

WURST, S. Effects of earthworms on above- and belowground herbivores. **Applied Soil Ecology,** v. 45, p. 123–130, 2010.

YEATES, G.W. Influence of earthworms on soil nematode populations**. Annual Abstracts Society of Nematologists, Journal of Nematology**, v. 12, n. 4, p. 242-242, 1980.

YEATES, G.W. Soil nematode populations depressed in the presence of earthworms. **Pedobiologia,** v. 22, p. 191–195, 1981.

ZERBINO, M.S. **Evaluación de la densidad, biomasa y diversidad de la macrofauna del suelo en diferentes sistemas de producción.** 92p. Tesis (Maestría en Ciencias Ambientales) - Facultad de Ciencias, Universidad de la República, Montevideo, 2005.

ZERBINO M.S.; ALTIER, N.; MORÓN, A.; RODRÍGUEZ, C. Evaluación de la macrofauna del suelo en sistemas de producción en siembra directa y con pastoreo. **Agrociencia Uruguay,** v. 1, p. 44-55, 2008.

ZERBINO M.S. Evaluación de la macrofauna del suelo en rotaciones cultivo-pasturas con laboreo. **Acta Zoológica Mexicana**, v 26, p. 189-202, 2010.

3: DESCRIPCIÓN DE COMUNIDADES DE LOMBRICES EN AGROECOSISTEMAS QUE INCLUYEN AL CULTIVO DE TRIGO

3.1. RESUMEN

Los lumbrícidos exóticos y varias especies del género *Amynthas* han invadido todos los continentes y parecen ser mejores competidores que las lombrices nativas en ecosistemas perturbados, como los que incluyen cultivos agrícolas. La escasa investigación sobre biodiversidad de lombrices en Uruguay ha registrado 19 especies hasta la fecha, de las cuales más de la mitad son exóticas. Con el objetivo de profundizar en el conocimiento de las especies de lombrices presentes en ecosistemas agrícolas, y determinar su relación con las características edáficas, se muestrearon suelos que incluyen trigo en su rotación, en dos localidades, una en el sur (Montevideo) y otra en el oeste (Paysandú). La identificación de las especies de lombrices se basó en la combinación de taxonomía morfológica con métodos moleculares. Se ajustó un método de identificación objetivo que complementará al morfológico, sin precedentes en el país. De esta manera se encontraron en total 17 especies de lombrices, cuatro de las cuales aún no han sido descritas para Uruguay, por lo que se enriquece la lista de especies descriptas para Uruguay. De las 12 especies exóticas encontradas, ocho fueron endógeas, dos anécicas y dos epígeas, mientras que las cinco nativas fueron endógeas. Se realiza, además, el primer reporte de especies exóticas en Paysandú, con la presencia de *Aporrectodea caliginosa*, la especie más abundante y omnipresente en este estudio. Se encontraron secuencias disponibles en GenBank para diez especies exóticas y dos de las nativas, lo cual evidencia la necesidad de continuar esta línea de investigación contribuyendo al repositorio con secuencias de especies nativas adecuadamente identificadas, a modo de facilitar el conocimiento cabal de la biodiversidad de lombrices en Uruguay.

3.2. EXOTIC OR NATIVE DOMINANCE IN EARTHWORM COMMUNITIES IN WHEAT CROPS IN URUGUAY?

Gabriella Jorge Escudero[a,b*], Carlos A. Pérez[c], Claudio Martínez[d], Mónica Cadenazzi[e], Jan Lagerlöf[f]

[a] Departamento de Sistemas Ambientales, Facultad de Agronomía, Universidad de la República, Garzón 780, 12900 Montevideo, Uruguay.

[b] Departamento de Suelos y Aguas, Facultad de Agronomía, Universidad de la República, Garzón 780, 12900 Montevideo, Uruguay.

[c] Departamento de Protección Vegetal, EEMAC, Facultad de Agronomía, Universidad de la República, Ruta 3 km 363, 60.000 Paysandú, Uruguay.

[d] Sección Bioquímica, Facultad de Ciencias; Núcleo Interdisciplinario Colectivo TÁ, Espacio Interdisciplinario, Universidad de la República, Iguá 4225 Esq. Mataojo C.P. 11400 Montevideo, Uruguay.

[e] Departamento de Biometría, Estadística y Cómputo, EEMAC, Facultad de Agronomía, Universidad de la República, Ruta 3 km 363, 60.000 Paysandú, Uruguay

[f] Department of Ecology, Swedish University of Agricultural Sciences (SLU), P.O Box 7044, SE-75007, Uppsala, Sweden

*Corresponding author: gjorge@fagro.edu.uy

Abstract

Exotic Lumbricids and several species of the genus *Amynthas* have invaded al continents and seem to be better competitors than natives in disturbed ecosystems as agricultural fields. The scarce earthworm biodiversity research in Uruguay has till the date registered 19 species to date, being more than half of them exotics. We hypothesized that (1) the exotic:native relation depends on location and on degree of disturbance; and that (2) exotics dominate in the south and in the disturbed lands. We sampled wheat rotations in Montevideo (in southern Uruguay) and Paysandú (in western Uruguay). Collecting included undisturbed sites close to each sampling plot, as control. Analyses were conducted to elucidate whether other variables, different from disturbance and location could be affecting variability in terms of species distribution, earthworm biomass and density. Out of 12 exotics species found, eight were endogeics, two anecics, and two epigeic, while the five natives were endogeic. This is the first report for Uruguay for the native species: *Microscolex phosphoreus* and *Glossodrilus parecis*; and the exotic species *Aporrectodea tuberculata* and *Murchieona minuscula.* The finding of *Ap. caliginosa* and *Mu. minuscula* in

Paysandú, represents the first report of exotic species in this location. In addition, ther former species was the most abundant and ubiquitous species in this study. The ratio exotic:native varied between locations being higher in Montevideo than in Paysandú. This might be attributed to the closeness of Montevideo to the natural seaport, main entrance for European and Middle East immigration. The second hypothesis was only true for Montevideo, where exotics dominated both in agriculture and control plots. Finding unrecorded species in such few and homogeneous sampled sites, evidences the poor coverage of sampling in anthropic and natural landscapes of the country. The short current earthworm list should be extended by increasing samplings, covering a wider range of locations and soil use systems. It will be a challenge to elucidate whether the native species have been displaced by exotics and/or disturbance, especially in western Uruguay, where some native species seem to have adapted to disturbed sites due to agricultural activity. The development of an appropriate local baseline of earthworm species present in undisturbed ecosystems, is necessary.

Keywords: Anthropochorous earthworms, Lumbricids, exotic:native relation

3.2.1. Introduction

Invasive non-native earthworms are the most seen earthworms worldwide, and this group may account for the greatest biomass per area compared to other invasive animals (James 2011). Anthropochorous earthworms, *i.e.* those spread (mostly unintentionally) by human activity (Maggenti et al. 2005), are generally adapted to agriculture conditions. As humans, these have expanded worldwide, in particular several members of the European Lumbricidae family and the Asian *Amynthas* genus (James and Hendrix 2004; James 2011). They have successfully invaded various disturbed ecosystems that either lacked earthworms due to the glacial history, or that in previous conditions held a native community with less competitive species (Hendrix et al. 2006). Moreover, exotic earthworms have also shown to be successful

invading certain apparently undisturbed indigenous ecosystems (Hendrix et al. 2006; Sánchez-de León and Johnson-Maynard 2009). There is plenty evidence that co-existence with natives takes place, but it is not always clear whether this may be a stable or transient state (Hendrix et al. 2006; Fragoso and Brown 2007).

Earthworm invasions in North America have been well documented (Fragoso 2007; Addison 2009; Callaham et al. 2016). Fewer studies have been conducted in Central and South America, with scarce research in the 20th century, although with an increasing trend since 1980, boosted by ELAETAO (The Portuguese/Spanish acronym for "Latin-American meeting on Ecology and Taxonomy of Terrestrial Oligochaete") with five consecutive events since 2003 (Fragoso and Brown 2007). Out of 14 surveyed countries/regions in Latin America, Uruguay presented the lowest number of earthworm species registered (19), and the highest proportion of exotics (58%) (Fragoso and Brown 2007).

At least 100 earthworm species have achieved distributions beyond their places of origin (Lee 1985; Fragoso et al. 1999). In Latin America, 66 exotics have been reported (Fragosso and Brown 2007). In Montevideo, capital of Uruguay, located in the south, and main seaport since colonial times, 10 species of the Lumbricidae family and two of the genus *Amynthas* of the Megascolecidae family, have been found. The two native species reported for Montevideo are *Microscolex dubius* and *Eukerria stagnalis*. In contrast, in studies performed in the northern central regions of Uruguay, only native Oligochaeta, belonging to the Ocnerodrilidae, Acantodrilidae and Glossoscolecidae families have been reported (Grosso and Brown 2007; Zerbino 2010). The contrast between these two zones in Uruguay may reflect the proximity to the naturally deep seaport, located in Montevideo, main entrance for European and Middle East immigration (Vidart and Pi Hurgarte 1969).

James (2011) hypothesized that exotics will dominate in sites with increasing intensity and frequency of disturbance. In Colombia, for example, it has been observed that intensive agriculture leads to the loss of native species (Feijoo 2011).

In the same way, in Argentina, *M. dubius* (native) was associated with natural pasture conditions, while *Aporrectodea caliginosa* (exotic) was associated to intensive agriculture characteristics. *Eukerria stagnalis* and *Ap. rosea*, native and exotic, respectively, coexist and were associated with system conditions of recent agriculture with high humidity, low pH and Ca (Falco et al. 2015; Masín et al. 2015).

Based on these findings, we hypothesized that (1) the exotic:native ratio depends on location and on degree of disturbance; and that (2) exotics dominate in the south and in disturbed lands. We sampled fields including wheat in the rotations in Montevideo and Paysandú, and undisturbed sites close to each wheat sampling plot were used as control. Analyses were conducted to elucidate whether other variables, besides disturbance and location could be influencing species distribution, earthworm biomass and density.

3.2.2. Methods

3.2.2.1. Study sites

Field data were obtained from two locations in southern and western Uruguay (Fig. 1). In the south, samples were collected from Organic Transitional Farms (OF) and Non-organic Farms (NF) close to Montevideo (34.5^{o}S; 56^{o}W). The OFs represent conventional tillage without agrochemicals input, whereas the NFs are based on no-till systems managed with herbicides and other agrochemicals (table 1). In the west, samples were collected from 10 m x 50 m plots in a long term crop rotation essay established in 1994, at the University Experimental Field belonging to "Mario A. Cassinoni"- Experiment Station of the Faculty of Agronomy, located in Paysandú (32.5^{o}S; 58^{o}W). The history of this site includes a period of crop-pasture rotation since 1970, preceded by 30 years of continuous cropping always with conventional tillage (Ernst et al. 2009). The different rotations include crops and pastures with

conventional tillage or no-till (table 2). All sampling fields were selected according to the criterion of having wheat as a current or prior crop, and a control site was selected in a nearby field with no disturbance level. Climatic variables of the sampled sites were registered by the wheather stations of Agronomy Faculty in Paysandú and Montevideo (Fig. 2).

Table 1. Sampled sites in farms in Montevideo

Site	Description	System
M-NF1-NT	Non-organic Farm	no-till
M-NF2-NT	Non-organic Farm	no-till
M-OF1-T	Organic transitional Farm	tillage
M-OF2-T	Organic transitional Farm	tillage
M-OF3-T	Organic transitional Farm	tillage
M-OF4-T	Organic transitional Farm	tillage
M-NF1-Ctrl	Non-organic Farm	control site
M-NF2-Ctrl	Non-organic Farm	control site
M-OF1-Ctrl	Organic transitional Farm	control site
M-OF2-Ctrl	Organic transitional Farm	control site
M-OF3-Ctrl	Organic transitional Farm	control site
M-OF4-Ctrl	Organic transitional Farm	control site

Note: M=Montevideo, OF=organic transitional farm, NF= non-organic farm, T=tillage, Cntrl=control site.

Table 2. Sampled sites at the University Experimental Field in Paysandú

Site	Description	Rotation (yrs)		System
		Pastures	Crops	
P-PC-T	Pasture-crop rotation	3.5	3.5	tillage
P-PC-NT	Pasture-crop rotation	3.5	3.5	no-till
P-CC-NT	Continuous crop	0	7	no-till
P-SPC-NT	Short Pasture-crop rotation	1.5	3.5	no-till
P-Cntrl	Control site	-	-	control site

Note: P=Paysandú, PC=pasture-crop rotation, CC=continuous crop, SPC=short pasture-crop rotation, T=tillage, NT=no tillage, Cntrl=control site.

3.2.2.2. Assessment of earthworm fauna

Sampling was done in autumn and spring of 2014 and in spring of 2015 (sampling dates in Fig 2), as the activity of earthworms increase with favorable temperature and humidity conditions (Edwards and Lofty 1976). In autumn of 2015 no sampling was carried out due to a long-lasting drought that hit the country (April to June 2015, Fig.1), and no earthworms were expected to be found in the upper 20 cm of the soil, according to their moisture requirements (Curry 1998). The sampling method was similar to that recommended by Anderson and Ingram (1993), with five sample units (soil monolith 25 cm x 25 cm and 20 cm depth) per plot or control site. Next to each sample unit, a soil sample was taken in duplicate of 0-20 cm for soil moisture content and chemical analysis; soil bulk density was determined using the core method described by Dane and Topp (2002). Moisture was determined by gravimetric method, drying at 105 ºC for 48 h, and for the chemical characterization soil was dried at 40 ºC for 48 h and grounded (Dane and Topp 2002).

Earthworms were hand sorted from the monoliths, counted and weighed (fresh weight). Before weighing, each individual was rinsed with distilled water and gently dried with paper napkins. They were then anesthetized gradually in alcohol until reaching a concentration of 20% for later fixation with 4% formaldehyde solution

(Righi 1990). All adult specimens were measured (length and width); classified by morphological taxonomy into species level, when possible, and categorized as natives or exotics, based on the worm list described for Uruguay by Grosso and Brown (2007), and following the available keys and taxonomic descriptions (Righi 1979; Sims and Gerard 1985; Reynolds 1996; Andersen 1997). In the absence of a specific key for Uruguay, these keys were not used strictly following all the dichotomous options, but rather as a guide. Species identity was confirmed by molecular methods by amplification and sequencing of a mitochondrial cytochrome oxidase I complex (COI) DNA region (barcoding). DNA was extracted from the rear section of representative earthworms for each morphological group, which was not fixed in formaldehyde, but preserved in anhydrous ethanol -20 ^{o}C. A standard DNA-extraction protocol was followed, which is a modified version of Dellaporta et al. (1983) (Jorge-Escudero et al. *in press*). The COI fragments were amplified with primers LCO1490 and HCO2198 (Folmer et al. 1994), applying a standard barcoding protocol adaptation (Huang 2007) based on George G. Brown's suggestion (com. pers.) (Jorge-Escudero et al. *in press*). PCR reactions were sent to Macrogen® (Korea) for amplicon purification and sequencing, and subjected to BLAST-N algorithm (Altschul et al. 1990) searching closely matching species.

3.2.2.3. Data analyses

The following community descriptors were calculated: Biomass (kg of earthworms per hectare); density (number of earthworms per square meter); adult abundance (A) and species richness (S) (absolute number of earthworms or species, respectively, present in the 5 samples of each plot or control site); Shannon-Wiener Diversity Index (H') ; where p_i is the number p of individuals of species i; and Evennes Index Pielou (E) (Margurran 1988). Coefficient of variation was calculated for biomass and density values grouped by sampling date and province.

Two Principal Component Analyses (PCA) were carried out to classify the plots per date according to the abundance of each species per plot, in the first place; and according to soil properties and community descriptors in the second place (soil texture, water content, bulk density, C, N and P; biomass, density, species richness, diversity and evenness). The correlation between Euclidean distance matrixes of sites according to species, and according to soil properties only (not including community descriptors, to avoid redundant information in both matrixes) was studied by a Mantel test. These analyses were performed with the statistical package Infostat®.

Fig. 1. Location of sampled sites in the provinces of Montevideo and Paysandú, and its distance to the seaport, main entrance for immigration since colonial times, Uruguay.

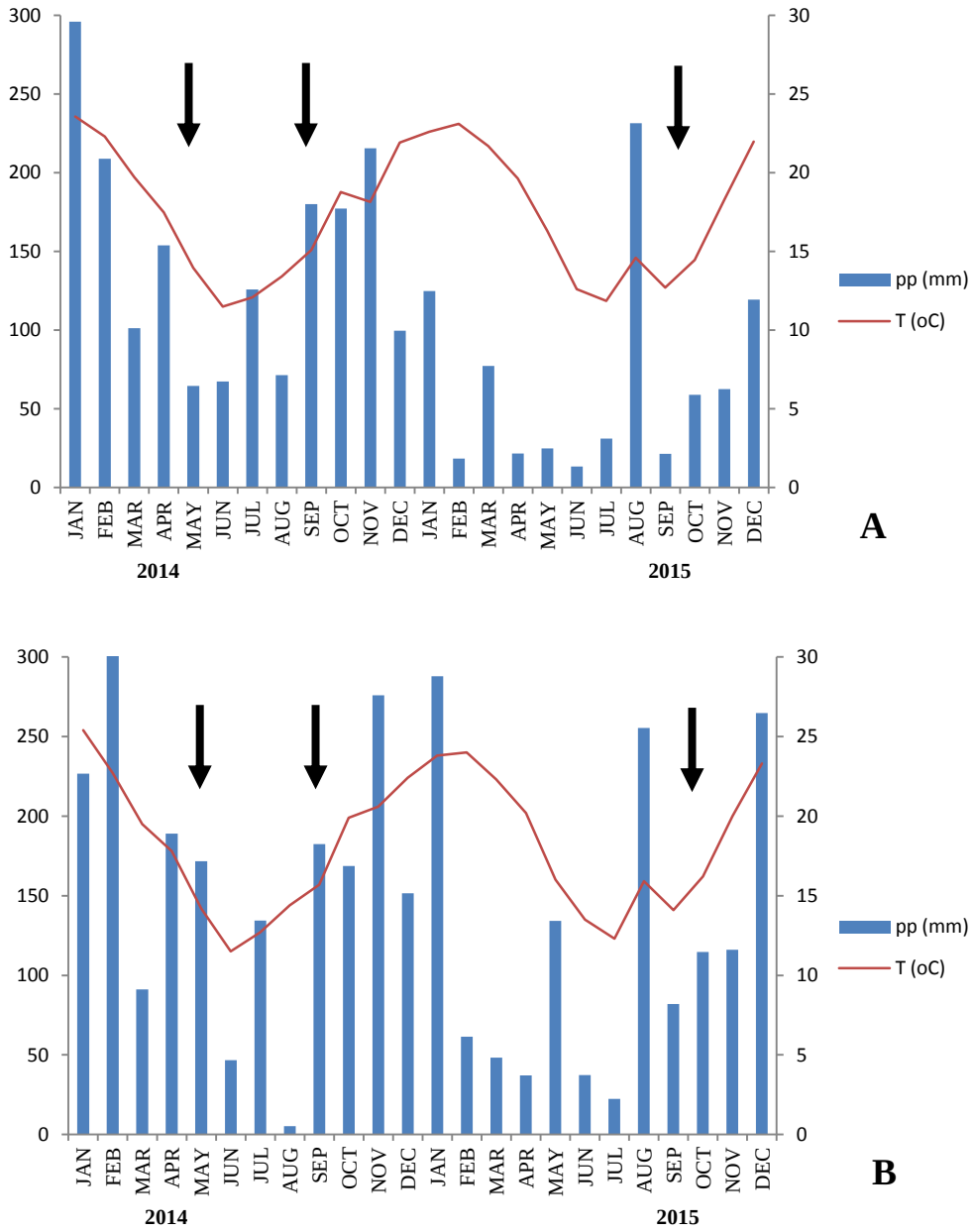

Fig.2. Monthly mean temperature (T) and accumulated precipitation (pp) for 2014 and 2015 in Montevideo (A) and Paysandú (B). Arrows indicate sampling dates.

3.2.3. Results

3.2.3.1. Sampling

A total of 1636 earthworms were collected, 56 % of which were < 1.5 mm-wide juveniles, 17 % were >1.5 mm- and < 2 mm-wide juveniles or subadults, and the rest (26%) were adults with developed genital features. Adult size ranged 20 – 150 mm and 1 - 8 mm, in length and width respectively. The largest adult specimens *(ca.* 150 mm length) were *Lumbricus terrestris* (Linnaeus 1758) only found in Montevideo The smallest adults found in both provinces, which presented a size range of 20-50 mm, did not coincide with the descriptions of any of the reported earthworms for Uruguay (Grosso et al. 2006; Grosso and Brown 2007), and were identified as *Murchieona minuscula* (Rosa 1905), of the Lumbricidae family

3.2.3.2.Earthworm density and biomass

An overall analysis per province showed high coefficients of variation (ranging from 50 % to 67 % in Paysandú, and 69 % to 148 % in Montevideo). However, the means for each sampling season showed that, in general, the biomass:density ratio was higher in Montevideo than in Paysandú (Fig. 3). The extreme values for earthworm density took place in the same Farm, M-OF1, with the highest values in the control site (M-OF1-Ctrl: >390 earthworms.m^{-2}), and the lowest in the wheat plot (M-OF1-T: <10 earthworms.m^{-2}). The biomass of M-OF1-Ctrl, M-OF4-Ctrl, and M-OF4-T had all > 1000 kg.ha^{-1} of earthworms.

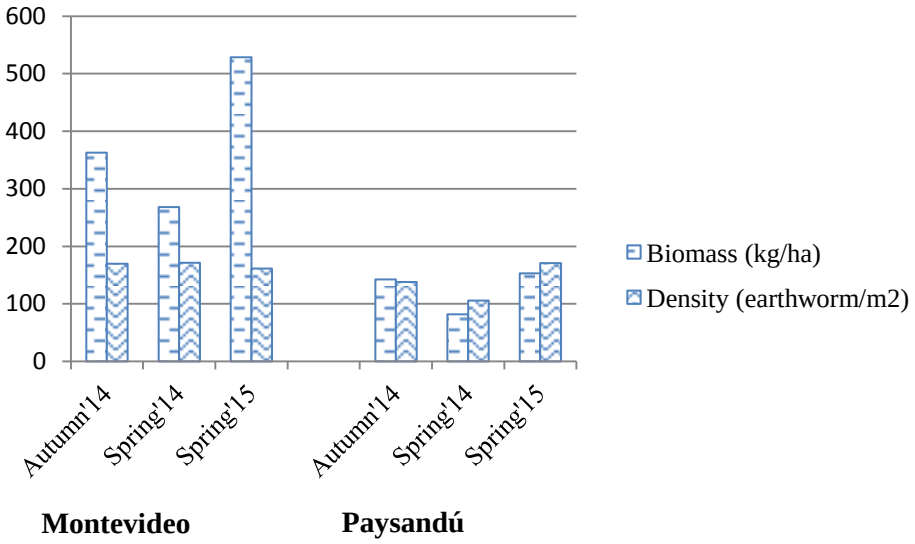

Fig. 3. Mean earthworm biomass and density for each sampling season in the provinces of Montevideo and Paysandú. The difference in the biomass:density ratio of the two provinces is represented graphically.

3.2.3.3. Species distribution and new records for Uruguay

Adults were classified by morphological features into 16 species, plus one group classified taxonomically up to family: Five of them belong to three families of Neotropical origin: *Microscolex dubius* (Fletcher, 1887) and *M. phosphoreus* (Dugés 1837) of the Acanthodrlidae family; *Glossodrilus parecis* (Righi and Ayres 1975) and *Glossoscolex* sp. (Leuckart 1835) of the Glossoscolecidae family; and specimens of the Ocnerodrilidae family. The other 12 species were grouped in 6 genera of the exotic family Lumbricidae and one Megascolecidae: *Allolobophora chlorotica* (Savigny 1893), *Aporrectodea caliginosa* (Savigny 1826), *Ap. rosea* (Savigny 1826), *Ap. trapezoids* (Dugés 1828), *Ap. tuberculata* (Eisen 1874), *Eisenia Andrei* (Bouché 1972), *Lumbricus terrestris* (Linnaeus 1758), *L. friendi* (Cognetii 1904), *Murchieona minuscula* (Rosa, 1905), *Octolasion cyaneum* (Savigny, 1826), *O. tyrtaeum* (Savigny 1826), and *Amynthas corticis* (Kinberg 1867). Species identity was confirmed by molecular methods based on the repository of GenBank and/or BOLD, in the case of most exotics, except for *Ap. tuberculata,* as the DNA segments of these samples failed to amplify accurately. Although similar to *Ap. caliginosa*, *Ap. tuberculata* was

recorded as a different species, based solely on morphological criteria, due to a different pattern of the genital marks following Andersen's description (1997). In the case of the natives, coinciding uploaded COI sequences in GenBank and BOLD, were only found for *Microscolex* genus, for the rest morphological features were used to identify the species following the descriptions of Righi and Ayres (1975) and Cordero (1943).

The maximum richness per plot (number of species) found was six, corresponding to the control sites of two Organic Transition Farms in Montevideo, and the first sampling in the pasture-crop rotation in Paysandú. In Montevideo, the agricultural soil had generally a lower richness than the control sites surrounding the wheat plots. In contrast, in Paysandú, the plots with crop rotation generally presented equal or higher richness than the control sites, which presented an important seasonal oscillation. The most common species was *Ap. caliginosa*, with 90 individuals found in 20 out of the 35 sampled sites. The rarest species were *E. andrei* and *Am. corticis*, both found in M-OF4. The six species found in Paysandú belong to the ecological endogeic group. Apart from 10 endogeic species, Montevideo also presented two epigeic species (*E. andrei* and *Am. corticis*), and two anecic species (*L. terrestris* and *L. friendi*), the four of them of exotic origin (tables 3 and 4).

A Principal Component Analysis (PCA) analysis where the variables considered were the abundance of each species per site required three axes to explain 57% of the variability. Although the first two axes only explain a 45 % of the variability among sites, a clear pattern could be observed in the biplot where M-OF4-T and M-OF4-Ctrl can be seen as a separated group on the right, highly associated to *E. andrei, Am. corticis, L. friendi, Ap. tuberculta*, and *Ap. trapezoides*. In autumn 2014, M-OF1-Ctrl, located in the central upper part of the biplot, is associated to *All. chlorotica, Ap. caliginosa, O. cyaneum, M. minuscula*, and *L. terrestris*. The four native species, associated to P-PC-T and P-PC-NT in autumn 2014, point to the lower left quadrant of the biplot (fig. 4, table 5).

3.2.3.4.Natives and Exotics

The native:exotic ratio, in terms of number of species, was 2:1 in the Experiment Station in Paysandú, and ranged from 1:1 to 1:6 in the different farms of Montevideo. In terms of the abundance of adult earthworms sampled by province, the native:exotic ratio was 1.8:1.0 in Paysandú; but in Montevideo it was 1.0:13.3 for the Organic Transition Farms and 1.0:5.3 for the Non-organic Farm (Table 3). In Paysandú the sites with continuous crops were dominated by exotics in number of species and earthworms, while in the control site exotics dominated in richness, but in abundance they only dominated in one sampling date (Table 4). In Montevideo, all control sites and all wheat plots with earthworms, except for two (M-OF2-Ctrl and M-NF2-Ctrl), were dominated by exotics. Samples M-OF2-T in autumn and spring 2014, and M-NF2-NT in spring 2015, presented very few earthworms, all of them being small-sized natives, Ocnerodilidae, *M. phosphoreus* and *M. dubius.*

3.2.3.5.Correlation with soil properties

The first two axes of the PCA carried out with soil parameters and earthworm community descriptors, explains 51 % of the variation, and three axes are required to achieve 65 % accumulated variation. The variables with highest weight on CP1 were richness, biomass, N, C, density, diversity and soil water content (w).

Richness, diversity, evenness, density, and biomass are strongly associated to the control sites in M-NF1-Ctrl, M-OF1-Ctrl, M-OF4-Ctrl, and the wheat plot in M-OF4-T, and increase in the same direction as C, N, and soil water content in the biplot generated by the first vs. second components (fig. 5, table 3). These community descriptors seem to be rather independent from soil texture.

The Mantel test showed a significant correlation between soil properties and species ($p= 0.002$), although the Pearson correlation coefficient (0.38) indicate a low magnitude for this association.

Table 3. Community descriptors and adult abundances for each species of the earthworm communities found in wheat farms in Montevideo

		RICHNESS			ABUNDANCE			DIVERSITY		NATIVES			EXOTICS										
Season	**Sampling Site**	**S NATIVES**	**S EXOTICS**	**S TOTAL**	**A. NATIVES**	**A. EXOTICS**	**A. TOTAL**	**H' Diversity INDEX (Shannon)**	**E Evennes INDEX (Pielou)**	*Microscolex dubius*	*M. phosphoreus*	Ocnerodrilidae	*Allolobophora chlorotica*	*Aporrectodea caliginosa*	*Ap. rosea*	*Ap. trapezoides*	*Ap. tuberculata*	*Eisenia andrei*	*Lumbricus terrestris*	*L. friendi*	*Murchieona minuscula*	*Octolasion cyaneum*	*O. tyrtaeum*	*Amynthas corticis*
Autumn	**Organic T. Farm 1**	**0**	**6**	**6**	**0**	**61**	**61**	**1.4**	0.79				**23**	**17**	**14**				**2**		**4**	**1**		
2014	M-OF1-Cntrl	**0**	**6**	6	**0**	**61**	61	**1.4**	0.79				23	17	14				2		4	1		
	M-OF1-T	**0**	**0**	0	**0**	**0**	0																	
	Organic T. Farm 2	**3**	**2**	**5**	**6**	**3**	**9**	**1.4**	0.89	**1**	**1**	**4**									**1**		**2**	
	M-OF2-Cntrl	**1**	**2**	3	**1**	**3**	4	**1**	0.95	1											1		2	
	M-OF2-T	**2**	**0**	2	**5**	**0**	5	**0.5**	0.72		1	4												
Spring	**Organic T. Farm 1**	**0**	**5**	**5**	**0**	**31**	**31**	**1.2**	0.72				**4**	**1**	**19**				**4**		**3**			
2014	M-OF1-Cntrl	**0**	**5**	5	**0**	**30**	30	**1.1**	0.70				4	1	19				4		2			
	M-OF1-T	**0**	**1**	1	**0**	**1**	1	**0.0**													1			
	Non-organic Farm	**1**	**4**	**5**	**4**	**16**	**20**	**1.4**	0.88			**4**		**8**	**5**	**2**						**1**		
	M-NF1-Cntrl	**0**	**2**	2	**0**	**8**	8	**0.7**	0.95					5	3									
	M-NF1-NT	**1**	**2**	3	**3**	**5**	8	**1.1**	0.99			3		3		2								
	M-NF2-Cntrl	**0**	**2**	2	**0**	**3**	3	**0.6**	0.92						2							1		
	M-NF2-NT	**1**	**0**	1	**1**	**0**	1	**0.0**				1												
	Organic T. Farm 3	**1**	**3**	**4**	**1**	**29**	**30**	**1.1**	0.82			**1**		**10**	**14**						**5**			
	M-OF3-Cntrl	**0**	**3**	3	**0**	**26**	26	**1.0**	0.91					7	14						5			
	M-OF3-T	**1**	**1**	2	**1**	**3**	4	**0.6**	0.81			1		3										

Note: M= Montevideo; OF= Organic Transitional Farm; NF= Non-organic Farm; T= Tillage; NT= Not-till; Cntrl= Control; S= Richness

Table 3 (cont). Community descriptors and adult abundances for each species of the earthworm communities found in wheat farms in Montevideo

		RICHNESS			ABUNDANCE			DIVERSITY		NATIVES			EXOTICS										
Season	**Sampling Site**	**S NATIVES**	**S EXOTICS**	**S TOTAL**	**A. NATIVES**	**A. EXOTICS**	**A. TOTAL**	**H' Diversity INDEX (Shannon)**	**E Evennes INDEX (Pielou)**	*Microscolex dubius*	*M. phosphoreus*	**Ocnerodrilidae**	*Allolobophora chlorotica*	*Aporrectodea caliginosa*	*Ap. rosea*	*Ap. trapezoides*	*Ap. tuberculata*	*Eisenia andrei*	*Lumbricus terrestris*	*L. friendi*	*Murchieona minuscula*	*Octolasion cyaneum*	*O. tyrtaeum*	*Amynthas corticis*
Spring	**Organic T. Farm 2**	**1**	**0**	**1**	**10**	**0**	**10**	**0.0**				**10**												
2015	M-OF2-Cntrl	**0**	**0**	0	**0**	**0**	0																	
	M-OF2-T	**1**	**0**	1	**10**	**0**	10	**0.0**				10												
	Organic T. Farm 4	**0**	**7**	**7**	**0**	**141**	**141**	**0.9**	0.46						**26**	**4**	**53**	**1**		**2**	**1**			**1**
	M-OF4-Cntrl	**0**	**6**	6	**0**	**38**	38	**0.9**	0.50						**3**	3	29			1	1			1
	M-OF4-T	**0**	**5**	5	**0**	**50**	50	**0.9**	0.59						**23**	1	24	1		1				
	Non-organic Farm	**2**	**6**	**8**	**9**	**53**	**62**	**1.9**	0.91	**5**	**4**		**11**	**19**	**9**		**7**				**5**	**2**		
	M-NF1-Cntrl	**1**	**4**	5	**1**	**25**	26	**1.2**	0.72	1				13	9						2	1		
	M-NF1-NT	**1**	**2**	3	**4**	**4**	8	**1.0**	0.89		4			3			1							
	M-NF2-Cntrl	**1**	**3**	4	**1**	**15**	16	**0.9**	0.66	1			11								3	1		
	M-NF2-NT	**1**	**2**	3	**3**	**9**	12	**1.0**	0.95	3				3			6							

Note: M= Montevideo; OF= Organic Transitional Farm; NF= Non-organic Farm; T= Tillage; NT= Not-till; Cntrl= Control; S= Richness

Table 4. Community descriptors and adult abundances for each species of earthworm communities found in wheat rotation plots in Paysandú.

		RICHNESS			ABUNDANCE			DIVERSITY		NATIVES					EXOTICS	
Season	**Sampling Site**	**S NATIVES**	**S EXOTICS**	**S TOTAL**	**A. NATIVES**	**A. EXOTICS**	**A. TOTAL**	**H' Diversity INDEX (Shannon)**	**E Evennes INDEX (Pielou)**	*Microscolex dubius*	*M. phosphoreus*	*Glossodrilus parecis*	*Glossoscolex sp.*	Ocnerodrilidae	*Aporrectodea caliginosa*	*Murchieona minuscula*
Autumn 2014	**TOTAL University Experimental Field**	5	2	**7**	47	20	**67**	**1.7**	**0.87**	**6**	**8**	**21**	**3**	**9**	**18**	**2**
	P-PC-T	3	0	**3**	13	0	**13**	**1.0**	**0.90**	2	4	7				
	P-PC-NT	5	1	**6**	22	1	**23**	**1.6**	**0.90**	3	7	9	3	3	1	
	P-CC-NT	1	2	**3**	2	8	**10**	**0.8**	**0.73**					2	7	1
	P-SPC-NT	3	1	**4**	7	2	**9**	**1.3**	**0.92**	1		2		4	2	
	P-Cntrl	1	2	**3**	3	9	**12**	**0.8**	**0.75**			3			8	1
Spring 2014	**TOTAL University Experimental Field**	3	2	**5**	24	10	**34**	**1.2**	**0.73**	**1**		**20**	**3**		**3**	**7**
	P-PC-T	3	1	**4**	6	2	**8**	**1.3**	**0.95**	1		3	2			2
	P-PC-NT	1	0	**1**	2	0	**2**	**0.0**				2				
	P-CC-NT	1	1	**2**	1	2	**3**	**0.6**	**0.92**			1			2	
	P-SPC-NT	2	0	**2**	5	0	**5**	**0.5**	**0.72**			4	1			
	P-Cntrl	1	2	**3**	10	6	**16**	**0.8**	**0.76**			10			1	5
Spring 2015	**TOTAL University Experimental Field**	2	1	**3**	7	14	**21**	**0.9**	**0.79**			**4**	**3**		**14**	
	P-PC-T	1	1	**2**	2	2	**4**	**0.7**	**1.00**			2			2	
	P-PC-NT	0	1	**1**	0	1	**1**	**0.0**							1	
	P-CC-NT	0	1	**1**	0	2	**2**	**0.0**							2	
	P-SPC-NT	2	1	**3**	5	3	**8**	**1.1**	**0.99**			2	3		3	
	P-Cntrl	0	1	**1**	0	6	**6**	**0.0**							6	

Note: P= Paysandú; PC= Pasture-crop rotation; SPC= Short pasture-crop rotation; CC= Continuous crop; T= Tillage; NT= Not-till; Cntrl= Control; S= Richness

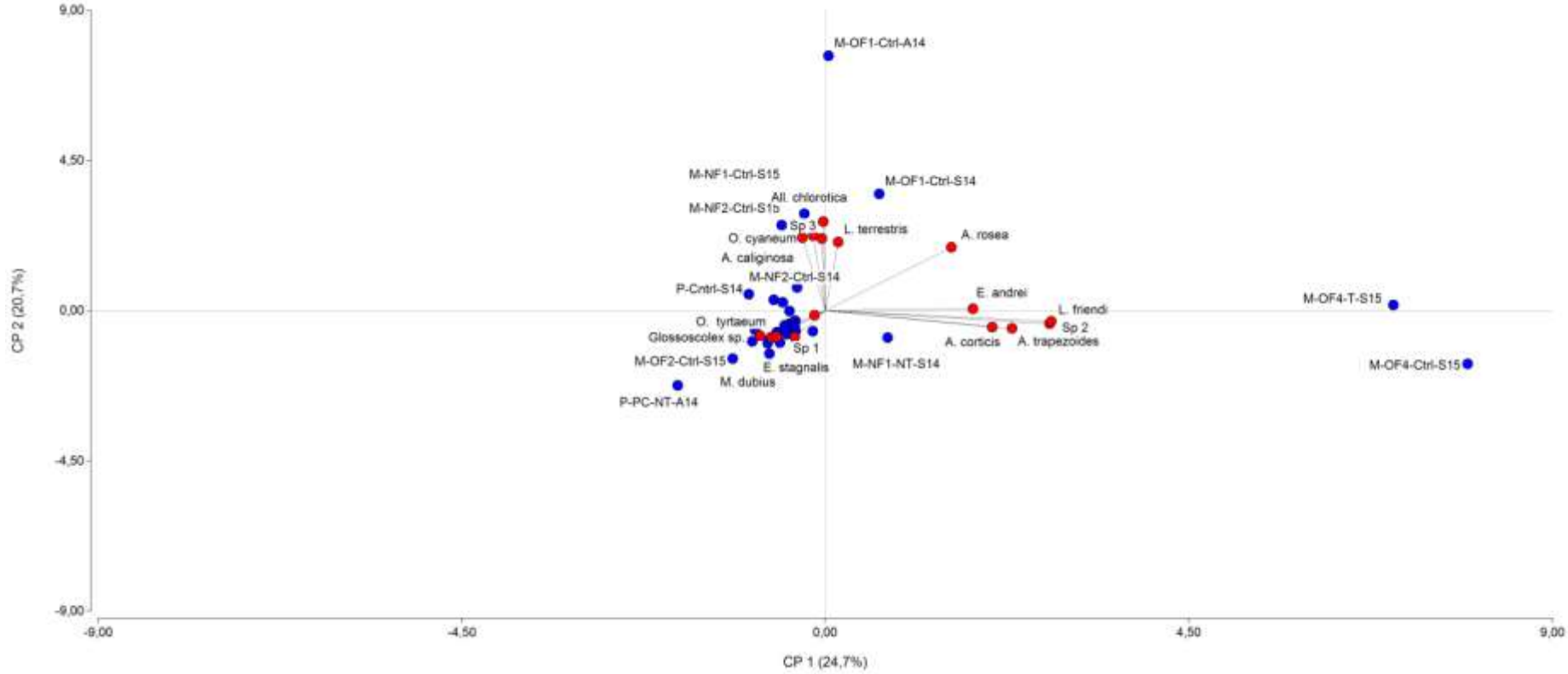

Fig 4. Biplot of the Principal Component Analysis of two axes, with the different plots per date as classification criterion, and the abundance of each species per plot as variables. Sample identification is explained in tables 1 and 2, the date of the sample was added at the end of each sample using S or A for spring and autumn, respectively, and 14 and 15 correspond to the sampling year (2014 or 2015).

Table 5. Principal component correlation with the original variables (earthworm species)

Variables	PC 1	PC 2	PC 3
M. dubius	-0..23	-0..25	0.67
Murchieona minuscula	-0.28	-0.24	0.79
Glossoscolex sp.	-0.21	-0.25	0.65
Ocnerodrilidae	-0.13	-0.25	-0.13
All. chlorotica	-0.01	0.85	0.13
Ap. caliginosa	-0.1	0.7	0.04
Ap. rosea	0.54	0.61	0.16
Ap. trapezoides	0.8	-0.17	0.02
Ap. tubercultata	0.97	-0.12	0.13
E. andrei	0.64	0.02	0.11
L. terrestris	0.06	0.65	0.09
L. friendi	0.97	-0.1	0.14
M. minuscula	-0.01	0.69	0.31
O. cyaneum	-0.05	0.71	0.03
O. tyrtaeum	-0.05	-0.04	-0.24
Am. corticis	0.72	-0.16	0.08

Note: PC= Principal Component

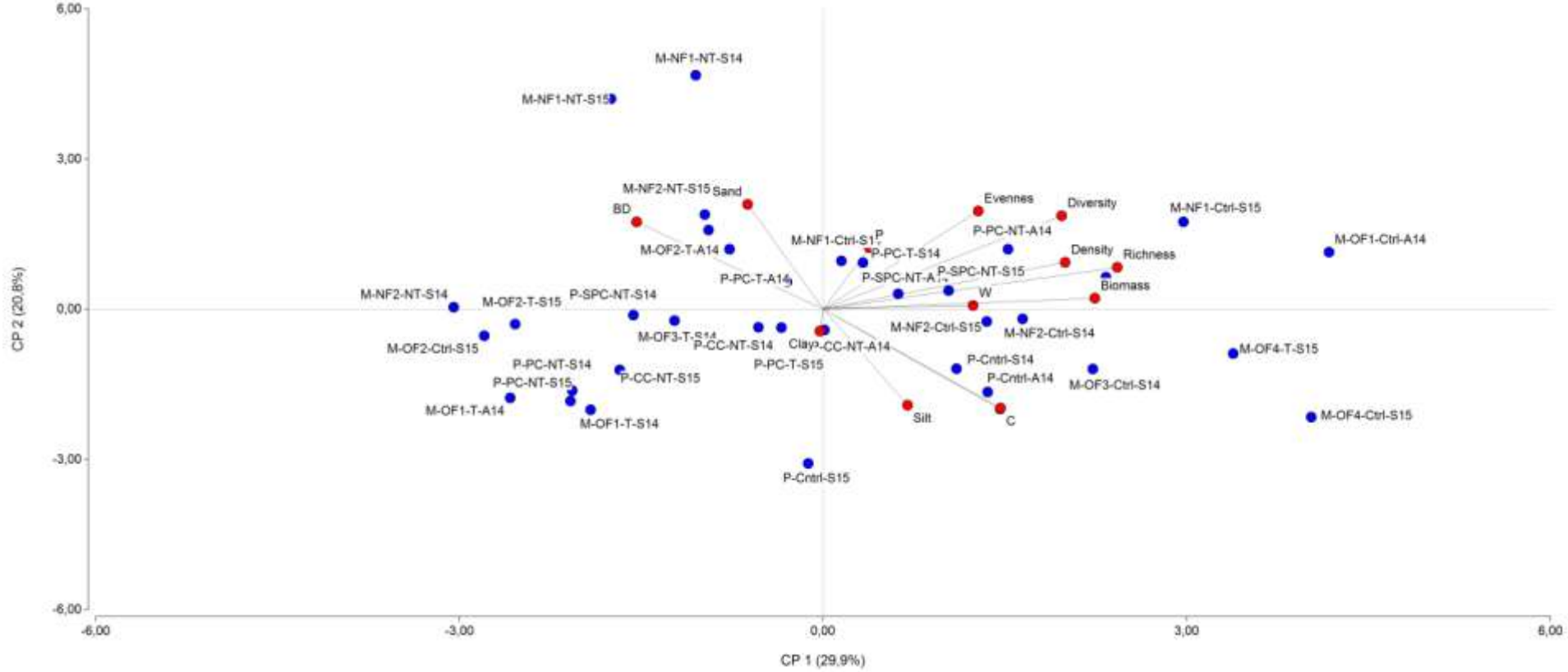

Fig 5. Biplot of the Principal Component Analysis of two axes, with the different plots per date as classification criterion and community descriptors, soil texture, water content, bulk density, C, N and P as variables. Sample identification is explained in tables 1 and 2, the date of the sample was added at the end of each sample using S or A for spring and autumn, respectively, and 14 and 15 correspond to the sampling year (2014 or 2015).

Table 6. Principal component correlation with the original variables (soil properties and community descriptors)

Variables	PC 1	PC 2	PC 3
BD	-0.56	0.53	0.31
W	0.45	0.02	-0.03
N	0.53	-0.6	0.28
C	0.53	-0.6	0.41
P	0.14	0.37	-0.23
Sand	-0.22	0.63	-0.29
Silt	0.25	-0.58	-0.52
Clay	-0.01	-0.13	0.86
Biomass	0.81	0.07	-0.42
Density	0.72	0.28	-0.03
Richness	0.88	0.25	-0.11
Diversity	0.71	0.56	0.27
Evennes	0.46	0.59	0.42

Note: PC= Principal Component; BD= Bulk density; W= soil water content; N= soil nitrogen content; C= soil carbon content; P= soil phosphorous content

3.2.4. Discussion

We confirmed our first hypothesis, since the exotic:native relation varied between locations and from disturbed land to less or non-disturbed land, and this ratio was higher in Montevideo than in Paysandú. However, the second hypothesis was only true for Montevideo, where exotics dominated both in agriculture and control plots. There was a different pattern in Paysandú: natives would dominate in terms of richness and abundance in most agriculture plots, particularly in three of the five treatments sampled, which were those corresponding to intermediate disturbance (rotations with pastures: P-PC-T, P-PC-NT, P-SPC-NT).

This unexpected result contrasts with the findings of Falco et al. (2015), where natives dominated in abundance in naturalized grasslands and recent agriculture sites, while exotics dominated in sites with a long history of intensive agriculture. With 80 years of agriculture (Ernst et al. 2009), the sampled sites in Paysandú should be considered to have a long history of intensive agriculture. On the other hand, compared to Montevideo, a lower intensity of invasion of exotic species was expected to this province, associated with lower immigration intensity through its River Port (Vidart and Pi Hurgarte 1969; Massolo 2015). Besides, temperature and precipitation regimes may also influence the invasion success, with milder temperatures in Montevideo than in Paysandú.

Another unexpected result was found in the control sites in Paysandú, which had a lower number of earthworms than in the agriculture rotations and were always dominated by exotics in terms of richness, and in terms of abundance this happened in two out of three sampling dates. Since the exotic anthropochourus species are adapted to agriculture, they were expected to dominate in the most disturbed environment, not so in the natural field. This unpredicted result may be due to the fact that "natural field" samples were taken from below the wire fences that separates plots and experiments, and plowing and agriculture was made 1 m from that point, hence, there may have been agrochemical contamination or possible soil compaction, which influences negatively on earthworm number and weight (Hansen and

Engelstad 1999; Paoletti 1999). The selection criterion was to take the less disturbed field nearby so that there were no major differences in soil type and water content. An adjacent riparian zone was the other alternative of control site given its low degree of disturbance, but was discarded because it had too many different factors that could influence earthworm fauna (soil type, topographic position, water content, woody vegetation). Therefore, this phenomenon must be further explored by expanding sampling in areas with varying degrees of disturbance and undisturbed areas. Prior to the present study, no exotics had been recorded for Paysandú (Grosso and Brown 2007), probably due to low sampling effort, so this becomes the first record of exotics for this province.

3.2.4.1.Species distribution

The PCA analysis was run to study whether sampling plots per date could be classified according the abundance of each species. Because the first two axes explained far less than 75 % of the variation we conclude that, species distribution is a complex phenomenon, only clearly separating one Organic Farm (M-OF4) from the rest of the sampling sites, highly associated to the fact that this site had the two rarest species and a very high number of *Ap. tuberculata*, which was only encountered in this site.

Aporrectodea caliginosa was the most abundant and ubiquitous species, being found in 4 out of 6 sampled Farms/Experimental Field. The sites in which it was not found, presented the peculiarity that, being both in Montevideo, were those that had the highest and the lowest extreme values in terms of abundance: OTF2 presented the lowest abundance, with a native Ocnerodrilidae as the dominating species. The site OTF4 registered the highest abundance, where the dominating species, *Ap. tuberculata* had not previously been reported for Uruguay, although probably belongs to the *Ap. caliginosa* complex (Pérez-Losada et al. 2009; Fernández et al. 2012). Momo et al. (1993) also found *Ap. caliginosa* to be the most ubiquitous and probably the first colonizer of recently disturbed soils. Lagerlöf et al. (2002) found

higher abundance of *Ap. caliginosa* in agricultural fields compared to the boundary and pastures. The rarest species in our study were *Eisenia andrei, Amynthas corticis* and *Octolasion tyrtaeum*. The first two, being epigeic species, were not expected to be found due to the features of the sites sampled (field crops and grasslands). However, they were found in an Organic Transition Farm, M-OF4, which add organic manure and compost to the fields: possible source for these epigeic earthworms.

The species of the native family Glossoscolecidae were only found in Paysandú, and those of the native family Ocnerodrilidae, only in Montevideo. The other families were represented in both provinces. *Glossoscolex* sp., *Microscolex dubius, M. phosphoreus, E. andrei* and Ocnerodrilidae were found only in cultivated fields, whereas *Allollobophora chlorotica, Am. corticis, O. cyaneum and O. tyrteaum* were found only in fields without recent disturbance (controls). *Lumbricus terrestris, L. friendi, E. andrei, O. tyrtaeum* and *Am. corticis* were only found in Organic Transitional Farms in Montevideo, not in the Non-organic Farm that uses a no-till system which includes the use of herbicides and other agrochemicals.

3.2.4.2.Earthworm density and biomass

In each sampled site, natives had generally a smaller size than exotics (with the exception of *M. minuscula*). This was first appreciated by eye when processing the sample and classifying the specimens, and later confirmed by establishing the biomass:density ratio which was higher in Montevideo, where exotics dominated, than in Paysandú, where natives dominated. This smaller size of natives may reduce earthworm significance in studies which only register earthworm biomass and not number, and may in certain cases hinder an accurate identification. In fact, in this study some Ocnerodrilidae worms had been misclassified in the first place as juveniles, due to their narrow breadth. After fixation, samples were reexamined and these specimens were reclassified as adults.

Mean density values per site (>100 ind/m^2), both in Montevideo and Paysandú, were higher than those reported by other authors for agroecosystems in similar latitudes and soils, e.g. different agriculture rotations in Colonia, in southwest Uruguay; soybean crops in Entre Ríos Province, Argentina, and agriculture systems with different levels of intensity in Buenos Aires Province, Argentina (Zerbino, 2010; Falco et al. 2015; Masín et al. 2015). The range of earthworm biomass per hectare found per site fell within the range reported by Kanianska et al. (2016) for arable lands and permanent grasslands in Central Europe, with their upper bond being higher, which is expectable, considering the appreciation regarding size made above and that all their sampled species belonged to the Lumbricidae family.

3.2.4.3.Richness, diversity and evenness

The control sites in Montevideo that presented the greatest richness (of six species) were those of the Organic Transitional Farms OF1 in autumn 2014 and OF4 in spring 2015, followed by one of the control sites of the Non- organic Farm, registering five species in spring 2014. In this province the cultivated soils in each Farm presented lower richness than the surrounding undisturbed field taken as control. In contrast, in Paysandú, the control site always presented equal or lower richness than the treatments with field crops, which presented an important seasonal oscillation.

Taking the maximum richness and the average abundance of both dates, these values were higher in rotations that included pastures (P-PC-T, P-PC-NT and P-SPC-NT) than in continuous crop rotation (P-CC-NT), in accordance with findings of Zerbino and Morón (2003) in the long-term experiment in INIA La Estanzuela. Nevertheless, in the present study this pattern was not verified for the biomass variable (Table 2 and Fig. 3). For changes in soil due to different managements, Zerbino and Morón (2003) proposed earthworm abundance as a more robust indicator than biomass.

Species diversity, although with a high variation from site to site, presented maximum values of H'=1.4 per plot and 1.8 per site in Montevideo; and H' = 1.4 per

plot and 1.6 per site in Paysandú. These values can be considered high in the context that the highest diversity value reported by Masín et al. (2011) and Falco et al. (2015) was H' = 0.33, and H' = 0.57, respectively. Evenness ranged from 0.50 to 1.00, a wider range than that reported by Masín (2011); it was lowest in OF4, where *Ap. tuberculata* was very numerous, strongly dominating the community, and highest in an agriculture plot with only two species in equal amounts.

3.2.4.4.Community structure

More juveniles (79 %) than adults (21 %) were collected, although this juvenile percentage was lower than those reported for Argentina in similar soils and latitudes. A range of 82 to 98 % of all earthworms sampled were juveniles, in samplings done in autumn and summer in different horticulture agroecosystems with or without till, and with or without organic fertilization (Masín et al. 2011); and 95% of the earthworms were juveniles in agriculture no-till soybean systems varying in chemical input (Masín et al. 2015).

All natives found belong to the endogeic ecological group, which mostly remain inside the soil, without emerging to the surface. Out of 12 exotics species found, eight were endogeics, two anecics, and two epigeic. In savannas or grassland ecosystems, endogeic species dominate (Fragoso and Lavelle 1995), since the greatest contribution of organic matter occurs within the soil by the short-cycle pastures roots, in contrast to forest environments where largest organic matter contribution takes place on the soil surface, mainly by falling leaves, which favors anecics and epigeics.

3.2.4.5.Correlation with soil properties

The fact that the first two axes of the PCA only explained 51 % of the variation (accumulating to 65 % when adding a third axis) showed that the phenomena regulating community characteristics is complex, depending on the interaction of several variables, possibly with some unmeasured variables influencing as well. This

coincides with previous studies which have found that, considering several soil properties as variables, the first two axes of a PCA explain 58 % (Falco et al. 2015), and 40 % (Singh et al. 2016) of the variation. This does not only depend on the soil properties considered, but also on how divergent the sampled soils are.

However, C, N and soil water content seem to be the measured soil properties to have the most influence on the first axis, in the same direction as diversity, richness, biomass, density. This coincides with previous studies which have found both earthworm abundance and biomass highly correlated to organic C and biomass also highly correlated to N (Hendrix et al. 1992; Zerbino and Morón 2003). Fragoso and Lavelle (1992) discussed that endogeic communities, as was the case of the present sampled sites, are typical of nutrient rich soils. When it comes to species distribution, the Mantel test revealed a significant but low correlation with soil properties, partly due to the wide dispersal of exotics in most sites, and also probably to the fact that sampled soils were not very contrasting in their properties, with similar usage.

3.2.5. Conclusion

Thirteen out of the 19 species described for Uruguay were found in agricultural soils and less disturbed surrounding areas of Montevideo and Paysandú. Besides, four other species, not yet registered in Uruguay, were found, and are reported in this paper: *Microscolex phosphoreus* and *Glossodrilus parecis* (natives); and *Aporrectodea tuberculata* and *Murchieona minuscula* (exotics). Finding unrecorded species in such few sampled sites evidences the poor coverage of sampling in anthropic and natural landscapes of the country. The present study also represents the first report of exotic earthworms in Paysandú: *Ap. caliginosa*, which was the most ubiquitous species in both provinces, and *Mu. minuscula*.

The earthworm exotic:native relation depended on location and on degree of disturbance when comparing agriculture plots and less disturbed surrounding areas in the Uruguayan provinces of Montevideo and Paysandú. Exotics dominated in

Montevideo, located closest to the main port, both in agriculture and control plots. Samples in Paysandú were dominated by natives, and interestingly, these dominated more strongly in agriculture plots than in the control plots. The fact that exotic species did not dominate in agricultural fields in Paysandú raises the question whether invasion success is limited by low entrance of exotics, by insufficient time to reach dominance, by climatic conditions, or by competition, given the case that native species present in Paysandú tolerate agriculture conditions, and can therefore coexist with exotics without being outcompeted.

Elucidating whether native species have been displaced by exotics and/or disturbance, represents a challenge since few researchers have studied the Oligochaetes in this country. A systematic study of native earthworms dwelling in different undisturbed ecosystems, which could generate a baseline is lacking. Most probably, with a greater effort of sampling and research, the short current earthworm list could be extended, particularly within the group of natives, since the majority of the studies have been carried out in agroecosystems, where it has been seen that the exotics are more competitive. The present results set a future challenge to continue discovering the local earthworm richness, which will involve not only a greater effort of sampling, but also to expand the diversity of environments and ecosystems sampled.

3.2.6. References

Addison JA (2009) Distribution and impacts of invasive earthworms in Canadian forest ecosystems. Biol Inv 11:59–79

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Molec Biol 215: 403-410

Andersen C (1997) Regnorme. Natur og Museum 36(4) Naturhistorisk Museum, Aarhus, Denmark.

Anderson JM, Ingram JSI (1993) Tropical soil biology and fertility. A handbook of methods, 2nd edn. CAB International, Wallingford

Callaham Jr MA, Snyder BA, James SW, Oberg ET (2016) Evidence for ongoing introduction of non-native earthworms in the Washington, DC metropolitan area. Biol Inv 18:3133–3136

Cordero EH (1943) Oligoquetos sudamericanos de la familia Glossoscolecidae, I. El género Glossoscolex en el Uruguay, con una sinopsis de las especies del grupo truncatus. Com Zool Museo Hist Nat, Montevideo, Uruguay 1(2):1-9

Curry JP (1998) Factors affecting earthworm abundance in soils. In: Edwards CA (ed) Earthworm ecology. St. Lucie Press - Boca Raton FL, London, pp 37-64

Dane JH, Topp GC (2002) Methods of Soil Analysis. Part 4. Physical Methods. Soil science Society of America, Inc. Madison, Wisconsin

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: Version II. Plant Molec Biol Rep 1(4):19-21

Edwards CA, Lofty JR (1976) Biology of Earthworms. Bookworm Publishers, Ontario

Ernst O, Siri Prieto G, Cadenazzi M. (2009) Influence of Crop-Pasture Rotation and Tillage System on Yields of Wheat, Soybean, Barley, Sorghum and Sunflower in Uruguay. J Agr Mach Sc, 53: 253 – 262

Falco LB, Sandler R, Momo F, Di Ciocco C, Saravia L, Coviella C (2015) Earthworm assemblages in different intensity of agricultural uses and their relation to edaphic variables. PeerJ 3:e979. ISSN 2167-8359.

Feijoo A, Carvajal AF, Zúñiga MC, Quintero H, Fragoso C (2011) Diversity and abundance of earthworms in land use systems in central-western Colombia. Pedobiol 54:69-75

Fernández R, Almodóvar A, Novo M, Simancas B, Díaz Cosín DJ (2012) Adding complexity to the complex: New insights into the phylogeny, diversification and origin of parthenogenesis in the *Aporrectodea caliginosa* species complex (Oligochaeta, Lumbricidae). Molec Phylogenet & Evol 64: 368–379

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochromec oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299

Fragoso C (2007) Diversidad y patrones biogeográficos de las lombrices de tierra de México (Oligochaeta, Annelida). In: Brown GG, Fragoso C (eds) Minhocas na América Latina: Biodiversidade e Ecologia, EMBRAPA Soja, Londrina, pp 109-124

Fragoso C, Brown GG (2007) Ecología y taxonomía de las lombrices de tierra en Latinoamérica: El primer Encuentro Latino-Americano de Ecología y Taxonomía de Oligoquetos (ELAETAO1). In: Brown GG, Fragoso C (eds) Minhocas na América Latina: Biodiversidade e Ecologia, EMBRAPA Soja, Londrina, pp 34-75

Fragoso C, Lavelle P (1995) Are earthworms important in the decomposition of tropical litter? In: Reddy MV (ed) Soil organisms and litter decomposition in tropics. Oxford & IBH Publishing Co, New Delhi, pp 103–112

Fragoso C, Lavelle P (1992) Earthworm communities of tropical rain forests. Soil Biol & Biochem 24(12): 1397-1408

Fragoso C, Lavelle P, Blanchart E, Senapati BK, Jiménez JJ, Martínez MA, Decaëns T, Tondoh J (1999) Earthworm communities of tropical agroecosystems: origin, structure and influence of management practices. In: Lavelle P, Brussaard L, Hendrix, PF (eds) Earthworm management in tropical agroecosystems. CAB International, Wallingford, K. pp 27-55

Grosso EG, Brown GG (2007) Biodiversidad y ecología de las lombrices de tierra en el Uruguay. In: Brown GG, Fragoso C (eds) In: Brown GG, Fragoso C (eds) Minhocas na América Latina: Biodiversidade e Ecologia, EMBRAPA Soja, Londrina, pp 287-296

Grosso EG, Jorge G, Brown GG (2006) Exotic and Native Earthworms in Various Land Use Systems of Central, Southern and Eastern Uruguay. Carib J of Sci 42(3):294-300

Hansen S, Engelstad F (1999) Earthworm populations in a cool and wet district as affected by tractor traffic and fertilisation. Appl Soil Ecol 13: 237-250

Hendrix PF, Baker GH, Callaham Jr MA, Damoff GA, Fragoso C, González G, James SW, Lachnicht SL,Winsome T, Zou X (2006) Invasion of exotic

earthworms into ecosystems inhabited by native earthworms. Biol Inv 8:1287–1300

Hendrix PF, Mueller BR, Bruce TRR, Langdale GW, Parolees RW (1992) Abundance and distribution of earthworms in relation to landscape factors on the Georgia Piedmont, U.S.A. Soil Biol & Biochem 24 (12):1357-13611

Huang J, Xu Q, Sun ZJ, Tang GL, Su ZY (2007) Identifying earthworms through DNA barcodes. Pedobiol 51:301-309

James SW, Hendrix PF (2004) Invasion of Exotic Earthworms into North America and Other Regions. In: Edwards CA (ed) Earthworm ecology, 2nd edn. Boca Raton: CRC Press, London, pp 75- 88

James, SW (2011) Earthworms. In: Simberloff D, Rejmánek M (eds) Encyclopedia of biological invasions. University of California Press, Los Angeles, pp 177-183

Jorge-Escudero G, Pérez C, Martínez C, Lagerlöf J (2018) Identification of earthworm species in Uruguay based on morphological and molecular methods. Agrociencia Uruguay. *in press*

Kanianska R, Jad'ud'ová J, Makovníková J, Kizeková M (2016) Assessment of relationships between earthworms and soil abiotic and biotic factors as a tool in sustainable agricultural. Sustainab 8(9):906; doi:10.3390/su8090906

Lagerlöf J, Goffre B, Vincent C (2002) The importance of field boundaries for earthworms (Lumbricidae) in the Swedish agricultural landscape. Agric Ecosyst Environ 89(1-2):91-103

Lee K (1985) Earthworms: Their Ecology and Relationships with Soils and Land Use. Academic Press, New York

Maggenti MAB, Maggenti AR, Gardner SL (2005) Online Dictionary of Invertebrate Zoology: A. On line Dictionary of Invertebrate Zoology. 16. http://digitalcommons.unl.edu/onlinedictinvertzoology/16 Accessed 06 October 2018

Magurran AE (1988) Ecological diversity and its measurements. Princeton University Press, New Yersey

Masín CE, Rodríguez AA, Maitre MI (2011) Evaluación de la abundancia y diversidad de lombrices de tierra en relación con el uso del suelo en el cinturón hortícola de Santa Fe (Argentina). Ci Suelo (Argentina) 29(1): 21-28

Masín CE, Rodríguez AA, Maitre MI, Cerana J, Hernández JP, Anglada M, Elizalde JH, Lallana MC (2015) Riqueza de lombrices de tierra en un argiudol bajo cultivo de soja (colonia ensayo, Entre Ríos). Rev Cient Agropec 19(1-2): 39-50

Massolo D (2015) La ruta de la inmigración. Barcos e Inmigrantes. http://www.barcoseinmigrantes.com/puertos.html. Accessed 06 October 2018

Momo FR, Giovanetti CM, Malacalza L. 1993. Relación entre la abundancia de distintas especies de lombrices de tierra (Annelida, Oligochaeta) y algunos parámetros fisicoquímicos en un suelo típico de la estepa pampeana. Ecol Austr 3: 7-14

Pérez-Losada M, Ricoy M, Marshall JC, Domínguez J (2009) Phylogenetic assessment of the earthworm Aporrectodea califinosa spcecies complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. Molec Phylogenet & Evol 52:293-302

Paoletti MG (1999) The role of earthworms for assessment of sustainability and as bioindicators. Agr Ecosys & Environ 74:137–155

Reynolds JW (1996) Earthworm Biology and Ecology. Course manual for Universidad Nacional de Córdoba. Facultad de Ciencias Exactas, Físicas y Naturales, Córdoba

Righi G (1990) Minhocas de Mato Grosso e de Rondônia. Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, Brasilia, DF

Righi G (1979) Introducción al estudio de las lombrices del suelo (oligoquetos megadrilos) de la Provincia de Santa Fe (Argentina). Rev Asoc Ci Nat Litoral Argentina Colección Climax, Santa Fé, Argentina 2:89–155.

Righi G, Ayres I (1975) Alguns Oligochaeta sul breasileiros. Rev. Brasil. Biol., 35(2):309-316

Sánchez-de León Y, Johnson-Maynard J (2009) Dominance of an invasive earthworm in native and non-native grassland ecosystems. Biol Inv 11:1393–1401

Sims RW, Gerard BM (1985) Earthworms. Keys and notes for the identification and study of the species. The Linnean Society of London and The Estuarine and Brackish Water Sciences Association-The Pitman Press, Bath

Singh S, Singh J, Vig AP (2016) Effect of abiotic factors on the distribution of earthworms in different land use patterns. J Basic & Appl Zool 74: 41–50

Vidart D, Pi Hurgarte R (1969) El legado de los inmigrantes. Ed. Nuestra Tierra, Montevideo

Zerbino MS (2010) Evaluación de la macrofauna del suelo en rotaciones cultivo-pasturas con laboreo convencional. Acta Zool Mex 26:189-202

Zerbino MS, Morón A (2003) Macrofauna del suelo y su relación con propiedades físicas y químicas en rotaciones cultivo-pastura. In: Morón A, Díaz R (eds) INIA La Estanzuela. 40 años de rotaciones ganaderas. Serie Técnica N^{o} 134 INIA, Colonia, Uruguay. pp 45 - 53

3.3. IDENTIFICATION OF EARTHWORM SPECIES IN URUGUAY BASED ON MORPHOLOGICAL AND MOLECULAR METHODS

Summary

Molecular techniques could aid earthworm species identification, especially when morphological characters are not taxonomically informative, or difficult to discern. There are no precedents of molecular-based methods for earthworm taxonomy in Uruguay. The present study aimed to make a first approach using DNA barcoding as a tool to smoothen the way towards determining the earthworm richness of Uruguay. This study was based on an earthworm collection generated from samplings in different agricultural soils in Montevideo and Paysandú, Uruguay. Earthworm species were identified both by morphological characters and molecular techniques. Adult individuals were identified by external morphology following available descriptions of earthworms present in the region. From each morphological group a representative sample was selected for genomic DNA extraction, the mitochondrial COI region was amplified and sequenced. Sequences obtained were subject to BLAST searches and compared to the sequences available in GenBank. Out of 11 sequenced exotic species, eight were fully identified and coincidence of morphological characters and molecular info was found; whereas two were less consistent, with lower similarity percentage; and one could not be fully identified due to lack of close related sequences in GenBank. While most of the exotic species had representative sequences annotated in GenBank, this was the case for only one native species, highlighting the need to develop this important area at the regional level. The scarce records of earthworm samplings in Uruguay, and the absence of national studies that have identified species by DNA sequences, make this study a kick-start for an innovative research program.

Key words: COI, sequencing, native and exotic earthworms

Identificación de especies de lombrices en Uruguay en base a métodos morfológicos y moleculares

Resumen

Las técnicas moleculares podrían ayudar a la identificación de especies de lombrices, especialmente cuando los caracteres morfológicos resultan difíciles de discernir. Basado en una colección de lombrices generadas a partir de muestreos en diferentes suelos agrícolas en Montevideo y Paysandú, el presente estudio representa un primer acercamiento al uso del *barcoding* del ADN como una herramienta para la determinación de la riqueza de lombrices en Uruguay. Las especies se identificaron tanto por caracteres morfológicos como por técnicas moleculares. Los individuos adultos se identificaron por morfología externa siguiendo las descripciones disponibles de las lombrices presentes en la región. De cada especie morfológica se obtuvo una muestra representativa para extracción de ADN genómico, con posterior amplificación y secuenciación de la región mitocondrial Citocromo Oxidasa I (COI). Las secuencias obtenidas se sometieron a búsquedas BLAST y fueron comparadas con las secuencias disponibles en GenBank. De 11 especies exóticas secuenciadas, ocho se identificaron completamente, encontrándose coincidencia entre caracteres morfológicos e información molecular; en tanto dos fueron menos consistentes, con un menor porcentaje de similitud; y una no logró ser identificada completamente debido a la falta de secuencias relacionadas cercanas en GenBank. Mientras la mayoría de las especies exóticas tenían secuencias representativas anotadas en GenBank, esto ocurrió sólo con una especie nativa, lo que demuestra la necesidad de desarrollar esta importante área a nivel regional. Los escasos registros de muestreos de lombrices en Uruguay, y la ausencia de estudios nacionales referidos a identificación de especies por secuencias de ADN, hacen de este estudio un puntapié inicial para una línea de investigación innovadora.

Palabras clave: COI, secuenciación, lombrices nativas y exóticas

3.3.1. Introduction

Earthworms, terrestrial oligochaetes (Annelida, Clitelata), have long been recognized as soil benefactors and their presence has been commonly associated with good quality soils (Chan, 2001). Recently, a comprehensive review of the effects of earthworms on the soil highlights their role in catalyzing ecosystem support services such as soil formation and nutrient cycling (Blouin et al., 2013). Several studies have proven how oligochaetes favour soil, by improving its physical properties (structure, porosity, bulk density), hydric properties (water regulation, infiltration and runoff), chemical properties (by accelerating N mineralization), and biological properties (they influence the structure of the microbial community, resulting in some cases in biological control of diseases and pests) (Blouin et al., 2013; Brown, 1995; Doube et al., 1994).

Although earthworms are sometimes referred to as a homogeneous group, more than 5000 different species are recognized in the world (http://taxo.drilobase.org; verified 2018, August 27). They vary in size - from barely some centimeters to several meters long- and in behavior- determining a particular depth of residence in the soil and levels of incidence on surface or in drilosphere (part of the soil influenced by earthworm secretions, Bouché, 1977). A comprehensive knowledge of the local earthworm biodiversity enables to predict the potential ecosystem services they could be offering (Blouin et al., 2013), as well as to identify potential threats caused by the introduction of exotic species (Hendrix et al., 2008).

The history of earthworm studies in Uruguay can be considered relatively brief. The first oligochaete researcher in Uruguay was Professor Ergasto Cordero, born at the end of the 19th century, who described several genera and native species. He made contributions to systematics, taxonomy and biogeography, in particular the Glossoscolecidae family, establishing their distribution and phylogeny (Cordero, 1931; 1943; 1945; in: Grosso, Jorge, & Brown, 2006). Unfortunately, as he had no followers, earthworm studies in Uruguay were interrupted, and resumed more than

half a century later by Grosso, Jorge, & Brown (2006) and Grosso & Brown (2007), Zerbino (2005; 2010; 2012), and Zerbino, Rodríguez and Altier (2006). It is difficult to determine if native species have been displaced, since there has been no systematic study of native earthworms present in natural ecosystems, which could be used as a baseline (Grosso, Jorge, & Brown, 2006), *i.e.* actual local earthworm richness (past and present) is not yet known. So far, 19 species of earthworms have been reported in Uruguay (and not all of them have been identified to the species level), more than half of these are exotic species (Grosso & Brown, 2007). Most probably with a greater sampling effort, this species list could be expanded, particularly within the group of natives, since the majority of the studies have been carried out in agroecosystems, where exotics are more competitive (Grosso, Jorge, & Brown, 2006; Zerbino, 2005; 2010; 2012; Zerbino, Rodríguez & Altier, 2006).

Ten out of the 11 exotic species found in Montevideo belong to the Lumbricidae family, Eurasian origin, and the remaining species of the genus *Amynthas*, belongs to the Megascolecidae. Only two native species have been recorded for this province: *Microscolex dubius* Fletcher, 1887 and *Eukerria stagnalis* Beddars, 1895 (Grosso & Brown, 2007). In surveys conducted in the northwest, only native Oligochaeta, belonging to the families Ocnerodrilidae and Glossoscolecidae, have been collected (Zerbino, 2007).

Morphological differences in earthworms have hitherto been the only elements to discern species. For instance, Sims and Gerard (1985) prepared a recognition key for British Lumbricids based solely on external characters such as setae arrangement, shape and position of genital pores, clitellum position and length, and position and shape of conspicuous genital marks. However, most external features are only observable in mature individuals and are often not sufficient to distinguish between species, particularly in the case of non-European species (Righi, 1990). Hence, the number, position and shape of internal organs have been further used to complete species identification (Blakemore, 2002; Righi, 1990). Dichotomous keys facilitate identification, under the assumption that the universe of earthworms for the sampled

area is already known and comprehended by the key. Consequently, these have local specificity, and cannot be used adequately in a different area from which they wereconceived for.

Currently, species identification by morphology can be complemented by molecular techniques, especially when inter-specific differences depend on internal characters or are distinguished only in sexually mature individuals. In particular, the use of "DNA barcoding" based on a standardized region of the mitochondrial cytochrome oxidase I gene (COI) has been widely used as a genetic marker to discriminate animal species (Hebert et al., 2003). This methodology has been successful for the identification of earthworm species in Asia, Europe and America (Chang, Rougerie, & Chen, 2009; Decaëns et al., 2013; Decaëns et al., 2016). However, when establishing phylogenetic relationships, it is not conclusive, and it is advisable to use more than one genetic marker (Decaëns et al, 2013; Fernández et al., 2016; James & Davidson, 2012). There are no precedents of identification of earthworms by molecular methods in Uruguay.

This study aimed to introduce the use of this new approach for identification of earthworm species by molecular techniques in Uruguay. It was based on an earthworm collection generated from samplings in different agricultural soils in Montevideo and Paysandú, Uruguay. Samplings covered a range of different managements, which included several cropping systems, tillage or no-till sowing and organic or non-organic production, to achieve variability and obtain a comprehensive range of species for COI sequencing, with which the applicability of molecular methods in Uruguay could be assessed.

3.3.2. Materials and methods

3.3.2.1. Sampling Sites

Field data were obtained from Typic Argiudolls (according to USDA soil taxonomy) of two provinces in western (Paysandú, 32.5ºS; 58ºW) and southern (Montevideo, 34.5ºS; 56ºW) Uruguay. In Paysandú, sample sites were plots from a long-term trial at a University Experiment Field, with different crop rotations (continuous crops and crop-pasture rotations). In Montevideo, sample sites were organic transition farms with tillage and no use of synthesized agrochemicals; and non-organic farms with no-till and use of synthesized fertilizers, herbicides and pesticides.

Sample management and morphological classificationSamplings were done in autumn and spring of 2014 and in spring 2015 with precipitations above the average (> 100 mm per month); no sampling was held during autumn 2015 due to a severe drought, since no earthworms were expected to be found in the first 20 cm of soil (Grosso, Jorge, & Brown, 2006). A similar method to that recommended by Anderson and Ingram (1993) was applied, with five sample units (soil monolith 25 cm x 25 cm and 20 cm depth) per site. In laboratory, earthworms were hand sorted from the monoliths, rinsed with distilled water and gently dried with paper napkins. They were then anesthetized gradually in alcohol until reaching a concentration of 20% for later fixation with 4% formaldehyde solution (Righi, 1990).

A total of 1636 earthworms were analyzed, 26% of which were adults. All adult specimens were grouped by morphology and identified at species level when possible. Classification as natives or exotics was based on the worm list described for Uruguay by Grosso & Brown (2007), and following the available keys and taxonomic descriptions (Andersen, 1997; Reynolds, 1996; Righi, 1979; Sims & Gerard, 1985). In the absence of a specific key for Uruguay, these keys were not used strictly following all the dichotomous options, but rather as a guide. For each site, one specimen per morphologic group was subject to molecular analysis. DNA

was extracted from the rear section of representative earthworms for each morphological group, which was not fixed in formaldehyde, but preserved in anhydrous ethanol at -20 ^{o}C.

3.3.2.2. Molecular techniques and DNA sequence processing

DNA extraction and polymerase chain reaction (PCR) amplification of the mitochondrial cytochrome oxidase I complex (COI) gene region was performed in LaTraMA laboratory, Sección de Bioquímica, Facultad de Ciencias, Universidad de la República, Uruguay. Total DNA was extracted from tissue samples, following a protocol of regular use in LaTraMA, which is a modification of Dellaporta, Wood, and Hicks (1983), where an additional standard chloroform: isoamylic alcohol (24:1) step was included to get rid of the excess proteins. Partial mitochondrial COI region was amplified with the primers LCO1490 and HCO2198 (Folmer et al., 1994), applying a standard barcoding protocol (Huang et al., 2007) with minormodifications: PCR amplifications were performed in an Axygen™ MaxyGene™ Gradient Thermal Cycler (Axygen Scientific THERM1001, USA) with the following conditions: denaturation step for 3 min at 94 ^{o}C, then 38 cycles of 30 s at 94 ^{o}C, 45 s annealing at 52 ^{o}C and 1 min at 72 ^{o}C, followed by a final elongation step of 10 min at 72 ^{o}C, and hold at 20 ^{o}C.

The PCR mix contained 2.5 uL of PCR buffer 10x, 1.25 uL $MgCl_2$ 50 mM, 0.25 uL dNTP 2 mM, 0.25 uL Taq 5U/uL, 1.25 uL of each primer, and 2 uL of DNA solution. A final volume of 25 uL was obtained by addition of mQ water. The PCR products were sent to Macrogen (Korea) for purification and sequencing. The DNA sequences were edited manually with the Chromas Lite software (http://technelysium.com.au/wp/chromas/; verified 2018, August 27) and subjected to Nucleotide Basic Local Alignment Search Tool (BLAST-N; Altschul et al., 1990) in NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 2018, August 27). The best matching species, published in indexed journals, was recorded for each query and included in Table 1. Similarity among sample sequences of the

same species was checked with Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2013), when these showed 100% similarity only one specimen per species was included in table 1.

3.3.3. Results and Discussion

By morphological taxonomy classification, four native earthworm species and 12 exotics were found, totalizing 16 earthworm species, three of which have apparently not been previously reported in Uruguay and need deeper analysis for an accurate taxonomical classification. Only two of the native species collected had the barcoding COI sequence annotated in the database, namely *Microscolex dubius* and *M. phsophoreus*, with two sequences deposited (Voua Otomo**,** Maboeta, & Bezuidenhout, 2013). This is a low number of accessions considering that *Aporrectodea caliginosa*, for example, has 357 COI sequences in GenBank, several of which are published in indexed journals. Natives were therefore not further analysed in the present study.

There is a reference database (RefSeq) with cured sequences, *i.e.* peer reviewed, with guarantee that the sequences are well annotated and not redundant. Although RefSeq has more than 55,000 organism sequences, up to date (verified 2018, August 27), only two earthworm COI sequences are deposited there: complete mitochondrion genomes for *Amynthas jiriensis* and *Lumbricus terrestris* (O'Leary et al., 2016). Therefore, RefSeq has still not become a reference database for terrestrial oligochaete studies.

The COI fragment of one of the unreported species failed to amplify accurately, reason why more samples of this species should be collected to repeat the DNA extraction and amplification. Out of the 11 successfully sequenced exotic species (35 specimens), eight were fully identified and coincidence of morphological characters and molecular info was found; two were less consistent, with lower percentage of

sequence similarity; and one (two specimens) could not be fully identified due to lack of close related sequences in GenBank. The fact that most exotic earthworm COI sequences were found in the available online database, the GenBank reference repository (https://www.ncbi.nlm.nih.gov/genbank/; verified 2018, August 27), is most probably because these belong to the Lumbricidae (originally Holarctic) and Megascolecidae (Asian) families, ranked as the first and second "most abundant and widely distributed invasives" in temperate zones (James, 2011). These species were *Allolobophora chlorotica* Savigny, 1893; *Amynthas corticis* Kinberg, 1867; *Aporrectodea caliginosa* Savigny, 1826; *Ap. rosea* Savigny, 1826; *Ap. trapezoides*, Dugès 1828; *Eisenia andrei* Bouché, 1972, *Lumbricus terrestris* Linnaeus 1758, *L. friendi* Cognetii, 1904, *Octolasion cyaneum* Savigny, 1826 and *O. tyrteum* Savigny, 1826 (table 1(fig. 1). It is the first time *Am. corticis* is reported for Uruguay, although this species is highly associated to *Am. gracilis* (Herrera & Mischis, 2007), which was reported for Montevideo already in the early studies conducted by Cordero (Grosso & Brown, 2007).

In the case of the genera *Allolobophora*, *Amynthas*, *Aporrectodea* and *Octolasion*, BLAST similarity analysis showed that COI fragments had over 97 % similarity with those sequences annotated in GenBank. Conversely, the genus *Lumbricus* showed lower similarity with the annotated sequences, between 74 % and 87 % (table 1). Only three specimens of the genus *Lumbricus* were DNA sequenced in this study, and the obtained sequences were rather short, probably due to degraded DNA (data not shown). Hence, DNA sequence data is not conclusive, and further investigation is needed to confirm if these low levels of similarity correspond to any differentiation involving the presence of cryptic species, as described for this genus in other invaded countries (Martinsson & Erseus, 2017; Spurgeon et al., 2016). Future studies should provide additional specimens, including COI sequences, as well as a complementary nuclear marker, such as Histone 3 or ITS2 (Martinsson, Rhodén, & Erséus, 2017), so as to get a robust phylogeny analysis and multi-locus species delimitation.

Based in multigene analysis, Martinsson & Erseus (2017) found the genus *Lumbricus* to be monophyletic, with maximum support in their H3 tree and some support in the COI tree, using sequences of European *Lumbricus castaneus, L. festivus, L. herculeus, L. rubellus,* and *L. terrestris*. They suggested that the morphospecies *L. rubellus* is composed of seven cryptic species. Their analysis also confirmed the previously suggested division between *L. terrestris* and *L. herculeus*, as two different species (James et al., 2010). According to size range attributed to *L. herculeus* (James et al., 2010), there was some suspicion that the small *Lumbricus* specimens in this study could belong to this species (fig.1 E and F). However, this fact was not confirmed by the BLAST similarity analysis, instead, some similarity appeared with *L. friendi*, but as the sequence of this species is unpublished, no certain conclusions can be drawn from that similarity, although external morphological characters coincide with the description of *L. friendi* (fig.1 F; Sims & Gerard, 1985).

Two cryptic linages of *Ap. caliginosa* were found (L2 and L3, table 1), which have also been reported for Europe and North America (Porco et al., 2013). Porco et al. (2013) highlight the importance of these cryptic lineages to detect earthworm invasive patterns, which morphological features could mask. For instance, in the present study *Ap. caliginosa* L2 was only found in Montevideo, while *Ap. caliginosa* L3 was found in both provinces, being the present study the first report of this species for Paysandú, which had previously only been found in the Uruguayan provinces of Montevideo, San José, Colonia and Treinta y tres (Grosso & Brown, 2007). However, due to the low number of samples, and that the samples are geographically concentrated, particularly in Paysandú, it is possible that *Ap. caliginosa* L2 might be found in further samplings in this province. Still, it could be a hypothesis for future studies that *Ap. caliginosa* L2 is absent from this province, where the present study could be seen as a preliminary survey.

Changes in the composition of the community of earthworms as a consequence of a certain use and management of the soil, is expected since different ecological groups are affected differently by agricultural activities. Species that feed on the surface and

bury fresh organic matter into greater depths in a system of vertical galleries (anecic species), such as *L. terrestris and L. friendi*, are more affected by agriculture management than other species that live and feed within the soil (endogenous species) such as *All. chlorotica, Ap. caliginosa, Ap. rosea, Ap. trapezoides, O. cyaneum* and *O. tyrtaeum* (Lavelle et al., 1989). Earthworms that only live superficially (epigeic species), are the most affected by tillage, but can survive under mulch by feeding on plant debris (Lavelle et al., 1989). The epigeics found in this study were *E. fetida* and *Am. corticis,* although not typical of agriculture land, they were most probably added to the soil with organic matter incorporation, since both can be found in compost piles. By showing different sensitivity to management according to their ecological group, it is interesting to use earthworms as bioindicators of soil quality not only in terms of variations in their density and biomass (Paoletti 1999, Bartz 2011, Zerbino, Rodríguez & Altier, 2006, Zerbino et al. 2008) but also to reach to species level and take advantage of the information provided by the possible changes in the community and ecological groups (Zerbino 2012).

In summary, molecular techniques along with morphology allowed a fully identification of several species found in two different localities. The possibility of identifying species by DNA barcoding has been made more accessible (Chang, Rougerie, & Chen, 2009; Pop, Wink, & Pop, 2003), with a repository of public sequences (e.g. GenBank). However, it relies on the fact that the sequences have previously been uploaded and published. A mayor constriction is the limited number of Uruguayan native species sequences available in GenBank, with only two species having its COI sequence annotated in RefSeq. Overcoming this limitation will require a detailed morphological study with a precise identification of morphospecies, generating the corresponding sequences, and expanding thus the database of native species.

Adding more native species to the online database represents a future challenge. Hence, it is essential to encourage earthworm taxonomy training, since the adoption

of molecular techniques does not imply that traditional taxonomy based on morphological characteristics can be dispensed with. The scarce records of earthworm samplings in Uruguay, and the absence of national studies that have identified species by DNA sequences, make this preliminary study a kick-start for a local innovative research program. Now that earthworms are recognised as a heterogeneous group, with different responses to agricultural management and potential to provide different and complementary ecosystem services, to be able to track changes in earthworm community composition, will help in the search of more sustainable management production systems, both by preserving biodiversity and by taking advantage of the ecosystem services earthworms may provide.

Table 1. Representative* specimens of exotic species collected in sampling sites in Montevideo and Paysandú with annotated sequences in GenBank**

ID	Assigned Morphological Species	Assigned Barcoding Species	Similarity with the online database*		Accession number	Accession number Reference	Sampling Site	Province
			bp	%				
23	***Allolobophora chlorotica***	***Allolobophora chlorotica***	501/518	97	JQ908733	Porco et al., 2013	Organic Transition Farm with tillage	Montevideo
69	***Amynthas cortices***	***Amynthas corticis***	615/617	99	KP214578	Novo et al., 2015	Organic Transition Farm with tillage	Montevideo
75	***Aporrectodea sp.***	***Aporrectodea caliginosa L2***	523/523	100	JQ908781	Porco et al., 2013	Organic Transition Farm with tillage	Montevideo
43	***Aporrectodea sp. (juvenile)***	***Aporrectodea caliginosa L3***	523/523	100	JQ908832	Porco et al., 2013	University Field: continuous crops	Paysandú
87	***Aporrectodea caliginosa***	***Aporrectodea caliginosa L3***	625/630	99	JQ908848	Porco et al., 2013	University Field: pasture-crops rotation	Paysandú
50	***Aporrectodea caliginosa***	***Aporrectodea caliginosa L3***	608/608	100	KT073940	Martinsson et al., 2015	Organic Transition Farm with tillage	Montevideo
20	***Aporrectodea rosea***	***Aporrectodea rosea***	641/646	99	JN869891	Klarica et al., 2012	Organic Transition Farm with tillage	Montevideo
51	***Aporrectodea rosea***	***Aporrectodea rosea***	614/615	99	KF441970	Fernández et al., 2016	Non-organic Farm with no tillage	Montevideo
71	***Aporrectodea sp.***	***Aporrectodea trapezoides***	546/549	99	KT073953	Martinsson et al., 2015	Organic Transition Farm with tillage	Montevideo
91	***Eisenia sp.***	***Eisenia fetida***	646/646	100	KX781372	Plytycz et al., 2016	Organic Transition Farm with tillage	Montevideo
70	***Lumbricus friend***	***Lumbricus friendi***	275/315	87	GU014034	Unpublished	Organic Transition Farm with tillage	Montevideo
22	***Lumbricus terrestris***	***Lumbricus terrestris***	440/514	86	HM388353	Porco et al., 2013	Organic Transition Farm with tillage	Montevideo
72	***Lumbricus sp. (juvenile)***	***Lumbricus terrestris***	342/403	85	KU888593	Souleman et al., 2016	Organic Transition Farm with tillage	Montevideo
76	***Octolasion sp.***	***Octolasion cyaneum***	616/616	100	JQ909151	Porco et al., 2013	Non-organic Farm with no tillage	Montevideo
30	***Octolasion tyrtaeum***	***Octolasion tyrtaeum***	280/281	99	JX531567	Shekhovtsov et.al.,2014	Organic Transition Farm with tillage	Montevideo
52	***Octolasion sp.***	***Octolasion tyrtaeum***	617/617	100	JQ909144	Porco et al, 2013	Non-organic Farm with no tillage	Montevideo

*Only one specimen per species is shown in this table when sequences within the same species had 100% similarity, more than one specimen of the same species is presented when sequence similarity was below 100%. **GenBank: online reference repository, https://www.ncbi.nlm.nih.gov/genbank/. bp= base pairs

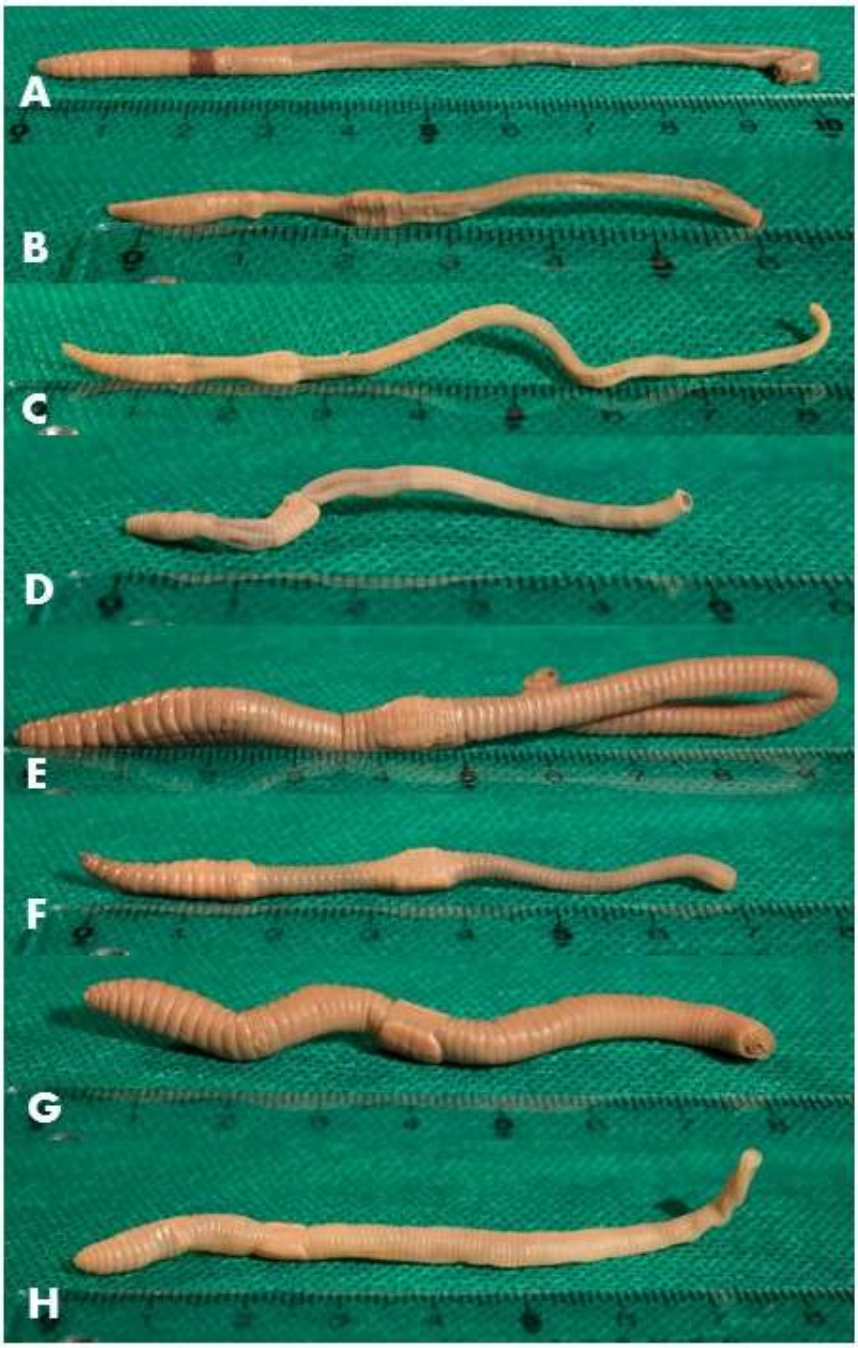

Fig. 1. Ventral / latero-ventral view of exotic earthworm specimens fixed in formaldehyde and preserved in 80% alcohol: A) ***Amynthas corticis, B) Allolobophora chlorotica,*** **C)** ***Aporrectodea caliginosa, D) Ap. rosea,*** **E)** ***Lumbricus terrestris,*** **F)** ***L. friendi,*** **G)** ***Octolasion tyrtaeum,*** **H)** ***O. cyaneum.*** **The rear end has been cut for DNA extraction. Numbers on the ruler correspond to centimeters. Photos: Gerardo Bentancur.**

Acknowledgements

We thank ANII (Uruguay) for PhD scholarship sponsoring. Financial support was given by the foundation Ekhagastiftelsen (Sweden), and CSIC-UdelaR (Uruguay). Many thanks to all the farmers and Oswaldo Ernst, responsible of the University Experimental Field in Paysandú, who kindly let us sample in their farms or experiment plots, respectively; and to workmates and students who helped with fieldwork. We are grateful to Elodie da Silva, Mailén Arleó y Martín Borteiro for lab assistance and to Gerardo Bentancur for taking the photos.

3.3.4. References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215, 403-410.

Andersen, C. (1997). Regnorme. *Natur og Museum*, 36(4), Naturhistorisk Museum, Aarhus, Denmark.

Anderson, J.M. & Ingram, J.S.I. (1993). *Tropical soil biology and fertility: A handbook of methods* (2nd. ed.) Wallingford, UK, CAB International.

Bartz, M.L.C. (2011). *Ocorrência e taxonomia de minhocas em agroecossistemas no Paraná, Brasil* (Doctoral Thesis) Universidade Estadual de Londrina, Londrina.

Blakemore, R.J. (2002). *Cosmopolitan Earthworms – an Eco-Taxonomic Guide to the Peregrine Species of the World*, (first CD ed. VermEcology), PO BOX 414 Kippax, ACT 2615, Australia.

Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussaard, L., Buttf, K.R., … & Brun, J-J. (2013). A review of earthworm impact on soil function and ecosystem services. *European Journal of Soil Science*, 62(4),161–182.

Bouché, M.B. (1977). Strategies lombriciennes, in: Lohm, U., Persson, T. (Eds.), *Soil organisms as components of ecosystems*. Proc. 6th Int. Coll. Soil Zool. Ecol. Bull. 25, Stockholm, pp. 157-163.

Brown, G.G. (1995). How do earthworms affect microfloral and faunal community diversity? *Plant and Soil*, 170(1), 209–231.

Chan, K.Y. (2001). An overview of some tillage impacts on earthworm population abundance and diversity - implications for functioning in soils. *Soil and Tillage Research*, 57, 179 -191.

Chang, C.-H., Rougerie, R. & Chen, J.H. (2009). Identifying earthworms through DNA barcodes: Pitfalls and promise. *Pedobiologia*, 52, 171–180.

Decaëns, T., Porco, D., James, S.W., Brown, G.G., Chassany, V., Dubs, F., …& Roy, V. (2016). DNA barcoding reveals diversity patterns of earthworm communities in remote tropical forests of French Guiana. *Soil Biology and Biochemistry*, 92, 171-183.

Decaëns, T., Porco, D., Rougerie, R., Brown, G.G. & James, S.W. (2013). Potential of DNA barcoding for earthworm research in taxonomy and ecology. *Applied Soil Ecology*, 65, 35-42.

Dellaporta, S.L., Wood, J. & Hicks, J.B. (1983). A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter*, 1, 19.

Doube, B.M., Stephens, P.M., Davoren, C.W. & Ryder, M.H. (1994). Interactions between earthworms, beneficial soil microorganisms and root pathogens. *Applied Soil Ecology*, 1, 3-10.

Fernández, R., Novo, M., Marchán, D.F. & Díaz Cosín, D.J. (2016). Diversification patterns in cosmopolitan earthworms: similar mode but different tempo. *Molecular Phylogenetics and Evolution*, 94, 701–708.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochromec oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294–299.

Grosso, E.G. & Brown, G.G. (2007). Biodiversidad y ecología de las lombrices de tierra en el Uruguay. In Brown GG, Fragoso C. [Eds.]. *Minhocas na América Latina: Biodiversidade e ecologia*. Londrina: EMBRAPA, 287-296.

Grosso, E.G., Jorge, G. & Brown, G.G. (2006). Exotic and Native Earthworms in Various Land Use Systems of Central, Southern and Eastern Uruguay. *Caribbean Journal of Science*, 42 (3), 294-300.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. B*, 270, 313–321.

Hendrix, P.F., Callaham, Jr. M.A., Drake, J.M., Huang, C.Y., James, S.W., Snyder, B.A. & Zhang, W. (2008). Pandora's Box Contained Bait: The Global Problem of Introduced Earthworms. *Annual Review of Ecology, Evolution, and Systematics*, 39, 593-613.

Herrera, J.A.D. & Mischis, C.C. (2007). Diversidad, abundancia y distribución de la oligoquetofauna (Annelida) en áreas protegidas de Selva Subtropical de Montaña: La Reserva Fiscal Parque la Florida (Tucumán, Argentina). In Brown GG, Fragoso C. (Eds.), *Minhocas na América Latina: Biodiversidade e ecologia*. (pp. 273-279). Londrina, Brazil: EMBRAPA.

Huang, J., Xu, Q., Sun, Z.J., Tang, G.L., Su, Z.Y. (2007). Identifying earthworms through DNA barcodes. *Pedobiologia*, 51, 301-309.

James, S.W. & Davidson, S.K. (2012). Molecular phylogeny of earthworms (Annelida: Crassiclitellata) based on 28S, 18S and 16S gene sequences. *Invertebrate Systematics*, 26, 213–229.

James, S.W. (2011). Earthworms. In: Simberloff, D. & Rejmánek, M. (Eds.), *Encyclopedia of biological invasions* (pp.177-183). Los Angeles, California USA: University of California Press.

James, S.W., Porco, D., Decaëns, T., Richard, B. & Rougerie, R. (2010). DNA Barcoding Reveals Cryptic Diversity in Lumbricus terrestris L., 1758 (Clitellata): Resurrection of L. herculeus (Savigny, 1826). *PLoS ONE*, 5(12): e15629. doi:10.1371/journal.pone.0015629

Lavelle, P., Barois, I., Martin, A., Zaidi, Z. & Schaefer, R. (1989). Management of earthworm population in agro-ecosystems: A possible way to mantain soil quality? In Clarbolm, M. & Bergström, L. (Eds.). *Ecology of arable land* (pp.109-122). Dordrecht, Netherlands: Kluwer Academic.

Klarica, J., Kloss-Brandstaetter, A., Traugott, M. & Juen, A. (2012). Comparing four mitochondrial genes in earthworms - Implications for identification,

phylogenetics, and discovery of cryptic species. *Soil Biology and Biochemistry*, 46, 23-30.

Martinsson, S. & Erseus, C. (2017). Cryptic speciation and limited hybridization within Lumbricus earthworms (Clitellata: Lumbricidae). *Molecular Phylogenetics and Evolution*, 106, 18–27.

Martinsson, S., Cui, Y., Martin, P.J., Pinder, A., Quinlan, K., Wetzel, M.J. & Erseus, C. (2015). DNA-barcoding of invasive European earthworms (Clitellata: Lumbricidae) in south-western Australia. *Biological Invasions*, 17 (9), 2527-2532.

Martinsson, S., Rhodén, C. & Erséus, C. (2017). Barcoding gap, but no support for cryptic speciation in the earthworm *Aporrectodea longa* (Clitellata: Lumbricidae), *Mitochondrial DNA Part A*, 28(2), 147-155.

Novo, M., Cunha, L., Maceda-Veiga, A., Talavera, J.A., Hodson, M.E., Spurgeon, D.,… & Kille, P. (2015). Multiple introductions and environmental factors affecting the establishment of invasive species on a volcanic island. *Soil Biology and Biochemistry*, 85, 89–100.

O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R,… & Pruitt, K.D. (2016). Reference sequence (RefSeq) database at NCBI:current status, taxonomic expansion, and functionalannotation. *Nucleic Acids Research*, 44, 733–745.

Paoletti, M.G. (1999). The role of earthworms for assessment of sustainability and as bioindicators. *Agriculture, Ecosystems and Environment*, 74,137–155.

Plytycz, B., Bigaj, J., Falniowski, A.F. & Morgan, A.J. (2016). Unexpected results and open questions from experiments on regeneration in lumbricid worms. *Invertebrate Survival Journal*, 13, 315-325.

Pop, A.A., Wink, M. & Pop, V.V. (2003). Use of 18S, 16S rDNA and cytochrome c oxidase sequences in earthworm taxonomy (Oligochaeta, Lumbricidae). *Pedobiologia*, 47, 428–433.

Porco, D., Decaëns, T., Deharveng, L., James, S., Skarzynski, D., Erséus, C.,… & Hebert, P.N. (2013). Biological invasions in soil: DNA barcoding as a

monitoring tool in a multiple taxa survey targeting European earthworms and springtails in North America. *Biological Invasions*, 15, 899–910.

Reynolds, J.W. (1996). *Earthworm Biology and Ecology: Course manual for Universidad Nacional de Córdoba.* Córdoba, Argentina: Facultad de Ciencias Exactas, Físicas y Naturales.

Righi, G. (1979). Introducción al estudio de las lombrices del suelo (oligoquetos megadrilos) de la Provincia de Santa Fe (Argentina). *Revista de la Asociación de Ciencias Naturales del Litoral*. Colección Climax, Santa Fé, Argentina 2, 89–155.

Righi, G. (1990). *Minhocas de Mato Grosso e de Rondônia*. Brasilia, DF. Brazil: Conselho Nacional de Desenvolvimento Cientifico e Tecnologico.

Shekhovtsov, S.V., Golovanova, E.V. & Peltek, S.E. (2014). Genetic diverity of the earthworm *Octolasion tyrtaeum* (Lumbricidae, Annelida). *Pedobiologia*, 57, 245-250.

Sims, R.W., Gerard, B.M. (1985). *Earthworms. Keys and notes for the identification and study of the species.* The Linnean Society of London and The Estuarine and Brackish water Sciencies Association. Bath, UK: The Pitman Press.

Souleman, D., Grumiaux, F., Frerot, H., Vandenbulcke, F. & Pauwels, M. (2016). Isolation and characterization of eight polymorphic microsatellites markers for the earthworm Lumbricus terrestris. *European Journal of Soil Biology*, 74, 76-80.

Spurgeon, D.J., Liebeke, M., Anderson, C., Kille, P., Lawlor, A., Bundy, J.G. & Lahive, E. (2016) Ecological drivers influence the distributions of two cryptic lineages in an earthworm morphospecies. *Applied Soil Ecology*, 108, 8–15.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, 30(12), 2725–2729.

Voua-Otomo, P., Maboeta, M.S. & Bezuidenhout, C. (2013). Inadequate Taxonomy and Highly Divergent COI Haplotypes in Laboratory and Field

Populations of Earthworms used in Ecotoxicology. A Case Study. *African Zooloy*, 48 (29), 290-297.

Zerbino, M.S. (2012). Efecto de rotaciones cultivo-pasturas en siembra directa, con pastoreo, sobre comunidades de Oligochaeta. *Agrociencia Uruguay*, 16(1),15-23.

Zerbino. M.S. (2010). Evaluación de la macrofauna del suelo en rotaciones cultivo-pasturas con laboreo convencional. *Acta Zoológica Mexicana*, 26, 189-202.

Zerbino, M.S. (2007). Evaluación de la biomasa de lombrices de tierra en diferentes sistemas de producción del Uruguay. In Brown GG & Fragoso C. (Eds.) *Minhocas na América Latina: Biodiversidade e ecología* (pp. 287-296). Londrina, Brazil: EMBRAPA.

Zerbino, M.S. (2005). *Evaluación de la densidad, biomasa y diversidad de la macrofauna del suelo en diferentes sistemas de producción*. [MSc thesis]. Montevideo, Uruguay: Facultad de Ciencias, Universidad de la República.

Zerbino, M.S., Altier, N., Morón, A. & Rodríguez, C. (2008). Evaluación de la macrofauna del suelo en sistemas de producción en siembra directa y con pastoreo. *Agrociencia Uruguay*, 1, 44-55.

Zerbino, M.S., Rodríguez, C. & Altier, N. (2006). Oligochaeta communities in Uruguayan Agro-ecosystems. *Caribbean Journal of Science*, 42, 315 – 324.

4: CONTRIBUCIÓN DE LAS LOMBRICES AL CONTROL BIOLÓGICO DE *Fusarium graminearum* EN RASTROJO DE TRIGO

4.1. RESUMEN

Las lombrices contribuyen a la sanidad vegetal mediante el control biológico de ciertos patógenos. *Lumbricus terrestris*, ha mostrado reducir *Fusarium graminearum*, principal causante de la fusariosis, una de las enfermedades más importantes en los cereales. Para saber si otras lombrices pudieran tener un efecto similar, se montaron tres experimentos, los dos primeros en la Universidad SLU, Uppsala, Suecia y el tercero en Facultad de Agronomía, UdelaR, Uruguay. En los dos primeros se evaluaron las especies *Aporrectodea longa, Lumbricus terrestris* (anécicas) y *L. rubellus* (epígea), creando condiciones sub óptimas y óptimas de humedad y alimentación para las lombrices, en el primer y segundo experimento, respectivamente. El rastrojo (con *Fusarium*, con microorganismos del suelo, o estéril) se colocó sobre el suelo de microcosmos llenos de tierra con o sin lombrices y se mantuvo en una cámara a temperatura constante durante varias semanas. Los resultados del análisis de qPCR revelaron que el inóculo de *F. graminearum* en el rastrojo fue reducido por ambas especies de lombrices en el primer experimento, mientras que no se encontraron diferencias significativas en el segundo. La cobertura de rastrojo en la superficie del suelo fue reducida por *L. rubellus* en ambos experimentos, mientras que *A. longa* sólo lo hizo en condiciones óptimas. En el tercer experimento, similar a los anteriores, se evaluaron las especies *Glossoscolex rione* (endógea, nativa) y *L. friendi* (anécica, exótica). *L. friendi* tuvo una actividad notoria en la superficie, reduciendo más del 30 % de la cobertura por rastrojo, pese a una alta mortalidad, mientras el tratamiento con *G. rione* no se diferenció del control. Se requerirá repetir el experimento para confirmar si los efectos también se extienden a la reducción del inóculo de *Fusarium* en el rastrojo, ya que, en la cuantificación del patógeno, éste se encontró por debajo de los niveles de detección. Las lombrices no fueron afectadas negativamente por *Fusarium*, ni sus toxinas. Tanto las lombrices anécicas como las epígeas mostraron potencialidad para contribuir al control biológico de *F. graminearum* en el rastrojo de trigo, ya sea reduciendo el rastrojo en superficie, reduciendo el inóculo patógeno sobre el rastrojo, o ambos, dependiendo de las condiciones ambientales y de su respuesta según el grupo ecológico al cual pertenece la lombriz. La lombriz endógea testeada no tuvo efecto sobre el rastrojo.

4.2. CONTRIBUTION OF ANECIC AND EPIGEIC EARTHWORMS TO BIOLOGICAL CONTROL OF FUSARIUM GRAMINEARUM IN WHEAT STRAW

Gabriella Jorge-Escudero[a,b,c*], Carlos A. Pérez[d], Hanna Friberg[e], Sara Söderlund[c], Silvana Vero[f], Gabriela Garmendia[f], Jan Lagerlöf[c]

[a] Departamento de Sistemas Ambientales, Facultad de Agronomía, Universidad de la República, Garzón 780, 12900 Montevideo, Uruguay.

[b] Departamento de Suelos y Aguas, Facultad de Agronomía, Universidad de la República, Garzón 780, 12900 Montevideo, Uruguay.

[c] Department of Ecology, Swedish University of Agricultural Sciences (SLU), P.O Box 7044, SE-75007, Uppsala, Sweden

[d] Departamento de Protección Vegetal, EEMAC, Facultad de Agronomía, Universidad de la República, Ruta 3 km 363, 60.000 Paysandú, Uruguay.

[e]Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences (SLU), P.O Box 7026, SE-75007, Uppsala, Sweden

[f] Cátedra de Microbiología, Departamento de Biociencias; Facultad de Química, Universidad de la República, Av. Gral. Flores 2124, 11800 Montevideo, Uruguay

*Corresponding author: gjorge@fagro.edu.uy

Abstract

Earthworms have proved to contribute to plant health through conservation biological control. Fusarium head blight, caused primarily by *Fusarium graminearum*, is one of the most important cereal diseases, with severe detrimental effects on yield and grain quality worldwide. Two experiments assess effects of anecic and epigeic earthworms on wheat straw and *Fusarium graminearum* inoculum. For both experiments a set of microcosms were arranged. PVC cylinders were filled with moist soil mix, wheat straw was evenly distributed on the soil surface (inoculated with *Fusarium*, soil microorganisms or sterile) and three earthworm species (*Aporrectodea longa*, *Lumbricus rubellus* or *L. terrestris*) were tested. The first experiment represented a sub-optimal situation for earthworms regarding soil moisture and feeding conditions, which was obtained by scarce

watering of the microcosms with a consequent decrease in soil water content during incubation, and by not adding a supplementary food source. In the second experiment, soil was enriched with cow manure as feed for the earthworms and soil moisture was maintained above 25%. Results from qPCR analysis revealed that *F. graminearum* inoculum on straw was reduced to undetectable amounts by *L. rubellus* and *A. longa* when the feeding conditions were limited, while no significant differences with the control without earthworms were found when earthworms had high food availability ($\alpha = 0.05$). Straw coverage on soil surface was reduced by *L. rubellus* (epigeic) in both experiments ($p<0.0001$), while *A. longa* (anecic) just did so under optimal conditions. *L. terrestris* (anecic), only tested under optimal conditions, reduced soil cover significantly more than the other two species ($p<0.0001$). This fungus or its toxins did not affect earthworms negatively. Both anecic and epigeic tested earthworms showed potential to contribute to biological control of *F. graminearum* in wheat straw, either reducing straw on surface, reducing the pathogen inoculum on straw, or both, depending on the environmental conditions and on their response according to their ecological group and species. The possibilities to optimize earthworms' capacity for biological control of fungal diseases in practical agriculture, under changing weather and organic matter supply conditions, are discussed.

Keywords:
Aporrectodea longa; Lumbricus rubellus; Lumbricus terrestris; Fusarium Head Blight; Conservation biological control.

4.2.1. Introduction

As it has been stated since Darwin's time, earthworms, terrestrial Oligochaeta (Annelida, Clitellata), have a positive effect on crop productivity, by enhancing soil quality through improvement of soil physical properties (structure, porosity, bulk density), hydrological properties (water regulation, increasing infiltration and reducing surface runoff), and chemical properties (accelerating N mineralization)

(Blouin et al., 2013). In addition, it has been found that earthworms also have a positive effect on plant health since they affect soil biological properties, changing the microbial community structure (Brown, 1995; Doube et al., 1994) and controlling certain pathogens (Bonkowski et al., 2000; Byzov et al., 2007). Greenhouse and field experiments have shown that earthworms can significantly reduce the incidence of several plant diseases caused by fungal pathogens such as *Gaeumannomyces graminis* var. tritici (Hume et al., 2015; Stephens and Davoren, 1995), *Rhizoctonia solani* (Stephens and Davoren, 1997), *Fusarium oxysporum and Verticillium dahliae* (Elmer, 2009), *F. culmorum* (Oldenburg et al., 2008; Wolfarth et al., 2011a; Wolfarth et al., 2011b), *Oculimacula yallundae* (Bertrand et al., 2015) generally with an associated increase in crop yields. Earthworms contribute to conservation biological control, as defined by Eilenberg et al. (2001), since soil conservation practices, which enhance their populations, may help to reduce the effects of residue-borne plant diseases.

Selective or preferential consumption is considered the main mechanism by which earthworms control plant pathogens (Bonkowski et al., 2000; Moody et al., 1995). This is primarily because earthworms prefer fungi of the early stages in the succession over the decomposition process rather than those of later stages and most plant pathogenic fungi belong to the former group (Bonkowski et al., 2000; Moody et al., 1995). Other suggested mechanisms of plant disease suppression include mechanical disruption of hyphae, and improvement of soil structures and nutrient availability, which would also be influencing indirectly through strengthening the plant health and defence system (Friberg et al., 2005). Moreover, production of antifungal compounds and stimulation of plant defences through production of IAA-like molecules are currently under research as contributing modes of action (Meghvansi et al., 2011; Puga-Freitas et al., 2012).

The mechanisms by which earthworms affect plant diseases are highly dependent on the ecology of both the earthworm and pathogen species. This may be directly related to the ecological group to which earthworms belong, which determines

differences in behaviour, influence zone, and their interaction with saprophytic microbiota (Brown et al., 2004, Friberg et al., 2008). For instance, recent studies showed that *Lumbricus terrestris* reduced *Fusarium culmorum* biomass in wheat straw by 98 % and 99.4 % after 8 and 11 weeks of incubation, respectively, whereas the effect of *Aporrectodea caliginosa* was not significant in this case (Oldenburg et al., 2008; Wolfarth et al., 2011a; Wolfarth et al., 2011b). However, *A. caliginosa* did reduce *Gaeumannomyces graminis* var. *tritici* disease severity by 25 % on wheat (Hume et al., 2015). These findings are coherent when analysed considering the ecology of earthworm and pathogen. The inoculum of *F. culmorum,* a causal agent for Fusarium Head Blight, is found in wheat straw on soil surface (Leplat et al., 2013). Hence surface dwelling earthworms, as *L. terrestris,* are expected to have greater effect than *A. caliginosa,* which belongs to the endogeic group and seldom moves to the soil surface (Bouché, 1977). On the other hand, *G. graminis* inoculum is found mainly in dead roots mostly remaining buried in the soil (Hornby, 1975), therefore more available for the action of the endogeic *A. caliginosa*.

Fusarium graminearum, is the predominant causal agent of Fusarium head blight (FHB), one of the main cereal diseases worldwide, which has severe consequences for grain quality and yield (Del ponte et al., 2017; Singh et al., 2016), with devastating consequences for the economy. As an example, the estimated economic impact of FHB in the US, from 1998 to 2000, reached $ 2.7 billion, combining direct and secondary economic losses (Nganje et al., 2004). It is a residue-borne pathogen, highly adapted to survive and sporulate on crop residues, being the main source of inoculum and the driving force for epidemic outbreaks. Thus, the importance of this disease has increased with the adoption of conservational or no-till systems in crop rotations highly dominated by cereals. These systems preserve the crop residues at the soil surface, allowing the pathogen to survive over time, resulting in production systems with high inoculum density of this pathogen (Leplat et al., 2013; Lindblad et al., 2013; Pereyra et al., 2004; Yuen and Schoneweis, 2007). The control of FHB has been limited given the lack of effective genetic resistance, and the unreliable efficiency obtained with fungicides (McMullen et al., 2012; Singh et al., 2016; Yuen

and Schoneweis, 2007). Thus, novel methods to minimize epidemic outbreaks are needed.

Since the inoculum of *F. graminearum* is primarily determined by the amount of infested wheat residues remaining on the soil surface (Leplat et al., 2013), as stated above, biocontrol by earthworms of this pathogen should rely on species whose activity is partly or mainly on soil surface, namely anecics or epigeics (Bouché, 1977). This is the case of *Aporrectodea longa* Savigny and *Lumbricus terrestris,* two anecic earthworms, and *Lumbricus rubellus,* epigeic. All three belong to the Eurasian Lumbricidae family, are common in agricultural soils in the temperate parts of Eurasia, and have also been introduced to similar climate regions of North and South America and Australia (Blakemore 2002; Fragoso and Brown 2007; DriloBASE Taxo). Due to their mentioned influence zone, these earthworms may reduce *Fusarium* inoculum on wheat straw, either by digging wheat straw pieces or by directly feeding on the fungus on the straw, which is the main source of infection for the following crop.

To our knowledge the epigeic *L. rubellus* has not been tested for biological control of pathogenic fungi, although it has been proved that together with *Octolasion cynaeum* they induce higher grass resistance to nematodes, by promoting root development (Wurst et al., 2008). There is more documented data for the other two selected species. Showing a very similar behaviour regarding selection of fungi decomposing wheat straw, *L. terrestris* and *A. longa* preferred a *Fusarium* species (*F. lateritum*) when offered this and several other decomposing fungi (Moody et al., 1995), and the *Fusarium* spores were not viable after passing through the guts of these earthworms (Moody et al., 1996). Moreover, as mentioned earlier, *L. terrestris* has been reported to reduce biomass of another FBH-causing species of *Fusarium, F. culmorum,* on wheat straw, mainly by directly feeding on the pathogen, and partly by microbial priming effect (Oldenburg et al., 2008; Wolfarth et al., 2011a) in which microbial activity is enhanced by the release of polysaccharide-rich cutaneous mucus (Brown, 1995). This has been proved in controlled and semi-controlled conditions with one

species of earthworm and fungus. It still remains unknown whether this pattern will be maintained when the earthworm and/or fungus species is changed, or if environmental conditions change. Whether the presence of alternative food or reduced soil water content might limit the effect of earthworms reducing pathogens inoculum needs to be researched.

The aim of this study was to evaluate the role of Lumbricidae earthworm species of two ecological groups, in different environmental conditions, simulating changing weather conditions and agriculture with or without addition of organic manure to the soil, on the amount of wheat residue at the soil surface and the level of infection by *Fusarium graminearum* of that residue. We hypothesized that anecic *(A. longa)* and epigeic earthworms *(L. rubellus)* (1) reduce the cover by wheat straw on surface, and (2) reduce the *Fusarium* inoculum on straw remaining on surface, both in optimal and sub-optimal moisture and feed conditions. We compared the effects of these two species in optimal moisture and feed conditions to that of *L. terrestris* (epigeic), previously reported to be effective in the control of another *Fusarium* species, *F. culmorum*.

4.2.2. Materials and Methods

The study was conducted at the Swedish University of Agricultural Sciences (SLU), Ultuna Campus, Uppsala (59°49′05″ N, 17°39′28″ E). Two experiments were held during the periods July - August 2014 and 2015. Because earthworms may present seasonal behaviour (Butt, 1991), the experiments were held during the same season of consecutive years. The first experiment would recreate sub-optimal conditions (limiting conditions in terms of food and water), while the second was considered in optimal conditions for earthworm performance (designed without any limitations regarding these factors).

4.2.2.1. Experiment set up

For both experiments a set of microcosms were arranged. PVC cylinders (30 cm high and 14.5 cm diameter) were filled with moist soil mix (described in 4.2.2.2). Wheat straw was evenly distributed on the soil surface (described in 4.2.2.3); and three earthworm species (*Aporrectodea longa*, *Lumbricus rubellus* or *L. terrestris*) were tested (described in 4.2.2.4). A nylon net (pore size 1 mm) was fixed to the bottom of each cylinder with a rubber band to retain the soil. Another net was fixed to the top of each container to prevent earthworms from escaping. Finally, all the cylinders were covered with plastic bags to avoid excessive evaporation, and watered weekly. In Experiment 2, water content was corrected weekly by weighing microcosms and adding an amount of water equivalent to the dried volume, while in Experiment 1, only soil surface was lightly sprayed with water once a week (table 1). Sticky-paper traps were placed next to the cylinders to control *Sciaridae* flies.

Both experiments were fully factorial with two factors: Experiment 1 had two levels in Factor 1 – Straw inoculation with microorganisms (no inoculation, *Fusarium* inoculation), and three levels in Factor 2 – Earthworm species (no earthworms, *L. rubellus, A. longa*), which made six treatments. Experiment 2 had three levels in Factor 1 – Straw inoculation with microorganisms (no inoculation, *Fusarium* inoculation, Soil microorganisms) and four levels in Factor 2 – Earthworm species (no earthworms, *L. terrestris, L. rubellus, A. longa*), which made 12 treatments. The experiments had a randomized complete block design. Each treatment with 6 replicates (6 cylinders) was incubated in the dark. The particular details for each experiment are listed in table 1.

Table 1. Information regarding amount and moisture of soil and straw used in each experiment and the conditions of incubation

	Experiment 1: Suboptimal moisture and feed conditions	Experiment 2: Optimal moisture and feed conditions
Soil (kg per mesocosm; fresh weight)	2.7	2.0
Initial soil water content (%ww)	25	25
Final soil water content (%ww)	20	25
Initial straw (g per mesocosm; fresh weight)	13	15
Initial straw water content (%ww):		
— Sterile straw	81	82
— Straw with *Fusarium*	84	87
— Straw with soil microorganisms	-	87
Incubation time (weeks)	6	5
Temperature (ºC)	18-22	18-19
Treatments	6	12
Feed	Nothing	Rehydrated pelletized cow manure
Weekly watering with deionized water	2-4ml/cylinder, sprayed on the straw and soil surface	80 – 120 ml/cylinder, poured to on the straw and soil surface to raise water content to 25%ww

4.2.2.2.Soil preparation

The soil was prepared as a mix of 60% (in volume) of clay-loam soil classified as Eutric cambisol (Kirchmann et al., 1994), 30% of sandy soil, both obtained from the Ultuna Campus field experiment, and 10% of a substrate with different organic matter content, according to the experiment, to provide structure improvement. In earlier experiments and pre-experiments, the pure clay soil was found to be difficult to handle since it became very hard if getting a little dry and smeared if too wet. In previous experiments, the mix with sandy soil and a substrate with organic matter was found easier to maintain (Lagerlöf et al., 2015). In Experiment 1, to simulate sub-optimal conditions in terms of food resources we did not offer extra food for earthworms, therefore organic matter was provided as commercial planting soil (sphagnum peat based Hasselfors P-jord®, pH: 6) without easily available earthworm food. In Experiment 2, we wanted both structure improvement and extra food for earthworms, therefore rehydrated pelletized organic cow manure (pelletized Weibulls concentrated®, NPK 2-1.5-1.7) was used. The particle size of the manure was on average less than 1 mm with no particles larger than 3 mm. Before mixing, the soils had been cleared from roots, debris, stones and macrofauna (e.g. earthworms and beetles) by hand and thereafter frozen at -20^{o}C for 24 h and thawed for 24 h, This process was repeated twice to reduce macro- and mesofauna, although at least a significant part of the nematodes, other microfauna, and microorganisms may not be affected (Sulkava and Huhta, 2003). In the first experiment, the final mix contained 3.48 % total C (including 0.036% carbonate C) and 0.20 % total N and pH- water was 7.73; the soil of the second experiment had 3.16 % C, 0.27 % N and pH was 6.54. Both substrates were initially moistened to 25 % water (fw/fw).

4.2.2.3.Straw preparation and inoculation

Winter wheat straw (*Triticum aestivum*) cv. 'Olivin', was collected from a field in Uppsala (Sweden) at harvest, *i.e.* at wheat ripening stage. It was then cut in aprox. 2.5 cm-long pieces and gamma sterilized at 25 kGy (CodanSteritex AB, Denmark) in the first experiment, and autoclaved in the second experiment. The straw was

transferred to sterile 1 L flasks, rewetted with 150 mL sterile malt broth (5 gL^{-1}) per portion of 20 g straw, and left overnight. Next, two of the bottles were inoculated with *Fusarium graminearum*, isolate VPE 104, which was isolated from wheat kernels harvested in Sweden in 2011, and kept at -80^{o}C until use. A second set of two bottles were left without inoculation. For inoculation, one agar plate (9 cm diameter, with potato dextrose agar) with one-week old culture of *F. graminearum* cut in pieces was added to each bottle. In experiment 2, another treatment inoculated with soil was also included. For this treatment, two bottles containing sterilized straw and malt broth were inoculated with 3 mL of soil suspension from the soils used in the microcosms. The soil suspension was prepared shaking soil particles (30 mL) in 100 mL sterile water. All bottles were incubated for two weeks at room temperature, preserved from direct sunlight, and shaken twice a week in order to stimulate an even colonization of the straw, which could be confirmed at the end of the two weeks as mycelia covered all the straw in the inoculated bottles.

4.2.2.4.Earthworm collection and laboratory adaptation

Adult earthworms of the species *A. longa* (2 individuals*), L. rubellus* (3 individuals) and *L. terrestris* (2 individuals), were placed into each of the corresponding cylinders. Adulthood was determined by the presence of clitellum. In the case of *A. longa*, some sub-adults were included, *i.e.* individuals with the same body length and width as adults, but without developed clitellum. We intended to use earthworms of similar size to assure equal consumption rate, which is generally reported in relation to bodyweight (Lavelle, 1988; Whalen and Parmelee, 1999). Earthworms were collected not more than one month before the experiment from agricultural and garden soils in the vicinity of Uppsala, Sweden.

Prior to the experiment, the earthworms were kept in 6 L boxes with the same moist soil mixture as described above, in a climate chamber of the SLU laboratory, Ultuna, at 18 °C for not more than 3 weeks, and were fed with cow manure wetted to 50 % moisture content, added at the soil surface regularly. At the beginning of the

experiments, the mean individual biomass (total fresh weight with gut content) of *A. longa* was 2.2 ± 0.6 g and that of *L. rubellus* 1.3 ± 0.3 g for the first experiment, and in the second experiment it was 2.1 ± 0.3 g, 1.0 ± 0.2 g, and 3.9 ± 0.4 g for *A. longa, L. rubellus* and *L. terrestris*, respectively. Both experiments in the present study were designed considering optimal stocking densities estimated for *A. longa and L. terrestris* (Lowe and Butt, 2005); no reference was found regarding *L. rubellus*.

4.2.2.5.Data collection

Soil surface cover

Soil covered by straw was determined based on photographs taken of each cylinder at the beginning and at the end of the experimental period. The data programme Matlab (Matlab® R2014b 8.4.0150421, Mathworks) was used for calculation of percentage straw cover of the soil surface. This software produced a binomial image for each photograph, assigning white for straw and black for soil, after several examples of random photographs where the user had to indicate what the total area of the surface was, what was considered straw and what was considered soil. Then, the white area (*i. e.* area covered by straw) was calculated by the software for each photograph as a ratio of the total area, which was then converted into percentage.

Straw, soil and casts sampling and sample processing

Sub-samples from the initial straw were taken for dry weight and *F. graminearum* inoculum determinations. At the end of the experiments, surface straw was collected, and weighed. Half of the amount of straw was ground to <0.5 mm and frozen (-20 ºC) for qPCR analysis, and the other half was oven-dried for 48 h at 105 ºC to calculate moisture content. For Experiment 1, also buried straw was collected, weighed, frozen and analysed. Samples of soil at 5 cm depth (Experiment 1) and surface casts (Experiment 1 and 2) were frozen for later qPCR analysis. Soil dry matter content was determined by drying the soil at 105 °C for 2 days. Casts were collected from the straw and soil surface when clearly distinguished from soil

particles, and preserved in 15 mL plastic centrifuge tubes. After that, the first 5 cm of soil were removed and 15 mL soil samples were collected from that depth using the same tubes where they would be preserved.

Quantification of F. graminearum in straw, soil and casts

The whole straw sample was ground and mixed with a rotary mill (SampleTek Model 200 Vial Rotator, Lincoln, Nebraska) to a fine powder, and from that mix sub-sample of 100 mg was taken to proceed to the DNA extraction using the DNeasy Plant Mini kit (Qiagen, Germany) according to the manufacturer's instructions.

DNA extraction was completed in a QiaCube (Qiagen) with the standard plant cells and tissues protocol. Two water controls were included during the DNA extraction. DNA from two extraction replicates was pooled before PCR. Soil and cast DNA was extracted using NucleoSpin® Soil (Macherey-Nagel, Germany) using three 300 mg aliquots of soil from each experimental plot. DNA extracts were stored at -20 °C. The amount of DNA of *F. graminearum* was quantified with real-time PCR (qPCR) using the FgramB379 and FgramB411 primers (Nicolaisen et al., 2009) at 500 nM. The PCR was performed on a CFX Thermal cycler (Biorad, Carlsbad, CA, USA) using a protocol with 2 min at 98 °C, 40 cycles of 98 °C for 5 s and 62 °C for 10 s followed by dissociation curve analysis at 68 to 95 °C. A 10-fold dilution series of DNA from mycelium of *F. graminearum* containing 0.0042-42 ng DNA per reaction was used to produce a standard curve. Efficiency was calculated according to Rebrikov and Trofimov (2006). Linearity of the curve and dynamic range as the DNA concentration range in between the curve, were also determined. All samples were run in duplicates, with reactions containing 2.5 µl template in EvaGreen master mix (Biotium, CA, USA). An appropriate dilution of one tenth of the sample DNA was tested to avoid matrix effect.

Earthworm survival growth and reproduction

Earthworms were counted, washed in tap water, dried with paper towels and then weighed for fresh biomass. Earthworm cocoons were retrieved by wet sieving of the soil (mesh size 2 mm).

4.2.2.6.Statistical analyses

Statistical analyses were performed with the statistical package Infostat®. Data were analysed by the Linear Mixed Model, with 2 fixed effect factors (earthworm species and straw condition) and one random factor (block: six levels). The earthworm species-factor had three levels in 2014 (without earthworms, *A. longa* and *L. rubellus*) and four in 2015 (*L. terrestris* was added); while the straw condition-factor had two levels in 2014 (sterile and *Fusarium*) and three in 2015 (soil microorganisms was added). When significant effects were found, means were compared by Fisher's test ($\alpha = 0.05$) with Sidak's p-value correction procedure. In all cases the assumption of normality of the residues was satisfied (Shapiro-Wilk's test).

4.2.3. Results

4.2.3.1. Experiment 1 – Sub-optimal conditions

Wheat straw on surface

At the end of the experiment, both factors, earthworm species and straw treatment, had a significant effect on surface cover; however, no interaction between these factors was observed (table 2). *Lumbricus rubellus* reduced 31 % of cover in relation to the initial cover, which was significantly more than the reduction made by *A. longa*, which did not make the cover differ from the control without earthworms ($\alpha = 0.05$) (Fig. 1A). Straw inoculated with *Fusarium* remained more on surface, compared to the sterilized straw (Fig. 1B).

Table 2. Linear Mixed Model analysis (LMM) of final soil-cover percentage with straw relative to the initial cover, considered as 100%, in Exp.1: after 6 weeks incubation under dry conditions and no extra feed, under influence of the factors earthworm species (*A. longa* or *L. rubellus*) and straw condition (with or without *Fusarium*); and Exp. 2: after 5 weeks incubation in optimal conditions of moisture and food, under influence of the factors earthworm species (*A. longa, L rubellus* or *L. terrestris*) and straw condition (with soil microorganisms, with or without *Fusarium*). Significant p-values at p< 0.05 are in bold.

	p-values	
Factor	**Exp. 1**	**Exp. 2**
Earthworm species	**<0.0001**	**<0.0001**
Straw condition	**<0.0001**	0.1116
Earthworm species * Straw condition	0.6619	0.4450

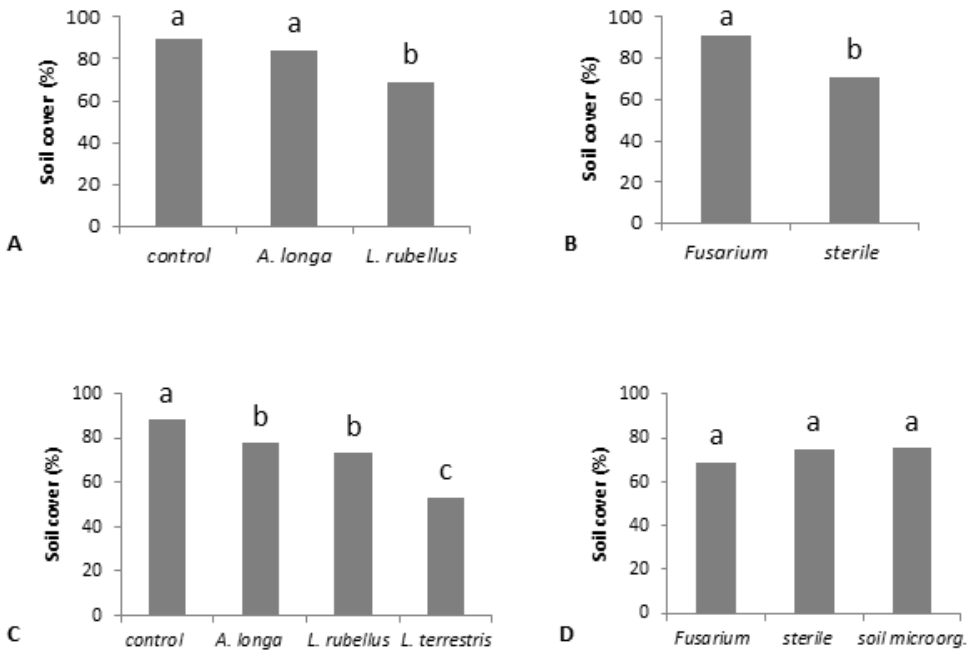

Fig. 1. Final soil-cover percentage with straw relative to the initial cover (100%), for Experiment 1, after 6 weeks' incubation under dry conditions and no extra feed, according to the factors earthworm species (A) and straw condition (B); and for Experiment 2, after 5 weeks' incubation in optimal conditions of moisture and food, according to the factors earthworm species (C) and straw condition (D). Different letters over each bar represent significant differences (Fisher's test with Sidak's correction; $p < 0.05$).

Quantification of F. graminearum *in straw, soil and casts*

The standard curve of the qPCR method used in this work revealed a strong linear relationship (R^2 = 0.998) between the logarithm of the amount of *Fusarium graminearum* DNA in each reaction and the corresponding CT values (CT stands for Cycle Threshold, which is the number of cycles required for the fluorescent signal to exceed the background level, *i.e.* to cross the threshold). Linearity was observed over the whole assayed range (42 ng and 4.2 pg of DNA per reaction), which constituted

the dynamic range of the method. The slope of the standard curve was -3.484, indicating an amplification efficiency of 94 %. The quantification limit was established in 4.2 pg, which corresponded to a CT of 33.7. Control samples not inoculated with *F. graminearum* exhibited in all cases CT values higher than 35, hence *F. graminearum* DNA content in these samples was lower than the quantification limit. Initial *Fusarium* DNA content measured with qPCR in inoculated straw was 201 ng/g wheat straw, while the sterile control presented no detectable amounts of the fungal DNA. At the end of the experiment, none of the treatments showed traces of *Fusarium* in the buried straw, casts or soil at 5 cm depth. The presence of both earthworm species caused the reduction of *F. graminearum* inoculum present on straw on the soil surface (Fig. 2 A).

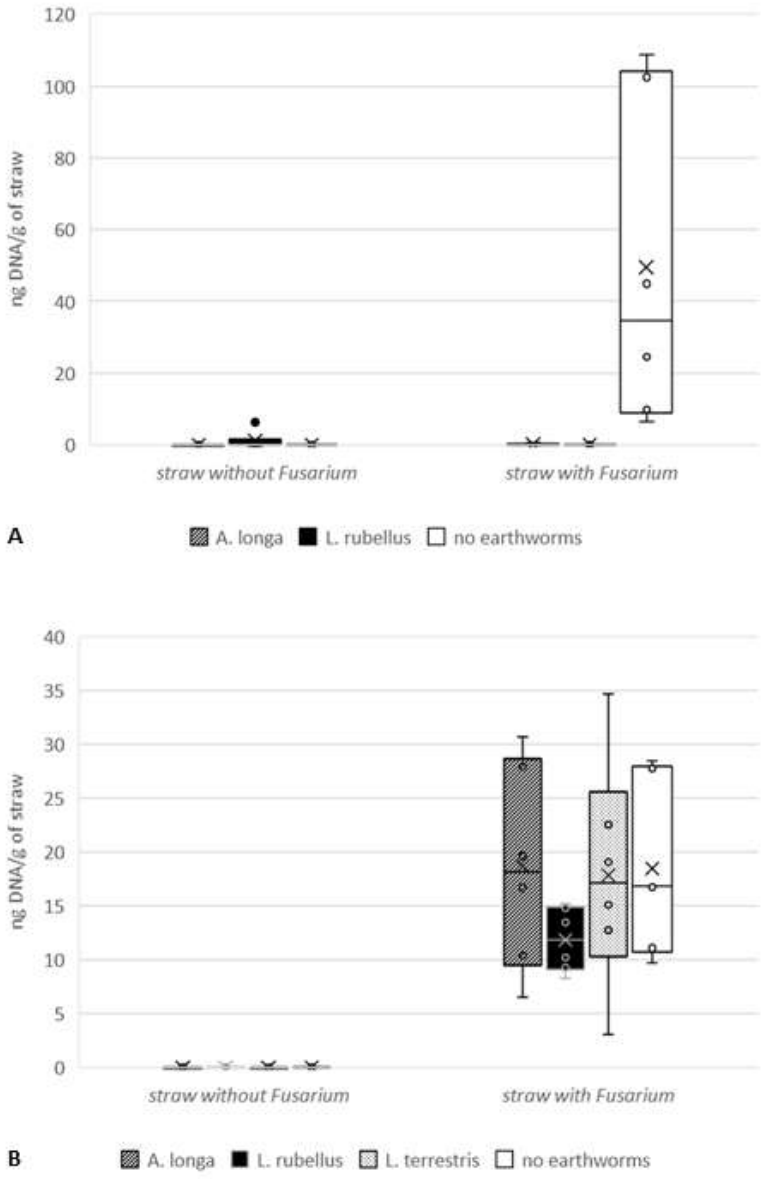

Fig.2. Final *Fusarium* DNA in wheat straw on the soil surface in (A) Experiment 1, after 6 weeks' incubation under dry conditions and no extra feed; and (B) Experiment 2, after 5 weeks' incubation in optimal conditions of moisture and food, with and without earthworms. The lower and upper quartile values are represented as a box, mean value is represented as a cross, median value as a horizontal line within the box, and the extreme values as whiskers.

Earthworm survival, growth, and reproduction

All *A. longa* earthworms survived and were mostly found in facultative diapause (Moreno and Borges, 2004) while *L. rubellus* remained active and their average survivorship was 83 % and 72% in treatments with and without *F. graminearum*, respectively. Both species decreased in weight during the 6 weeks of incubation, irrespective of the presence or not of *F. graminearum* ($p=0.1923$). *L. rubellus* was the species with a significantly ($p < 0.0001$) more marked decrease in weight (table 3). *Aporrectodea longa* produced no cocoons, while *L. rubellus* laid a mean of 2.9 cocoons per earthworm during the 6 weeks, not showing significant differences according to straw condition ($p=0.47$). The soil water content decreased from 25% to 20% on wet weight basis.

Table 3. Experiment 1. Mean individual earthworm biomass (across the different straw treatments) before and after 6 weeks' incubation under dry conditions and no extra feed. Different letters in each plot represent significant differences (Fisher's test with Sidak's correction; $p<0.05$).

Earthworm species	Initial weight (g)	Final weight (g)	Delta weight (%)	Std Dev	n
A. longa	2,24	1,95	-12.75 **a**	7,05	12
L. rubellus	1,29	0,69	-45..6 **b**	13.54	12

4.2.3.2.Experiment 2 – Optimal conditions

Wheat straw on surface

Results from the linear mixed model analysis performed on data of Experiment 2 only showed a significant effect of earthworm species on surface cover reduction, whereas the straw condition factor and the interaction between both factors were not significant (Table 2). All earthworm species had a significant effect on the reduction of straw on surface when compared to the control ($\alpha = 0.05$), where *L. terrestris* reduced soil cover by almost a half (Figs.1 C and 2 D). Percentage of soil cover was not affected by the straw condition (Fig. 1 D).

Quantification of F. graminearum *in straw, soil and casts*

The initial Fusarium DNA content measured with qPCR was 36 ng/g of wheat straw for inoculated straw, and not detectable for the treatment inoculated with soil microorganisms and the sterile control. At the end of the experiment no significant differences were observed in the Fusarium DNA content in surface straw, when comparing the different treatments inoculated with *Fusarium,* with or without earthworms ($\alpha = 0.05$). No detectable Fusarium DNA was found in casts.

Earthworm survival, growth, and reproduction

Earthworms had a high survival rate of 100 %, 94 % and 92 % for *A. longa, L. rubellus* and *L. terrestris,* respectively, in treatments without *Fusarium,* while all the three species had a 100 % of survival in the treatments with *Fusarium*. Mean biomass increased during the five weeks of the experiment, showing significant differences according to earthworm species ($p < 0.0001$) but not according to straw treatments (sterile or inoculated with *Fusarium* or soil microorganisms) ($p = 0.1577$) (table 4). *Lumbricus terrestris* in presence of soil microorganisms-inoculated straw presented a high variability in biomass due to a heterogeneous response to the treatment by the different replicates (while two replicates showed more than 30% increase in weight, the other four showed decrease in the range 6 % - 25 %). *Aporrectodea longa, L. terrestris,* and *L. rubellus* had a final mean weight of 3.54 g,

4.87 g, and 1.39 g; and laid a mean of 0.1, 1.8, and 11.1 cocoons per earthworm, respectively, showing significant differences among species ($p<0.0001$), but were not affected by straw condition ($p=0.9170$).

Table 4. Experiment 2. Mean individual earthworm biomass (across the different straw treatments) after 5 weeks' incubation in optimal conditions of moisture and food. Different letters in each plot represent significant differences ($p<0.05$).

Earthworm species	Initial weight (g)	Final weight (g)	Delta weight (%)		Std Dev	n
L. terrestris	3.86	4.87	+25.88	**a**	25.25	18
A. longa	2.11	3.54	+75.58	**b**	22.09	18
L. rubellus	1.01	1.39	+39.13	**a**	27.48	18

4.2.4. Discussion

4.2.4.1. Earthworm response to suboptimal and optimal conditions

Under sub-optimal conditions the epigeic earthworm tested *(L. rubellus)* did not cease its activity, but presented an increased mortality and a strong decrease in weight. However, the anecic earthworm (*A. longa)* reduced its activity by entering in facultative diapause, thus avoided mortality and showed a lower decrease in weight. Experiments held for longer periods with anecics (11 weeks) report reduction in biomass, most probably due to shortage of food (Oldenburg at al., 2008; Schrader et al., 2009). Similar to these studies, soil had been cleared from all vegetable residues and no additional food source was included. Food shortage may have made wheat straw with a very high C:N ratio, and the microbiota developed on it, main source of food available for earthworms. The high temperature could also have been a factor that depressed the earthworms, since temperature rose above 20^oC, when 15^oC is

recommended (Lowe and Butt, 2005). Besides, water content at the end of the experiment was 5% below the optimum 25% and the value of pH was 7.7, slightly above the optimum range (Baker and Whitby, 2003; cited byLowe and Butt, 2005).

Under optimal conditions (soil enriched with cow manure, moisture above 25 %, temperature below 20^{o}C), earthworms increased in survival rate, gained weight and had higher cocoon production. Of the two anecics tested *A. longa* had a 100% of survival rate, with only one individual found in facultative diapause, while and *L. terrestris* presented a similar survival rate to the epigeic *L. rubellus*, which was higher than that presented in suboptimal conditions. Individual fresh weight was within the reported ranges: 1.82- 4.51 g for *A. longa* (Lagerlöf et al., 2015); 0.4 – 1.3 g for *L. rubellus* (Ma, cited in Klok and de Roos, 1996) and for *L. terrestris* 4.07 - 5.40 g (Schrader et al., 2009) or 4 to 7 g (Butt et al., 1994). Weekly cocoon production of *A. longa*, 0.01 cocoons earthworm^{-1} week^{-1}, was below previously reported for this species: 0.03-0.73 cocoons earthworm^{-1} week^{-1} in lab conditions at similar temperatures (Lagerlöf et al., 2015), or 0.54-1.05 cocoons earthworm^{-1} week^{-1} in field conditions (Holmstrup, 1999). *Lumbricus rubellus* also produced less cocoons per week (2.22) compared to the reported range: 3 - 3.3 cocoons earthworm^{-1} week^{-1}, although this report was under lower temperature, 15^{o}C (Ma and Bonten, 2011). *Lumbricus terrestris* produced more cocoons (0.36) than the reported mean for similar temperatures; 0.20 cocoons earthworm^{-1} week^{-1} (20$\pm$2^{o}C) (Butt, 1991). Interestingly our experiments were held in the northern hemisphere summer, when Butt (1991) described a seasonal decrease in cocoon production, even in lab cultures set at constant temperature conditions and in darkness.

Studying the response of earthworms under sub-optimal conditions of humidity and food availability, might be a closer reflection of field conditions in soils without organic amendments under periods with scarce precipitation. These variables are important to include in laboratory studies, particularly in the case of *F. graminearum*, since it is able to survive in a wide range of environmental conditions (Burgess and Griffin, 1968; Ramirez et al., 2006).

4.2.4.2.Soil cover reduction

Surface straw was significantly reduced by the presence of both epigeic and anecic earthworms under optimal conditions, while the epigeic earthworm tested *(L. rubellus)* also reduced surface straw significantly under sub-optimal conditions. *A. longa* did not have an effect on soil cover under sub-optimal conditions, probably because it entered into facultative diapause. Similarly, in a study conducted by Wolfarth et al. (2011a; 2011b), the two tested earthworm species used (*L. terrestris and A. caliginosa*) also had contrasting results regarding surface straw removal depending on the species and ecological group.

Hence, our first hypothesis was true for the epigeic species in sub-optimal conditions of food and moisture, and for both the epigeic and anecic species tested in optimal conditions, although *L. terrestris* had a more marked effect on the reduction of surface wheat straw than the other two species. The first earthworm described and named by Carl Linnaeus in 1758, *Lumbricus terrestris*, is also the first earthworm to be reported as contributing to biological control in orchards, removing sources of pathogenic fungi by its litter-burying activity (Niklas and Kennel, 1981; Kennel 1990; Laing et al., 1986), and has since then been studied for several other pathogens (Jorge-Escudero et al. *in press*).

A significantly lower reduction of surface straw cover occurred in treatments inoculated with *F. graminearum* compared to those with sterile straw in sub-optimal conditions, regardless of the presence of earthworms. Possibly, *F. graminearum*, which is able to grow in a wide range of humidity conditions, outcompeted decomposing microflora in the dry conditions (Burgess and Griffin, 1968; Ramirez et al., 2006), since this effect was not observed under optimal conditions of humidity, which is in concordance with results obtained by Wolfarth et al. (2011a; 2011b), considering their experiment did not present water as limiting factor.

4.2.4.3.Fusarium graminearum inoculum reduction

Although the presence of *F. graminearum* significantly braked surface straw reduction under sub-optimal conditions, this environment did not impede epigeic or anecic earthworms from reducing the amount of *F. graminearum* on straw to undetectable levels. This coincides with the reported effect of the anecic *L. terrestris* on *F. culmorum* on wheat straw (Oldenburg et al., 2008; Wolfarth et al., 2011a). However, earthworms had no significant effect on *F. graminearum* under optimal conditions. Probably, the presence of an alternative and more attractive food source already within the soil in the optimal conditions may have hindered earthworms from direct feeding on the inoculum of *F. graminearum*. This was so despite that the level of activity of the earthworms proved to be lower in sub-optimal than in optimal conditions, and the initial level of infestation in the first case was far above that of the second case. In experiments that report a positive effect of the anecic *L. terrestris* on *Fusarium culmorum* reduction on straw remaining on surface, no extra food source was offered to the earthworms, and organic plant residues were deliberately removed from the soil, which had rather low organic matter content (2.1 %; Oldenburg at al., 2008; Wolfarth et al., 2011a).

Hence, our second hypothesis was confirmed for both epigeic and anecic earthworms in sub-optimal conditions, where lack of food may have forced earthworms to feed on *F. graminearum*. The cleaner aspect of inoculated straw where earthworm activity was evident due to straw mobility (personal observation) indicated that earthworms may have browsed the straw feeding from the fungi, which, together with protozoa, make the main supplies of their diet (Brown, 1995). Moreover, *Fusarium* species, as early successional fungi, have been shown to be preferred by earthworms over fungi of later stages of succession, often belonging to Basidiomycota (Bonkowski et al., 2000).

The absence of *F. graminearum* in buried straw, soil and casts reinforces the ideas that: (1) burial of *Fusarium* infested straw reduces the level of infestation due to a greater microbial activity in soil (Pereyra et al., 2004); hence, anecic earthworms (*i.e.*

litter-burying species) could be contributing to *Fusarium* reduction by their burying behaviour (Oldenburg et al., 2008; Wolfarth et al., 2011a); (2) anecic earthworms' ingestion of *F. graminearum* infested straw or *Fusarium* colonization seems to contribute to degradation of the straw, as stated by Oldenburg et al. (2008) and Wolfarth et al. (2011a). Probably, *F. graminearum* spores lose viability after passing through the gut, as shown for spores of a related species, *F. lateritium*, with *L. terrestris* and *A. longa* (Moody et al., 1996), although this is not a rule for all types of fungal spores (Brown and Doube, 2004).

According to the results of the present study, *Fusarium* reduction ability through straw burial or ingestion, already proved for anecic species, can also be extended to epigeic species, *i.e.* a surface dwelling and litter-fragmenting earthworm, as *L. rubellus*. Earthworm functional plasticity according to site has been evidenced with isotopic analysis (Neilson et al., 2000). Particularly, Eisenhauer et al. (2008) also found anecic behaviour in *L. rubellus*.

4.2.4.4.Fusarium graminearum effect on earthworms

The presence of *F. graminearum* on wheat straw had no effect on earthworm survival, growth or reproduction, regardless of earthworm species or soil conditions. This agrees with previous reported results for *A. caliginosa* and *L. terrestris* with *F. culmorum*, another fungal pathogen causing FHB in wheat (Oldenburg et al., 2008; Schrader et al., 2009; Wolfarth et al., 2011a). Negative effects of *Fusarium* on earthworm survival, over a similar period of time, has been reported for the species *Eisenia fetida* in the case of incubation on pure culture of *Fusarium* sp. (species not reported, Edwards and Fletcher, 1988).

However, feeding behaviour varies among earthworm species, according to the earthworms' ecological group (Brown and Doube, 2004). *Lumbricus terrestris* has been reported to prefer straw infested with *F. culmorum*, rather than non-infested straw (Wolfarth et al., 2011a); also to prefer agar disks inoculated with *F.*

oxysporum, rather than agar disks with other fungi (Cooke, cited in Edwards and Fletcher, 1988); and to feed on *F. nivale* cultures, rather than other eight fungal species (Bonkowski et al., 2000). Besides, Schrader et al. (2009) found that, although DON (a mycotoxin produced by *F. culmorum* and *F. graminearum)* was assimilated by the earthworms and incorporated to their tissue, it was not accumulated over time.

Earthworm preference for straw with *F. graminearum* compared to sterile or inoculated with soil microorganisms could not be proved in the present study, since a similar effect was observed regardless straw treatment. Probably the sterile straw was rapidly colonized by a rich variety of soil microflora when put on the soil surface (observed as mycelium development on surface), which made it just as attractive as the previously inoculated straw.

4.2.4.5.Soil microorganism control

The soil microorganism control was included in Experiment 2 (optimal conditions) to verify if the two weeks of microorganism incubation, with *Fusarium* or soil microorganisms, presented any advantage or disadvantage in the palatability of the straw. Although sterilized straw was most probably rapidly colonized by soil microorganisms when the straw was laid on the soil at the experiment set up, these two weeks of prior inoculation could have been a differing factor. However, no significant effect was found in Experiment 2 for the straw type factor on final soil-cover percentage with straw, or on earthworm weight. This means that two weeks of prior inoculation did not mean any advantage in terms of preference of straw for the earthworms, which confirmed that the sterilized control used in Experiment 1 (sub-optimal conditions) was robust enough.

4.2.4.6. How to optimize the effect of earthworms on F. graminearum with soil management

As mentioned in the introduction, superficial placement of crop residues due to reduced tillage or no-till, stimulates plant pathogens (*F. graminearum*, among others), resulting in an increase in crop diseases caused by residue-borne pathogens (Arvidsson, 1998).

Although rotary cultivation and reduced tillage cause substantial earthworm mortality (Boström, 1995), earthworm abundance is in most cases larger in agricultural soil under reduced soil cultivation as compared to soil under moldboard ploughing (Lagerlöf et al. 2012). Earthworms of different ecological groups can therefore, according to our results contribute to the reduction of the amount of superficial straw by consumption and burial of fungal colonized straw. The nutritional value of straw material for earthworms increases when the straw is fragmented into smaller pieces (Boström and Lofs-Holmin, 1986). This is due to faster colonization by microorganisms and easier handling and ingestion of the straw by earthworms (Boström and Lofs-Holmin, 1986). Amount of input of crop residues is important for maintaining a dense population of earthworms. It has been proved that 1 g of dry matter of crop residue could result on 0.01-0.12 g of earthworm biomass (Andersen, cited in Boström and Lofs-Holmin, 1986).

In no-till agriculture, crop residues that can be infested by *F. graminearum* will inevitably remain on the soil surface if not taken away. In such systems, *L. rubellus* and other epigeic earthworm species can be abundant (Lagerlöf, et al., 2012). Weather fluctuations could eventually hinder earthworm effect on the reduction of *Fusarium* due to a decrease in earthworm activity during sporadic periods of low soil water content. In these situations, according to our results, *L. rubellus* will probably maintain more active on surface than *A. longa*, achieving a significant effect on *Fusarium* inoculum reduction despite its high mortality. Therefore, according to our results, earthworms could play a significant role reducing residue-borne pathogens

such as *F. graminearum*, in conservational systems where earthworms are favoured and pathogen inoculum management is limited.

Thus, earthworms become a novel alternative to be included in an integrated pest management, focused on conservational biological control, contributing to additional strategies by reducing the inoculum density present on the straw.

4.2.5. Conclusions

This study represents the first work investigating the contribution of earthworm to the biological control of *F. graminearum* under contrasting environmental conditions and gives important information for applied understanding of this novel approach. Epigeic *(L. rubellus)* and anecic earthworms (*L. terrestris* and *A. longa),* whose growth or activity were unaffected by *Fusarium graminearum* including its toxins, contributed to the reduction of *F. graminearum* in crop residues by decreasing the amount of wheat straw on surface in optimal moisture and feed conditions, hence reducing the substrate for the pathogen survival. The epigeic earthworm also showed the ability to reduce wheat straw on the soil surface when such conditions were sub-optimal. Besides, *L. rubellus*, and *A. longa* reduced the inoculum of the pathogen in straw, most probably through consumption of the fungus, when no other food source was offered. Therefore, we conclude that anecic and epigeic earthworms have potential to contribute to reduction of *F. graminearum* inoculum in wheat straw, both in optimal and sub-optimal conditions. Their mechanisms of action, or the magnitude of this contribution will depend on the earthworm species and their response to environmental conditions. Earthworm effect on *F. graminearum* can be further maximized with environmental friendly and sustainable soil management, which favours earthworm populations, as well as practical hints like chopping crop residue to facilitate earthworm to feed on them and other saprophytic fungi to colonize them and outcompete *F. graminearum* and other plant pathogens.

Acknowledgments

Many thanks to:

Paula Persson for providing the *Fusarium* strain.

Helena Bötker, Jamlek Muturi John, Fredrick Ayuke, Nyashadzashe Marecha and Boitshepho Chube for lab assistance.

Anders Larsolle, for developing a special procedure on MATLAB for the surface straw cover analysis.

Claudia von Brömssen, Jorge Franco and Andrés Beretta for statistical advice.

ANII, Uruguay, for sponsoring PhD scholarship.

The foundation Stiftelsen Olle Engkvist Byggmästare, Sweden, and CSIC-UdelaR, Uruguay for financial support.

4.2.6. References

Arvidsson, J., 1998. Effects of cultivation depth in reduced tillage on soil physical properties, crop yield and plant pathogens. Eur. J. Agron. 9, 79–85.

Bertrand, M., Blouin, M., Barot, S., Charlier, A., Marchand, D., Roger-Estrade, J., 2015. Biocontrol of eyespot disease on two winter wheat cultivars by an anecic earthworm (*Lumbricus terrestris*). App. Soil Ecol. 96, 33-41.

Blakemore, R.J., 2002. Cosmopolitan Earthworms – an Eco-Taxonomic Guide to the Peregrine Species of the World, first CD ed. VermEcology, PO BOX 414 Kippax, ACT 2615, Australia.

Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussaard, L., Butt, K.R., Dai, J., Dendooven, L., Peres, G., Tondoh, J.E., Cluzeau, D., Brun, J.-J., 2013. A review of earthworm impact on soil function and ecosystem services. Eur. J. Soil Sci. 62(4), 161–182.

Bonkowski, S., Griffiths, B.S., Ritz, K., 2000. Food preference of earthworms for soil fungi. Pedobiol, 44, 666–676.

Bouché, M.B., 1977. Strategies lombriciennes, in: Lohm, U., Persson, T. (Eds.), Soil organisms as components of ecosystems. Proc. 6th Int. Coll. Soil Zool. Ecol. Bull. 25, Stockholm, pp. 157-163.

Boström, U., 1995. Earthworm populations (Lumbricidae) in ploughed and undisturbed leys. Soil Till. Res. 35, 125-133.

Boström, U., Lofs-Holmin, A., 1986. Growth of earthworms (*Allolobophora caliginosa*) fed shoots and roots of barley, meadow fescue and lucerne studied in relation to particle size, protein, crude fiber content and toxicity. Pedobiol. 29, 1-12.

Brown, G.G., 1995. How do earthworms affect microfloral and faunal community diversity? Plant Soil. 170(1), 209–231.

Brown, G.G., Doube, B.M., 2004. Functional Interactions between Earthworms, Microorganisms, Organic Matter, and Plants, in: Edwards C.A. (Ed.), Earthworm ecology (2nd ed). Boca Raton: CRC Press, London, pp. 213–239.

Brown, G.G., Edwards, C.A., Brussaard, L., 2004. How earthworms affect plant growth: Burrowing into the mechanisms, in: Edwards C.A. (Ed.), Earthworm ecology (2nd ed). Boca Raton: CRC Press, London, pp.19–49.

Burgess, L.W., Griffin, D.M., 1968. The recovery of *Gibberella zeae* from wheat straws. Aust. J. Exp. Agric. Anim. 8, 364-370.

Butt, K.R., 1991. The effects of temperature on the intensive production of *Lumbircus terrestris* (Oligochaeta: Lumbricidae). Pedobiol. 35, 257-264.

Butt, K.R., Frederickson, J., Morris, R.M., 1994. The life cycle of the earthworm *Lumbircus terrestris* L. (Oligochaeta: Lumbricidae) in laboratory culture. Eur. J. Soil Biol. 30, 49-54.

Byzov, B.A., Khomyakov, N.V., Kharin, S.A., Kurakov, A.V., 2007. Fate of soil bacteria and fungi in the gut of earthworms. Eur. J. Soil Biol. 43, 149-156.

Del ponte, E.M., Valent, B., Bergstrom, G.C., 2017. A special issue on Fusarium head blight and wheat blast. Trop. plant pathol. 42,143–145.

Doube, BM., Stephens, PM., Davoren, C.W., Ryder, M.H., 1994. Interactions between earthworms, beneficial soil microorganisms and root pathogens. App. Soil Ecol. 1, 3-10.

Edwards, C.A., Fletcher, K.E., 1988. Interactions between Earthworms and Microorganisms in Organic-matter Breakdown. Agric. Ecosyst. Environ. 24, 235-247.

Eilenberg, J., Hajek, A., Lomer, C., 2001. Suggestions for unifying the terminology in biological control. BioContr. 46, 387-400.

Eisenhauer, N., Marhan, S., Scheu, S., 2008. Assessment of anecic behavior in selected earthworm species: Effects on wheat seed burial, seedling establishment, wheat growth and litter incorporation. App. Soil Ecol. 38, 79-82.

Elmer, W.H., 2009. Influence of earthworm activity on soil microbes and soilborne diseases of vegetables. Plant Dis. 93(2), 175–179.

Fragoso, C., Brown, G.G., 2007. Ecología y taxonomía de las lombrices de tierra en Latinoamérica: El primer Encuentro Latino-Americano de Ecología y Taxonomía de Oligoquetos (ELAETAO1), in: Brown, G.G., Fragoso, C. (eds.), Minhocas na América Latina: Biodiversidade e Ecologia. EMBRAPA Soja, Londrina, pp. 34-75.

Friberg, H., Lagerlöf, J. Hedlund, K., Rämert, B., 2008. Effect of earthworms and incorporation of grass on *Plasmodiophora brassicae*. Pedobiol. 52, 29-39.

Friberg, H., Lagerlöf, J., Rämert, B., 2005. Influence of soil fauna on fungal plant pathogens in agricultural and horticultural systems. Biocontrol Sci. Technol. 15(7), 641-658.

Holmstrup, M., 1999. Cocoon production of *Aporrectodea longa* Ude and *Aporrectodea rosea* Savigny (Oligochaeta; Lumbricidae) in a Danish grass field. Soil Biol. Biochem. 31, 957-964.

Hornby, D., 1975. Inoculum of the take-all fungus: nature, measurement, distribution and survival. EPPO Bull., 5, 319-333.

Hume, E.A., Horrocks, A.J., Fraser, P.M., Curtin, D., Meenken, E.D., Chng, S., Beare, M.H., 2015. Alleviation of takeall in wheat by the earthworm *Aporrectodea caliginosa* (Savigny). App. Soil Ecol. 90, 18–25.

Jorge-Escudero, G., Lagerlöf, J., Pérez, C.A., 2018. Control biológico de enfermedades y plagas promovido por lombrices. Revista Brasileria de Agroecologia. 13 (4), *in press*. ISSN: 1980-9735.

Kennel, W., 1990. The role of the earthworm *Lumbricus terrestris* in integrated fruit production, Acta Horticulturae. 285, 149-156.

Kirchmann, H., Persson, J., Carlgren, K., 1994. The Ultuna long-term soil organic matter experiments, 1956-1991. Reports and Diss, Dept. of Soil Sc. SLU, Uppsala.

Klok, C., de Roos, A. M., 1996. Population level consequences of toxicological influences on individual growth and reproduction in *Lumbricus rubellus* (Lumbricidae, Oligochaeta). Ecotoxicol. Environ. Saf. 33, 118–127.

Lagerlöf, J., Ayuke, F., Bejai, S., Jorge, G., Lagerqvist, E., Meijer, J., Muturi John, J., 2015. Potential side effects of biocontrol and plant-growth promoting *Bacillus amyloliquefaciens* bacteria on earthworms. App. Soil Ecol. 96, 159 - 164.

Lagerlöf, J., Pålsson, O., Arvidsson, J., 2012. Earthworms influenced by reduced tillage, conventional tillage and energy forest in Swedish agricultural field experiments. Acta Agric.Scand. Sect. B Soil Plant Sci. 62, 235-244.

Laing, J.E., Heraty, J.M., Corrigan, J.E., 1986. Leaf burial by the earthworm, *Lumbricus terrestris* (Oligochaeta: Lumbricidae), as a major factor in the population dynamics of *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) and its parasites, Environ. Entomol. 15, 321-326.

Lavelle, P. 1988. Earthworms and the soil system, Biol. Fertil. Soils, 6:237.251.

Leplat, J., Friberg, H., Abid, M., Steinberg, C., 2013. Survival of *Fusarium graminearum*, the causal agent of Fusarium head blight. A review. Agron. Sustain. Dev. 33, 97–111.

Lindblad, M., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., Fredlund, E., 2013. Deoxynivalenol and other selected Fusarium toxins in Swedish wheat - Occurrence and correlation to specific Fusarium species. Int. J. Food Microbiol. 167, 284–291.

Lowe, C.N., Butt, K.R., 2005. Culture techniques for soil dwelling earthworms: A review. Pedobiol. 49, 401-413.

Ma, W.C., Bonten, L.T.C., 2011. Bioavailability pathways underlyingzinc-induced avoidance behavior and reproduction toxicity in *Lumbricus rubellus* earthworms. Ecotoxicol. Environ. Saf. 74, 1721–1726.

McMullen, M., Bergstrom, G., De Wolf, E., Dill-Macky, R., Hershman, D., Shaner, G., Van Sanford, D., 2012. A unified effort to fight an enemy of wheat and barley: *Fusarium* head blight. Plant Dis. 96, 1712-1728.

Meghvansi, M. K., Singh, L., Srivastava, R. B., Varma, A., 2011. Assessing the Role of Earthworms in Biocontrol of Soil-Borne Plant Fungal Diseases, in: Ayten Karaca (Ed.), Biology of Earthworms, Soil Biol. Springer, New York, pp.173-190.

Moody, S.A., Briones, M.J., Piearce T.G., Dighton, J. 1995. Selective consumption of decomposing wheat straw by earthworms. Soil Biol. Biochem. 27(9), 1209-1213.

Moody, S., Piearce, T.G., Dighton, J., 1996. Fate of some fungal spores associated with wheat straw decomposition on passage through the guts of *Lumbricus terrestris* and *Aporrectodea longa*. Soil Biol. Biochem. 28(4/5), 533-537.

Moreno, A.G., Borges, S. (Eds.), 2004. Avances en taxonomía de lombrices de tierra / Advances in Earthworm Taxonomy (Annelida:Oligochaeta). Editorial Complutense, Madrid.

Nicolaisen, M., Suproniene, S., Nielsen, L.K., Lazzaro, I., Spliid, N.H., Justesen, A.F., 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. J. Microbiol. Meth. 76, 234-240.

Neilson, R., Boag, B., Smith, M., 2000. Earthworm $d^{13}C$ and $d^{15}N$ analyses suggest that putative functional classifications of earthworms are site-specific and may also indicate habitat diversity. Soil Biol. Biochem. 32, 1053-1061.

Nganje W.E., Bangsund, D.A., Leistritz, F.L., Wilson, W.W., Tiap, N.M., 2004. Regional Economic Impacts of Fusarium Head Blight in Wheat and Barley. Rev. of Agr. Econ. 26(3), 332-347.

Niklas, J., Kennel, W., 1981. The role of the earthworm, *Lumbricus terrestris* (L.) in removing sources of phytopathogenic fungi in orchards, Gartenbauwissenschaft, 46, 138-142.

Oldenburg, E., Kramer, S., Schrader, S., Weinert J., 2008. Impact of the earthworm *Lumbricus terrestris* on the degradation of *Fusarium*-infected and

deoxynivalenol-contaminated wheat straw. Soil Biol. Biochem. 40, 3049-3053.

Pereyra, S.A., Dill-Macky, R., Sims, A.L., 2004. Survival and inoculum production of *Gibberella zeae* in wheat residue. Plant Dis. 88, 724-730.

Puga-Freitas, R., Barot, S., Taconnat, L., Renou, J.P., Blouin, M., 2012. Signal molecules mediate the impact of the earthworm *Aporrectodea caliginosa* on growth, development and defence of the plant *Arabidopsis thaliana*. Plos One. 7 (12), 1-10.

Ramirez, M.L., Chulze, S., Magan, N., 2006. Temperature and water activity effects on growth and temporal deoxynivalenol production by two Argentinean strains of *Fusarium graminearum* on irradiated wheat grain. Int. J. Food Microbiol. 106(3), 291–296.

Rebrikov, D.V., Trofimov, D., 2006. Real-time PCR: approaches to data analysis (a review). Prikl. Biokhim. Mikrobiol, 42, 520–528.

Schrader, S., Kramer, S., Oldenburg E., Weinert J., 2009. Uptake of deoxynivalenol by earthworms from *Fusarium*-infected wheat straw. Mycotoxin Res. 25, 53-58.

Singh, R.P., Singh, P.K., Rutkoski, J., Hodson, D.P., He, X., Jørgensen, L.N., Hovmøller, M.S., Huerta-Espino, J., 2016. Disease Impact on Wheat Yield Potential and Prospects of Genetic Control. Ann. Rev. Phytopathol. 54, 303-322.

Stephens, P.M., Davoren, C.W., 1995. Effect of the lumbricid earthworm *Aporrectodea trapezoides* on wheat grain yield in the field, presence or absence of *Rhizoctonia solani and Gaumannomyces graminis var tritici.* Soil Biol. Biochem. 33(11), 1531–1538.

Stephens, P.M., Davoren, C.W., 1997. Influence of the earthworm *Aporrectodea trapezoides* and *A. rosea* on the disease severity of *Rhizoctonia solani* on subterranean clover and ryegrass. Soil Biol. Biochem. 29, 511-516.

Sulkava, P., Huhta, V., 2003. Effects of hard frost and freeze-thaw cycles on decomposer communities and N mineralization in boreal forest soil. App. Soil Ecol. 22, 225-239.

Whalen JK, Parmelee RW. 1999. Quantifcation of nitrogen assimilation effciencies and their use to estimate organic matter consumption by the earthworms *Aporrectodea tuberculata* (Eisen) and *Lumbricus terrestris* L. App. Soil Ecol. 13, 199-208.

Wolfarth, F., Schrader, S., Oldenburg, E., Weinert, J., Bruttone, J., 2011a. Earthworms promote the reduction of *Fusarium* biomass and deoxynivalenol content in wheat straw under field conditions. Soil Biol. Biochem. 43, 1858-1865.

Wolfarth, F., Schrader, S., Oldenburg, E., Weinert, J., 2011b. Contribution of the endogeic earthworm species *Aporrectodea caliginosa* to the degradation of deoxynivalenol and *Fusarium* biomass in wheat straw. Mycotoxin Res. 27, 215–220.

Wurst, S.; Allema, B.; Duyts, H.; Van Der Putten, W.H. 2008. Earthworms counterbalance the negative effect of microorganisms on plant diversity and enhance the tolerance of grasses to nematodes. Oikos, 117, 711–718.

Yuen, G.Y., Schoneweis, S.D., 2007. Strategies for managing *Fusarium* head blight and deoxynivalenol accumulation in wheat. Int. J. Food Microbiol., 119, 126-130.

Websites

DriloBASE Taxo. http://taxo.drilobase.org (accessed 10 June 2018).

4.3. CONTRIBUTION OF NATIVE AND EXOTIC EARTHWORM SPECIES TO THE INOCULUM REDUCTION OF FUSARIUM IN WHEAT STRAW IN URUGUAY

Abstract

Earthworms can enhance plant health directly or indirectly, mediated by soil, microorganisms or plant immune system. Particularly, three Lumbricid earthworm species have shown to significantly reduce *Fusarium* spp. on wheat straw, responsible for one of the main diseases that affect wheat production and cereals in general, Fusarium Head Blight. To elucidate whether any Uruguayan earthworm species could be fulfilling a comparable role, we selected two species to test in a multifactorial experiment. The selected species were: *Lumbricus friendi*, anecic, exotic, similar to *L. terrestris*, but smaller; and *Glossoscolex rione*, endogeic, native, of similar size to *Aporrectodea caliginosa.* These species were incubated in soil microcosms covered with *Fusarium* inoculated –or sterile- straw for six weeks at $20^{o}\pm2$. The notorious actividy of *L. friendi* on the surface, reduced more than 30% of the straw on soil surface, despite high mortality during the experiment. We concluded that *L. friendi* showed potential to contribute to the biological control of *F. graminearum* in the wheat straw. However, the treatment with *G. rione* did not differ from the control, probably due to the fact that this earthworm seldom comes up to the soil surface. Being this the first reported experiment with *G. rione* its high survival was recorded as a success. The earthworms were not negatively affected by *Fusarium*, or its toxins. Because the quantities of the pathogen DNA were below the detection levels in the present study, a repetition of the experiment with highly infected residue will be necessary to confirm the reduction of the *Fusarium* inoculum in the straw.

Keywords:

Glossoscolex rione; Lumbricus friendi; Fusarium Head Blight; Conservation biological control.

4.3.1. Introduction

Among the various ecosystem services provided by life on earth and biodiversity, earthworms have mostly been associated to those belonging to the supporting category due to their influence on nutrient cycling, soil formation and structure, and indirectly on primary production by enhancing plant growth. However, with their activity, earthworms also provide ecosystems services grouped in the regulating category, as climate regulation (by C sequestration), water regulation and purification (by enhancing infiltration and pollution remediation), and plant disease regulation (Blouin et al., 2013; Lavelle, 2006; Reid et al., 2005; Brown, Edwards & Brussaard, 2004). The earthworm effect on plant health, was first discovered in the 40's, but only recently has research in this matter gained strength and developed, elucidating direct and indirect mechanisms of action (Jorge-Escudero, Lagerlöf & Pérez, *in press*).

Earthworms can enhance plant health indirectly through their positive effects on soil physical, chemical, and biological properties, creating a healthier environment for plant growth, i.e. soil with more pores, nutrients and plant growth promoting bacteria, hence, making plants less vulnerable to hazards (de la Peña, 2009; Friberg, Lagerlöf & Rämert, 2005; Brown, Edwards & Brussaard, 2004). Besides, it has been proved that earthworms induce differential expression of certain genes related to stress response (Jana et al., 2010; Blouin et al., 2005) and that in their presence AAI-like compounds are released, although it is not clear yet if it is by themselves or by associated PGPR (Puga-Freitas et al., 2012; Canellas et al., 2011; Jana et al., 2010). Direct mechanisms include ingestion and digestion, with a preference for pathogenic rather than beneficial fungi (Bonkowski, Griffiths & Ritz, 2000), as well as chemical effects with antimicrobial molecules as part of earthworm immune system (Wang et al., 2007; Cho et al., 1998), and possibly a mechanical disturbing effect on root pathogens due to earthworm activity in rhizosphere. The mechanism by which

biological control occurs, and its efficiency are case-specific depending on the ecology of the pathogen, plant and earthworm species involved[5].

Most earthworms used in biological control studies belong to the Lumbricidae family, originally from Europe, but currently spread all over the world due to human migration and to the adaptation of certain species of this family to agricultural practices (James, 2011)[6]. Less is known about earthworms belonging to other families, although a few experiments have been conducted with Megascolecids and *Pontoscolex corethrurus* (Boyer et al., 2013; Lafont, et al., 2007; Blouin, et al., 2005; Boyer et al., 1999; Senapati, 1992).

Several species of the Lumbricidae family are widespread in South America, particularly associated to soil disturbance and agriculture (Fragoso & Brown, 2007), and in the south of Uruguay they dominate over the natives[7]. However, some native species of the Glossoscolecidae family have also shown certain adaptation to agriculture since they have been found in wheat rotations in western Uruguay, where natives still dominate in the oligochaeta community[8].

One of the main diseases that affect wheat production and cereals in general, is Fusarium Head Blight, caused by *Fusarium* species that survive saprophytically on wheat straw (e.g. *Fusarium graminearum, F. culmorum*) and represents a threat for the following crop (Umpiérrez et al., 2013). Recent studies have shown that *Lumbricus terrestris* can reduce *Fusarium culmorum* biomass in soil and in wheat straw inoculated with *Fusarium* (Wolfarth et al., 2011a; Oldenburg et al., 2008). However, the effect was not significant for the species *A. caliginosa* (Wolfarth et al. 2011b). The inoculum of *F. graminearum* was reduced by the earthworm species *L. rubellus* and *Aporrectodea longa* when no other food source (e.g. manure) was offered; *L. rubellus* also reduced *Fusarium* inoculum indirectly by reducing straw on

[5] For the purposes of this Thesis, see section 4.2.
[6] For the purposes of this Thesis, see section 4.2.
[7] For the purposes of this Thesis, see section 3.2
[8] For the purposes of this Thesis, see section 3.2

surface[9]. It was concluded that anecic earthworms may contribute to *Fusarium* reduction by their burying behaviour and by ingestion while being on the surface, and that the size of the earthworm may affect their effectiveness. These studies with *Fusarium* spp. mentioned above were conducted in Europe, and the species tested all belong to the Lumbricidae family.

To elucidate whether any Uruguayan earthworm species could be fulfilling a comparable role, we first considered the theoretical potentiality of the earthworms found in Uruguayan wheat cultures, so as to select candidates to be tested. Of the previously earthworms proved to have a significant effect on *Fusarium* reduction, only the anecic *L. terrestris* have been found in Uruguay. One of the earthworms selected for the present study was a similar but slightly smaller species, *L. friendi*, since it is anecic and has been found in southern Uruguay wheat farms. This species, as *L. terrestris*, is exotic[10].

Regarding native earthworm species that could affect *Fusarium* on straw, we encountered two aspects for consideration. Hitherto, no native anecics or epigeics have been found in Uruguay, and a direct effect of endogeics on surface straw is less probable (Wolfarth et al., 2011b). Besides, natives tend to be less abundant and smaller than exotics[11], so in the case there is an effect, this should be expected to be relative to size and density. However, it has been shown that earthworms may have indirect effects on plant health (Jorge-Escudero, Lagerlöf & Pérez, *in press*), and there are some native species that reach considerable size. For instance, *Glossoscolex rione*, found in wheat rotations has a body size similar to *A. caliginosa*, therefore this species was selected for experimentation. The following specific questions were addressed: (1) Does *L. friendi* and/or *G. rione* reduce *Fusarium* inoculum in wheat straw? (2) Do they reduce the straw cover on surface? We hypothesized that (1) The anecic *L. friendi* would have a significant effect reducing both infected straw on surface and *Fusarium* inoculum on remaining surface straw; (2) the effect of the

[9] For the purposes of this Thesis, see section 4.2.
[10] For the purposes of this Thesis, see section 3.2
[11] For the purposes of this Thesis, see section 3.2

endogeic *G. rione* would be minor or neutral in controlling *Fusarium* on wheat straw, as that proved for the endogeic *A. caliginosa*.

4.3.2. Materials and methods

4.3.2.1. Soil preparation

Loamy soil (5 % C; 0.4 % N) was obtained from the Agronomy Faculty Campus in Sayago, Montevideo, Uruguay. It was cleared from straw, roots debris, stones and macrofauna by hand (e.g. earthworms and beetles) and thereafter frozen for 48 h and thawed, twice, to reduce macro- and mesofauna, although nematodes, other microfauna and microorganisms may remain (Sulkava & Huhta, 2003).

4.3.2.2.Earthworm collection and laboratory adaptation

Earthworms were collected at Agronomy Faculty sites in Uruguay: the native species *G. rione* was collected from the Experiment Station Mario A. Cassinoni, Paysandú, while the exotic Lumbricidae species, *L. friendi*, was collected in Sayago Campus, Montevideo (fig. 1). Prior to the experiment, the earthworms were kept in three 20 L boxes with moist soil mixture of the same quality as was used in the experiments (see description of soil preparation above), at constant temperature (19 ± 1°C) for 1 to 8 weeks, and were fed with mixed vegetables slightly buried under the soil surface every fortnight. At the beginning of the experiments, the mean biomass (total fresh weight with gut content) of *G. rione* worms was 0.84 g and that of *L. friendi* was 1.02 g.

Fig. 1. Images of adult specimens of *Lumbricus friendi* (above) and *Glossoscolex rione* (below) used in the experiment

4.3.2.3. Straw preparation

Winter wheat straw (*Triticum aestivum*) cv. 'INIA Don Alberto' was collected in December 2015 from a field experiment in INIA La Estanzuela, which had been artificially inoculated *Fusarium graminearum* one month prior to bloom, with maize grain colonized by a pool of *Fusarium graminearum* strains from the INIA collection. The straw was air dried at 40 ºC for 48 h, and then cut in pieces of 2.5 cm long. The straw for the control treatment was gamma sterilized at 25 kGy at the Radiation Unit in LATU (Technological Laboratorium of Uruguay) with an Irradiation Equipment EMI-9, with a Cobalt-60 source and 23-litre cylindrical containers. Dose was measured with alanine-dosimeters, based on ISO/ASTM 51607:2013 using a Paramagnetic Resonance Spectrometer model Miniscope MS400 (Magnetech®, Argentina).

4.3.2.4. Experiment setup

The experiment had a completely randomized block design (RCBD). Six treatments with six repetitions were established according to table 1, where two earthworm species (*G. rione* and *L. friendi*) were tested with straw with and without *F. graminearum*, plus controls (without earthworms), totalizing 36 experimental units (fig. 2).

A total of 36 PVC cylinders (30 cm high and 16 cm diameter) were filled with 2 kg of the soil described above (moisture = 20 % w/w), and were later moistened to 25 % w/w water content.

Two adult earthworms were placed into each of the cylinders following table 1. Adulthood was determined by the presence of clitellum. In the case of *G. rione*, some sub-adults were included, *e.i.* individuals with the same body length and width as adults, but without developed clitellum.

Six grams of air dried wheat straw, cut into 2.5 cm-long pieces, with *Fusarium* or sterilized were evenly distributed on the soil surface, simulating straw soil coverage on field after harvest.

A plastic net (pore size 1 mm) was fixed to the bottom of each cylinder with rubber bands to retain the soil. Another net was fixed to the top of each container to prevent earthworms from escaping. Finally, all the cylinders were covered with plastic bags to avoid excessive evaporation, and were incubated for 6weeks at 19±1°C in the dark.

Table 1. Treatments combining the presence/absence of earthworms and pathogen.

Treatment	Earthworms		Pathogen
	G. rione	*L. friendi*	*F. graminearum*
1	+	-	-
2	+	-	+
3	-	+	-
4	-	+	+
5	-	-	-
6	-	-	+

Fig. 2. Microcosms composed of 16 cm-wide PVC cylinders, filled with soil and with 6 g of wheat straw on the soil surface. Treatments were determined by whether the straw was sterilized or contained *Fusarium*, combined with the addition or not of earthworms.

4.3.2.5.Data collection

Soil surface coverage

Soil covered by straw was visually determined based on photos taken for each cylinder at the beginning and at the end of the experimental period. The circular section of the photograph corresponding to the surface of the soil in each cylinder was divided radially in eight equal sections. The percentage of the area covered by straw in each section was estimated by eye and the average of all the sections made the cover percentage value used for each cylinder.

Straw sampling and processing

At the end of the experiment, surface straw was carefully collected by hand, so as to minimize the amount of soil particles adhered to it. All the straw collected from each

microcosms was stored in the freezer (-20 ºC), and later it was lyophilized, weighed and, sampled for *Fusarium* quantification.

Quantification of F. graminearum *in straw*

DNA was extracted from samples with the ZR Fungal/Bacterial DNA MiniPrep™ (ZymoResearch, USA) using 20 mg of lyophilized and ground straw to a fine powder with a rotary mill (SampleTek Model 200 Vial Rotator, Lincoln, Nebraska). The obtained extract was diluted to tenth in order to avoid inhibition of PCR by matrix effect. *Fusarium graminearum* was quantified by qPCR in a Rotor-Gene 6000™, Corbett Life Science according to Nicolaisen et al. (2009). Briefly, reactions were performed in duplicate with total volumes of 10 µl, consisting of 5 µl of Rotor-Gene™ SYBR® Green PCR Master Mix (Qiagen, Venlo, Netherlands), 0.5 µl of each primer (25 µM), 1 µl of template DNA and 3 µl of sterile miliQ water. The PCR thermal cycling consisted of 2 min at 95°C and 40 cycles of 95°C for 5 seconds and 62°C for 15 seconds (during which the fluorescence was acquired). Following the final amplification cycle, a dissociation curve between 50°C and 99°C was constructed in order to confirm the specificity of the amplification. A standard curve was generated by duplicate analysis of 10-fold serial dilutions (between $5.2\ 10^{-1}$ and $5.2\ 10^{-5}$ ng/µL.) of a known amount of *F. graminearum* DNA purified and quantified using a QubitdsDNA HS Assay Kit (Invitrogen, USA) in a Qubit Fluorometer (Invitrogen, USA). The mean cycle threshold (Ct) values of each dilution were plotted against log of the corresponding DNA concentration. PCR efficiency was calculated from the slope of the standard curve with the formula Efficiency = $[10^{(-1/slope)}]$-1 (Dorak, 2007). Quantitation limit and dynamic range were set in the linear range. Data analysis was carried out in Rotor-Gene 6000 cycler software (Qiagen, Venlo, Netherlands). Absolute quantification of DNA in the experimental samples was determined by interpolation of the threshold cycle values (Ct) of each sample in the corresponding standard curve.

Previous to the analysis of the samples by real time PCR, matrix effect on amplification was evaluated. Total DNA of 20 mg of of lyophilized and ground straw without *F. graminearum* inoculation was extracted with the aid of ZR Fungal/Bacterial DNA MiniPrep^{TM} (ZymoResearch, USA). The obtained extract, and the corresponding dilutions in water to half, and tenth were amended with a known amount of *F. graminearum* DNA to reach a concentration of 0.52 ng/µl. The same quantity of *F. graminearum* DNA solubilized in deionized water, instead of in matrix extract (positive control) and two blank controls containing only deionized water or matrix extract were also included in each run. All amplification reactions were performed in duplicate. In each case, the mean CT and the corresponding confidence intervals were determined. The minimal dilution of matrix extract in which the CT value was not significantly different to the corresponding to the positive control in water was chosen to continue the study. *F. graminearum* DNA concentration in each sample was determined by real time PCR as described above.

Earthworm survival growth and reproduction

Earthworms were counted, washed in deionized water, dried with paper napkins and then weighed for fresh biomass with gut content. Earthworm cocoons were retrieved by wet sieving of the soil (mesh size 2 mm).

4.3.2.6.Statistical analyses

Statistical analyses were performed with the statistical package Infostat®. When the assumption of normality of the residues was satisfied, data was analyzed by the Linear Mixed Model, with 2 fixed effect factors (earthworm species and straw condition) and one random factor (block: six levels). The earthworm species-factor had three levels (*G. rione, L. friendi*, and control without earthworms); while the straw condition-factor had two levels (with *Fusarium* and sterilized). When significant effects were found, means were compared by Fisher's test (alpha= 0.05).

4.3.3. Results

4.3.3.1. Earthworm effect on wheat straw

Soil cover by straw differed according to the earthworm factor, but was not affected by the straw condition, i.e. whether the straw was infected with *Fusarium* or sterile (table 2). *Lumbricus friendi* reduced soil cover significantly, while treatments with *Glossoscolex rione* did not differ from the control (fig. 3). However, earthworm effect on *Fusarium* inoculum could not be assessed because *Fusarium* DNA quantities were below detection level in the qPCR analyses.

Table. 2. General Linear Mixed models output for soil coverage according to two fixed factors (straw condition and earthworm species)

	numDF	denDF	F-value	p-value
(Intercept)	1	27	1946.72	<0.0001
Straw	1	27	0.38	0.5447
Earthworm	2	27	19.67	<0.0001

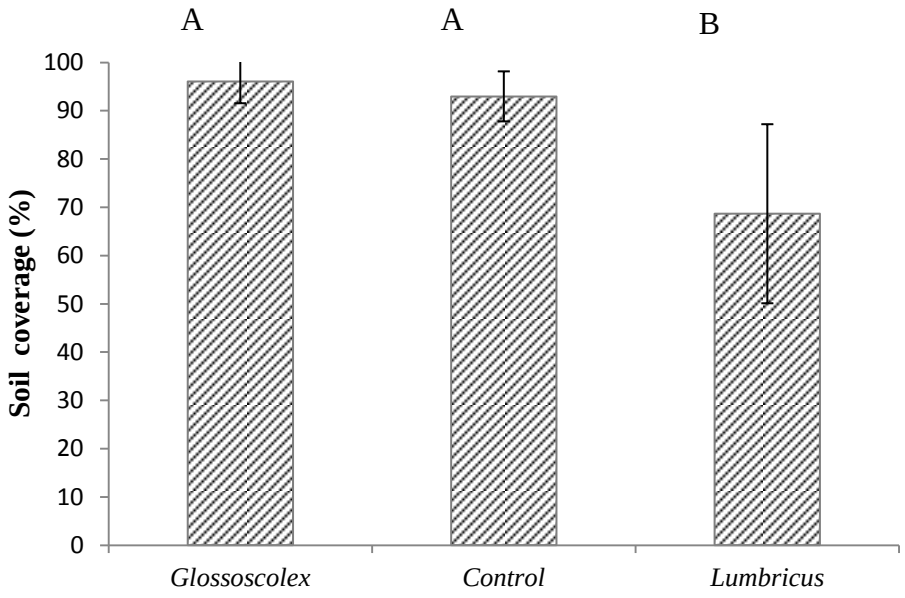

Fig. 3. Earthworm species effect on soil cover. Different letters indicate significant differences (Fisher's test, alpha= 0.05); bars indicate standard deviation.

4.3.3.2.Earthworms survival and reproduction

Survival was higher for *G. rione* (79%) than for *L. friendi* (29%). No significant effect of straw condition was found on biomass of this species (p= 0.3536), although it owrth mentioning that surviving *G. rione* specimens showed high coefficient of variation (186 %) in weighte. The maximum cocoon production for *G. rione* occurred in the treatment with sterile straw, while the maximum cocoon production for *L. friendi* occurred in the treatment with straw with *Fusarium* (table 3). Mean cocoon production was in all cases approximately half the maximum amount found in each case.

Table. 3. Mean and maximum number of cocoons produced per individual per week for the tested earthworms under different straw conditions (with *Fusarium* infection or sterile)

	Mean	Maximum	Std dev
G. rione			
Fusarium	0.15	0.33	1.47
Sterile	0.24	0.42	1.47
L. friendi			
Fusarium	0.65	1.17	3.87
Sterile	0.40	0.92	3.19

4.3.4. Discussion

Lumbricus friendi had a marked effect reducing surface straw, whereas *G. rione* had no effect on surface straw, probably due to its endogeic behaviour. (Jorge-Escudero, Lagerlöf & Pérez, *in press*; Wolfarth et al. 2011a; Wolfarth et al. 2011b). Pathogen infection of straw on surface may likely be controlled by surface dwelling earthworms anecics (or epigeics), which come to the surface in search of organic material to bury, while pathogens of the roots may be controlled by endogeics, which remain in the soil close to the rhizosphere (Jorge-Escudero, Lagerlöf & Pérez, *in press*)[12]. Since high quantities of crop residues enhance *Fusarium* survival (Leplat et al., 2013), the reduction of the residues on soil surface effected by the earthworms, contributes to the control of this pathogen.

The fact that there were no detectable amounts of *Fusarium* in any of the treatments could be attributed to degradation caused by problems in sample handling, for which the cold chain broke, remaining by accident at 4 ^{o}C for a period prior to liofilization. Hence, we strongly recommend repeating the test in the same conditions to confirm

[12] For the purposes of this Thesis, see section 4.2.

the earthworm effect, reducing the amount of crop residue at the soil surface, but also reducing *Fusarium* inoculum on the remaining residue.

Both the high and low mortality percentage of *L. friendi* and *G. rione*, respectively, were unexpected. This study represents the first recorded experience of experimentation with *G. rione*, thus, no information regarding optimal culture techniques was available. However, a mean survival of 79% suggests its suitability for experimentation. In the case of *L. friendi*, Butt and Briones (2011) obtained 98 % survival at 15 ºC. Although in the present experiment the incubation was at 19 ºC, we expected a higher survival than that obtained (29 %). In addition to temperature, manipulation prior to experiment set could have influenced a high mortality, since selected earthworms were kept out of the soil, and maintained humid in couples in petri dishes, for approximately one hour before being introduced in the corresponding cylinders.

In average, the native *G. rione* produced one cocoon per earthworm every 4.2 and 6.5 weeks, in sterile and *Fusarium*-inoculated straw, respectively. *Lumbricus friendi*, which every week produced 2.5 cocoons in sterile straw and 1.5 cocoons when straw was inoculated with *Fusarium.* In these experimental conditions *G. rione* had a lower reproduction rate than *L. friendi*, which in turn, had a far lower reproduction rate than that reported for Spanish *L. friendi* at 15 ºC (more than 2 cocoons individual^{-1} wk^{-1}; Butt & Briones, 2011). Different geographical sites may determine cryptic lineages of one species, with possible different ecological features (Spurgeon et al., 2016). However, the present results should not be taken as optimal values, since not all earthworms were adults, and high mortality may have influenced the reproduction results, as it has been shown that no cocoons are produced when individuals of *L. friendi* are maintained in isolation (Butt & Briones, 2011). Therefore, lower values than previously reported were expected. Mortality may have had a minor incidence on cocoon production of *G. rione*, both because more of them survived, and because they are assumed to be parthenogenic (Ljungström, 1972). Hence, even when one of

the earthworms in the cylinder died, the other may have been able to continue producing cocoons.

To sum up, *L. friendi* had a notorious activity on the surface, reducing more than 30% of the straw on soil surface, despite a high mortality, while the treatment with *G. rione* did not differ from the control. A repetition of the experiment will be necessary to confirm if this pathogen controlling effect does also extend to the reduction of the *Fusarium* inoculum in the straw, since the quantities of the pathogen DNA were below the detection levels, probably due to degradation caused by conservation. The earthworms were not negatively affected by *Fusarium*, or its toxins. The exotic anecic earthworm showed potential to contribute to the biological control of *F. graminearum* in the wheat straw, by reducing the straw on soil surface, while the native endogeic earthworm tested had no effect on straw reduction.

4.3.5. References

Blouin M, Hodson ME, Delgado EA, Baker G, Brussaard L, Butt KR, Dai J, Dendooven L, Peres G, Tondoh JE, Cluzeau D, Brun J-J. 2013. A review of earthworm impact on soil function and ecosystem services. European Journal of Soil Science. 62(4): 161–182.

Blouin M, Zuily-Fodil Y, Pham-Thi A-T, Laffray D, Reversat G, Pando A. 2005. Belowground organism activities affect plant aboveground phenotype, including plant tolerance to parasites. Ecology Letters. 8: 202-208.

Bonkowski S, Griffiths BS, Ritz K. 2000. Food preference of earthworms for soil fungi. Pedobiologia. 44: 666–676.

Boyer J, Michellon R, Chabanne A, Reversat G, Tibere R. 1999. Effects of trefoil cover crop and earthworm inoculation on maize crop and soil organisms in Reunion Island. Biology and Fertility of Soils. 28(4): 364–370.Boyer J, Reversat G, Lavelle P, Chabanne A. 2013. Interactions between earthworms and plant-parasitic nematodes. European Journal of Soil Biology. 59: 43-47.

Brown GG, Edwards CA, Brussaard L. 2004. How earthworms affect plant growth: Burrowing into the mechanisms. In: Edwards CA. (Ed.). Earthworm ecology (2nd ed). London: Boca Raton. CRC Press. 19–49.

Butt KR, Briones MJI. 2011. Life cycle studies of the earthworm Lumbricus friendi (Cognetti, 1904). Pedobiologia. 54: 27– 29.

Canellas LP, Dantas DJ, Aguiar NO, Peres LEP, Zsögön A, Olivares FL, Dobbss LB, Facanha AR, Nebbioso A, Piccolo A. 2011. Probing the hormonal activity of fractionated molecular humic components in tomato auxin mutants. Annals of Applied Biology. 159(2): 202-211.

Cho JH, Park CB, Yoon YG, Kim, SC. 1998. Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization. Biochimica et Biophysica Acta. 1408(1): 67-76.

de la Peña E. 2009. Efectos de la biota edáfica en las interacciones planta-insecto a nivel foliar. Ecosistemas. 18 (2): 64-78.

Dorak MT. 2007. Real-Time PCR. New York: Taylor and Francis Group.

Fragoso C, Brown GG. 2007. Ecología y taxonomía de las lombrices de tierra en Latinoamérica: El primer Encuentro Latino-Americano de Ecología y Taxonomía de Oligoquetos (ELAETAO1). In: Brown GG, Fragoso C. (Eds.). Minhocas na América Latina: Biodiversidade e Ecologia. Londrina: EMBRAPA Soja. 34-75.

Friberg H, Lagerlöf J, Rämert B. 2005. Influence of soil fauna on fungal plant pathogens in agricultural and horticultural systems. Biocontrol Science and Technology. 15(7): 641-658.

James SW. 2011. Earthworms. In: Simberloff D, Rejmánek M. (Eds.). Encyclopedia of biological invasions. Los Angeles: University of California Press. 177-183.

Jana U, Barot S, Blouin M, Lavelle P, Laffray D, Repellin A. 2010. Earthworms influence the production of above- and belowground biomass and the expression of genes involved in cell proliferation and stress responses in *Arabidopsis thaliana*. Soil Biology and Biochemistry. 42: 244–252.

Jorge-Escudero G, Lagerlöf J, Pérez CA. 2018. Control biológico de enfermedades y plagas promovido por lombrices. Revista Brasileria de Agroecologia. 13 (4): *in press*. ISSN: 1980-9735.

Lafont A, Risede JM, Loranyer-Merciris G, Clermont-Dauphin C, Dorel M, Rhino B, Lavelle P. 2007. Effects of the earthworm Pontoscolex corethrurus on banana plants infected or not with the plant-parasitic nematode Radopholus similis. Pedobiologia. 51: 311–318.

Lavelle P, Decaëns T, Aubert M, Barot S, Blouin M, Bureau F, Margerie P, Mora P, Rossi J.-P. 2006. Soil invertebrates and ecosystem services. European Journal of Soil Biology. 42: 3–15.

Leplat J, Friberg H, Abid M, Steinberg C. 2013. Survival of *Fusarium graminearum*, the causal agent of Fusarium head blight. A review. Agronomy for Sustainable Development. 33: 97–111.

Ljungström PO. 1972. Biology of *Glossoscolex uruguayensis* (Glossoscolecidae, Oligochaeta). A new species for Argentina. Studies on Neotropical Fauna and Environment. 7: 195-205.

Reid WV, Mooney HA, Cropper A, Capistrano D, Carpenter SR, Chopra K, Dasgupta P, Dietz T, Duraiappah AK, Hassan R, Kasperson R, Leemans R, May TRM, McMichael AJ, Pingali P, Samper C, Scholes R, Watson RT, Zakri AH, Shidong Z, Ash NJ, Bennett E, Kumar P, Lee MJ, Raudsepp-Hearne C, Simons H, Thonell J, Zurek M. 2005. The Millennium Ecosystem Assessment: ecosystems and human well-being: synthesis. Washington, DC: World Resources Institute.

Nicolaisen M, Suproniene S, Nielsen LK, Lazzaro I, Spliid NH, Justesen AF. 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. Journal of Microbiological Methods. 76: 234-240.

Oldenburg E, Kramer S, Schrader S, Weinert J. 2008. Impact of the earthworm *Lumbricus terrestris* on the degradation of *Fusarium*-infected and deoxynivalenol-contaminated wheat straw. Soil Biology and Biochemistry. 40: 3049-3053.

Puga-Freitas R, Barot S, Taconnat L, Renou JP, Blouin M. 2012. Signal molecules mediate the impact of the earthworm *Aporrectodea caliginosa* on growth, development and defence of the plant *Arabidopsis thaliana*. Plos One. 7 (12): 1-10.

Senapati BK. 1992. Biotic interactions between soil nematodes and earthworms. Soil Biology and Biochemistry. 24: 1441–1444.

Spurgeon DJ, Liebeke M, Anderson C, Kille P, Lawlor A, Bundy JG, Lahive E. 2016. Ecological drivers influence the distributions of two cryptic lineages in an earthworm morphospecies. Applied Soil Ecology. 108: 8–15.

Sulkava P, Huhta V. 2003. Effects of hard frost and freeze-thaw cycles on decomposer communities and N mineralization in boreal forest soil. Applied Soil Ecology. 22: 225-239.

Umpiérrez M, Garmendia G, Cabrera M, Pereyra S, Vero S. 2013. Diversity of Pathogen Populations Causing Fusarium Head Blight of Wheat in Uruguay. In: Alconada Magliano MT, Chulze SN. (Eds.). Fusarium Head Blight in Latin America. Dordrecht: Springer. 32-44.

Wang C, Sun Z, Liu Y, Zhang X, Xu G. 2007. A novel antimicrobial vermipeptide family from earthworm Eisenia fetida. European Journal of Soil Biology. 43: 127-134.

Wolfarth F, Schrader, S, Oldenburg E, Weinert J, Bruttone J. 2011a. Earthworms promote the reduction of *Fusarium* biomass and deoxynivalenol content in wheat straw under field conditions. Soil Biology and Biochemistry. 43: 1858-1865.

Wolfarth F, Schrader S, Oldenburg E, Weinert J. 2011b. Contribution of the endogeic earthworm species *Aporrectodea caliginosa* to the degradation of deoxynivalenol and *Fusarium* biomass in wheat straw. Mycotoxin Research. 27: 215–220.

5: EFECTO TOXICOLÓGICO DE LOS FUNGICIDAS USADOS PARA CONTROLAR *Fusarium graminearum* SOBRE LA ACTIVIDAD DE LAS LOMBRICES

5.1. RESUMEN

El efecto cóctel de los formulados comerciales de agroquímicos, que combinan varios principios activos sumados a otros componentes (como ser solventes y adyuvantes), no es habitualmente estudiado. Los servicios ecosistémicos provistos por las lombrices en los suelos podrían verse afectados por el uso de tecnologías que incluyan agroquímicos. Existen evidencias de que las lombrices, beneficiadas por la reducción del laboreo, tienen el potencial de contribuir en el control biológico de enfermedades de plantas, por lo cual resulta de interés conocer cómo afectan a las lombrices los fungicidas utilizados en cultivos de cereales. El objetivo de este estudio fue evaluar los efectos (1) subletales sobre *E. fetida* de dos fungicidas comerciales utilizados para el control de la fusariosis de la espiga del trigo; y (2) agudos de mortalidad de un fungicida comercial sobre *G. rione* (especie nativa) y *E. fetida* (especie de referencia). Se colocaron 10 individuos de *E. fetida* o cinco de *G. rione* en recipientes de vidrio con 600 g (peso seco) de suelo artificial previamente humedecido a 60% capacidad de campo y rociado con concentraciones seriadas de los fungicidas. Se incubaron a 20 ^{o}C por 2 y 8 semanas para los ensayos letal y subletal, respectivamente. Los ensayos con *E. fetida* cumplieron con los criterios de validación de las normas ISO 11268. La concentración de inhibición de 25% de la progenie de *E. fetida* se estimó en 212 L/ha para Swing Plus y 700 L/ha para Prosaro. Ambos presentaron un efecto de hormesis sobre la progenie, es decir, de estimulación a bajas concentraciones. *G. rione* mostró mayor sensibilidad al fungicida Prosaro que *E. fetida*, con valores preliminares de dosis letal a los 14 días de 174 L/ha para la primera, y de >1000 L/ha para la segunda. Siendo el presente, el primer trabajo de toxicidad que se realiza con esta especie nativa, los estudios deben continuarse de modo de ajustar las condiciones de cría y sobrevivencia optimas en laboratorio y poder determinar la repetibilidad de los resultados. Este tipo de estudios permite estimar el impacto de los agroquímicos sobre individuos no-blanco, y aporta información con un enfoque de sistema que contribuye a un manejo integrado de las distintas tecnologías en la búsqueda de una producción sustentable.

5.2. TOXICOLOGICAL EFFECT ON EARTHWORMS OF FUNGICIDES USED TO CONTROL *Fusarium graminearum*

Abstract

Although marketing regulations require earthworm toxicity studies of the active ingredients in agrochemicals, the cocktail effect of commercial formulations that combine several active ingredients, with other components (such as solvents and adjuvants), is not usually studied on this group. The ecosystem services provided by the earthworms are lost when land management reduce their populations. Such is the case with the use of agrochemicals, which can have lethal or sub-lethal effects on them. Hence, an increase in the use of fungicides, due to a larger area with no-till systems, where straw on surface favors the incidence of Fusarium Head Blight (FHB), could counteract the minimization of the environmental impacts that this soil conservation management offers. Earthworms, which are benefited by the reduction of tillage, have the potential to contribute to the biological control of FHB. Therefore, it is of interest to know how this group is affected by the fungicides used to control this pathology. The objective of this study was (1) to evaluate sublethal effects of two commercial fungicides on *Eisenia fetida*; (2) evaluate acute mortality effects of a commercial fungicide on *Glossoscolex rione* and *E. fetida*. The results of the tests with *E. fetida* met the validation criteria of the ISO 11268 standards. The inhibition concentration of 25% of the progeny of *E. fetida* was estimated at 212 L / ha for Swing Plus and 700 L / ha for Prosaro. Both fungicides showed an effect of hormesis on the progeny, *i.e.* stimulation at low concentrations. Being the present, the first work of toxicity that is carried out with the native species *G. rione*, it was found that this is probably much more sensitive to the fungicide Prosaro than *E. fetida*, with preliminary values of lethal dose at 14 days of 174 L / ha for the first, and> 1000 L / ha for the second. However, studies with *G. rione* should be continued in order to adjust breeding and survival conditions in laboratory conditions.

Keywords:

Eisenia fetida; *Glossoscolex rione*, Fusarium Head Blight, cocktail effect

5.2.1. Introduction

Earthworms play a fundamental role in soils, contributing to their physical, chemical and biological properties. Several studies have proved that earthworms favor plant development and health (Blouin et al., 2013). For these reasons, those agricultural managements that are detrimental to earthworm populations would also be affecting the long-term production and productivity of the soil (Scheu, 2003; Brown et al., 1999). Agrochemicals can have acute effects on worms, forcing them to move away or causing their death, which reduce their population in the short term. Besides, sub lethal effects on growth, reproduction rate and/or offspring development, may also reduce their population in the long-term (Domínguez et al., 2016; Santadino, Coviella & Momo, 2014;).

The toxicity of active ingredients of agrochemicals has been tested for various animals, including earthworms as international requirements for the safety sheets, following the United Nations' standard classification and labelling for hazardous material, the *Globally Harmonized System* of Classification and Labelling of Chemicals (GHS). In the European Union it is regulated by the Registration, Evaluation, Authorization and Restriction of Chemicals regulation (REACH) (ECHA, 2018); and in the United States by the U.S. Government's Occupational Safety and Health Administration (OSHA) communication standards (OSHA, 2018). However, field application of agrochemicals is carried out with commercial formulations, which contain other ingredients as solvents, adjuvants, etc.; moreover, these may combine several active ingredients. To register a commercial formulation of agrochemicals in Uruguay, the Ministry responsible for Agricultural Issues (MGAP) requires toxicity for humans, fish, domestic animals and bees, to be declared on the label (MGAP, 2017). Toxicity for earthworms is not included in these requirements; therefore, the combined effect on earthworms of more than one active ingredient, or their effect in combination with solvents and adjuvants, is not known.

Standardized toxicity tests are performed mostly with *Eisenia fetida* or *E. andrei* given the convenience of their short reproductive cycle, and their ease of breeding and management (ISO, 2012; ISO, 2008; Environment Canada, 2004; OECD, 1984). However, this approach has been questioned since these species, due to their ecology, are hardly found in agricultural fields where agrochemicals are actually applied, since they are *epigeic* and inhabit sites with high accumulation of organic matter on surface, as manure (Pelosi, Joimel & Makowski, 2013). In addition, *endogeic* species, such as *Aporrectodea caliginosa*, which do inhabit cultivated fields, have shown to be more sensitive than the previous ones (Pelosi, Joimel & Makowski, 2013). Buch et al. (2013) found that the acute lethal and avoidance behavior of *Pontocolex corethrurus*, widespread in Brazil, are comparable to those obtained with *E. fetida*, so the latter would be a good test species in this case.

The three first lumbricid species mentioned above have been reported for Uruguay, as exotic, introduced involuntarily by immigrants and their agricultural and cattle breeding activities (Grosso & Brown, 2007). In the south of the country the exotics dominate, especially in disturbed environments as agricultural fields. However, in the west, with fewer exotics species, natives dominate even in disturbed soils (see chapter 3). One of the native species found in wheat fields in western Uruguay is *Glossoscolex rione* (Ljungström, 1972)[13]. There is very little information about the ecology of this species (Cordero, 1943), and there are no previous reports regarding breeding or toxicity testing. *Glossoscolex rione* had a very high survival percentage in an experiment held for 6 weeks in moist soil cores at 19 $\pm$ 2ºC[14].

Since this native worm has been reported for wheat fields[15], it is of interest to know how it is affected by agrochemicals used in this crop to control Fusarium Head Blight, one of the main cereal diseases in Uruguay and worldwide. These agrochemicals include mostly triazole-based fungicides which are applied by spraying at full flowering and 10 days later (Machado & Del Ponte, 2016).

[13] For the purposes of this Thesis, also see chapter 3.
[14] For the purposes of this Thesis, see chapter 4.
[15] For the purposes of this Thesis, see section 3.2.

We hypothesized that fungicides would have a detrimental effect on *G. rione* survival and that these earthworms would be more sensitive than the standard test organisms, *E. fetida*. The use of avoidance tests has been recommended as they are more sensitive than lethality tests (García et al., 2008), but in the case of native species, the former may have erratic results (De Silva & van Gestel, 2009) perhaps due to a lower mobility in the case of native worms compared to *E. fetida*. The aims of this study were (1) to assess the sublethal effect of two commercial fungicides on *E. fetida*; (2) to assess the acute lethal effect of one commercial fungicide on both *G. rione* and *E. fetida*.

5.2.2. Materials and methods

5.2.2.1. Test facilities

Experiments were held in LATITUD laboratory, in the Uruguayan Technological Laboratory (LATU), Montevideo.

Test organisms

Individuals of *Eisenia fetida* were obtained from the Composting Demonstration Unit of the Faculty of Agronomy, Montevideo, and kept in combined substrate of organic manure and loose peat of 70 % Sphagnum sp. (Kekkilä ®, Finland) in the ratio 1:1 (v/v). Individuals of *Glossoscolex rione* were collected in Mario Cassinoni Experiment Station of the Faculty of Agronomy, Paysandú, and kept in soil obtained from the very same collection site and fed with ecologically bred (organic) cow manure. All earthworms were acclimated in their laboratory cultures at 20 ºC for at least one month before the tests.

Test substances

Two commercial fungicides with different toxicologic properties (IUPAC, Table 1) were tested: Swing Plus, BASF® (active ingredients: Metconazole 27.5% and

Epoxiconazole 37.5%), and Prosaro, BAYER® (active ingredients: Tebuconazole 12.5% and Prothioconazole 12.5%). Cibencarb, CIBELES® (active ingredient Carbendazim 500 g L^{-1}) was used as positive control (reference substance); deionized water was used as negative control. These fungicides were selected due to official recommendations to control Fusarium head blight. Cibencarb was chosen as a positive control since it contains carbendazim, which is the positive control suggested by the ISO standards (ISO, 2008).

Table 1. Acute lethal and sub lethal reproduction toxicities and their corresponding toxicity category of the active ingredients in the tested fungicides (Source: IUPAC).

Commercial fungicide	Active substance	Acute toxicity, 14-day LD_{50} (mg kg-1)	Acute toxicity category	Sub lethal toxicity, 56-day reproduction NOEC (mg kg-1)	Sublethal toxicity category
Swing Plus	metconazole	> 500	MODERATE	0.9	MODERATE
	epoxiconazole	> 500	MODERATE	0.084	HIGH
Prosaro	tebuconazole	1381	LOW	10	MODERATE
	protioconazole	> 1000	LOW	1.33	MODERATE

Note: NOEC= No Observable Effect Concentration

Test substrate

Artificial soil was prepared following ISO 11268 international standards with 10% air-dried peat of *Sphagnum* sp., 20% kaolin and 70% sand. Afterwards, the mix was enriched with 1 % of rehydrated dried organic cow manure added as feed. $CaCO_3$ was used to neutralize the mix. Water-holding capacity was 84% on a dry weight basis.

5.2.2.2. Experiment set up

A set of 1.8 L glass containers were used as experimental units. These were filled with 600 g (dry weight) artificial soil which was wetted to with 50% moisture (dry weight basis) corresponding to 60 % of water holding capacity. In order to simulate field application, each container was sprayed with 3 mL of fungicide emulsions (prepared with deionized water and the corresponding concentration of fungicide, see tables 2 and 3) or deoinized water for control, left 1 hour under a fume hood, covered with a plastic film and left overnight for the fungicide to spread uniformly on the soil. The following day ten *E. fetida* or five *G. rione* were added according to treatment. After the addition of the earthworms the film was perforated with a needle. Vessels were incubated at 20 $\pm$ 2 ºC with fluorescent lighting, 600 lux with 16L:8D photoperiod.

5.2.2.3.Toxicity test 1: Sub lethal effects - growth and reproduction

This test was done following ISO 11268-2 international standards, using *E. fetida* (mean weight 368 $\pm$ 25 mg) to test Swing Plus® (SP) and Prosaro® (P) in five different concentrations each, following a logarithmic series which included one concentration lower by one order of magnitude than that recommended by the trade companies for field application, the concentration recommended by the trade companies, and the three other concentrations were greater by one, two and three orders of magnitude; the concentration used for Cibencarb® (reference substance for positive control, based on carbendazim) was equivalent to that recommended by the company for field application (table 2). Experimental units were prepared according to the *Experiment set up* section for 12 treatments (which included the five concentrations for each fungicide plus a negative and a positive control). Each treatment had five replicates, totalizing 60 glass containers. All earthworms had developed clitellum.

Total test duration was 56 days. Weekly monitoring consisted of adding 1% manure as feed and watering in order to maintain moisture levels. At day 28 adult worms

were removed from the vessels, washed in tap water and gently dried with paper tissues. Earthworm number and fresh biomass was recorded. At the end of the experiment offspring and cocoons were retrieved, counted and weighed.

Table 2. Test substance concentration for treatments with Swing Plus (SP), Prosaro (P), water (Cntrl -), or Cibencarb (Cntrl +) in Toxicity test 1. $SP10^0$ and $P10^0$ indicate recommended application dozes.

Treatment		Test substance dose
Number	Label	(L/ha)
1	SP 10^{-1}	0.15
2	SP 10^0	1.5
3	SP 10^1	15
4	SP 10^2	150
5	SP 10^3	1500
6	P 10^{-1}	0.1
7	P 10^0	1
8	P 10^1	10
9	P 10^2	100
10	P 10^3	1000
11	Cntrl -	0
12	Cntrl +	0.5

5.2.2.4.Toxicity test 2: Acute effects - lethality

A 14-day lethality test was performed for both *E. fetida* (individual mean weight 336 $\pm$ 25 mg) and *G. rione* (individual mean weight 410 $\pm$ 55 mg) with Prosaro® (P), which was, according to Test 1, the fungicide with the strongest effect on earthworms. It was tested in five different concentrations following a logarithmic series which ranged between the two highest concentrations used in the previous test

(table 3). The selected concentration for Cibencarb®, used as reference substance for positive control, was within the range suggested by ISO 11268 (1-5 mg /kg of soil). Experimental units were prepared according to the *Experiment set up* section, for 14 treatments, each with five replicates, totalizing 72 glass containers. Treatments included the five concentrations of the fungicide, plus a negative (deionized water) and a positive control (carbendazim), for each earthworm species. All earthworms were adults (with clitellum) or subadults (similar size as adults but lacking clitellum). At day 7 and 14, live worms were counted, washed in tap water and gently dried with paper tissues, and weighed.

Table 3. Test substance concentration for treatments with Prosaro (P), water (Cntrl -) or Cibencarb (Cntrl+) and the earthworm species *E. fetida* (E) or *G. rione* (G) in Toxicity test 2

Treatment		Test substance concentration
Number	Label	(L/ha)
1	E P 1	100
2	E P 1.8	180
3	E P 3.2	320
4	E P 5.6	560
5	E P 10	1000
6	E Cntrl -	0
7	E Cntrl +	2.96
8	G P 1	100
9	G P 1.8	180
10	G P 3.2	320
11	G P 5.6	560
12	G P 10	1000
13	G Cntrl -	0
14	G Cntrl +	2.96

Statistical analysis

Quantitative (growth and reproduction) and quantal (lethality) data analyses were performed with MedCalc® (v.17.9.4) software. The 25% inhibition dose (ID_{25}) was calculated for both fungicides by non-linear regression, fitting to the Gompertz model. Dose values were log-transformed. Prosaro 50 % lethal dose (LD50(14d)) for each earthworm species, and their 95% confidence limits, were estimated with Probit regression, using the method of maximum likelihood, given that in both cases data provided at least two partial effects. Dose values were log-transformed and replicates were pooled.

A linear mixed model was used to assess the effect of the different doses on the assessed parameters (growth, number and biomass of juveniles) with "dose" as fixed factor, and "block" (replicate) as a random factor. When significant differences were found, LSD Fisher was used as post hoc test. Normality of residuals was checked with Shapiro–Wilks' test, modified by Mahibbur and Govindarajulu (1997, cited by: Balzarini, 2008). These analyses were performed with the Infostat® (v.2016) software, powered by R through DCOM®.

Outliers were defined by Grubbs' rule, as those where the difference between the value in question and the mean value, divided by the standard deviation, exceeds a critical tabulated value -1.67, in the case of 5 replicates and a significance level of 5% (Grubbs, 1969). Given the case, this value was replaced by the mean value of the other replicates.

5.2.3. Results

5.2.3.1.Toxicity test 1: Sub lethal effects - growth and reproduction

The results of this test satisfied validity requirements of ISO 11268-2, mortality of adults in the control was lower than 10%, and all control containers produced more than 30 juveniles. Reproduction in the negative control has one value which was identified as outlier according to Grubbs' rule (Grubbs 1969). After eliminating this value, the resulting coefficient of variance for reproduction in the negative control was 20 %.

Eisenia fetida mortality after 28 days of exposure to the fungicides was $\leq$ 4% in the control, as well as in the four lower doses ($x10^{-1}$ to $x10^{2}$) of both fungicides. The highest dose produced 84 11% and 92 8% of adult mortality for SP and P, respectively. Individual biomass of surviving adults in the spiked treatments did not differ significantly (p= 0.253) from control (0.46 g).

At day 56, the estimated 25% inhibition dose (ID_{25}) for reproduction was 212 L/ha for SP and 700 L/ha for P. Both fungicides generated a hormetic effect, producing more juveniles at lower doses than in the control: up to +58% for P, + 44 % for SP (Fig 1). Mean juvenile weight ranged from 9.79 mg to 15.37 mg in treatments with SP; and from 10.00 mg to 13.91 mg in treatments with P. No treatment showed significant differences in mean juvenile weight (p=0.100 for P; p=0.130 for SP) compared to the control, 12.98 2.94 mg. Positive control did not show significant differences with control in any of the measured variables.

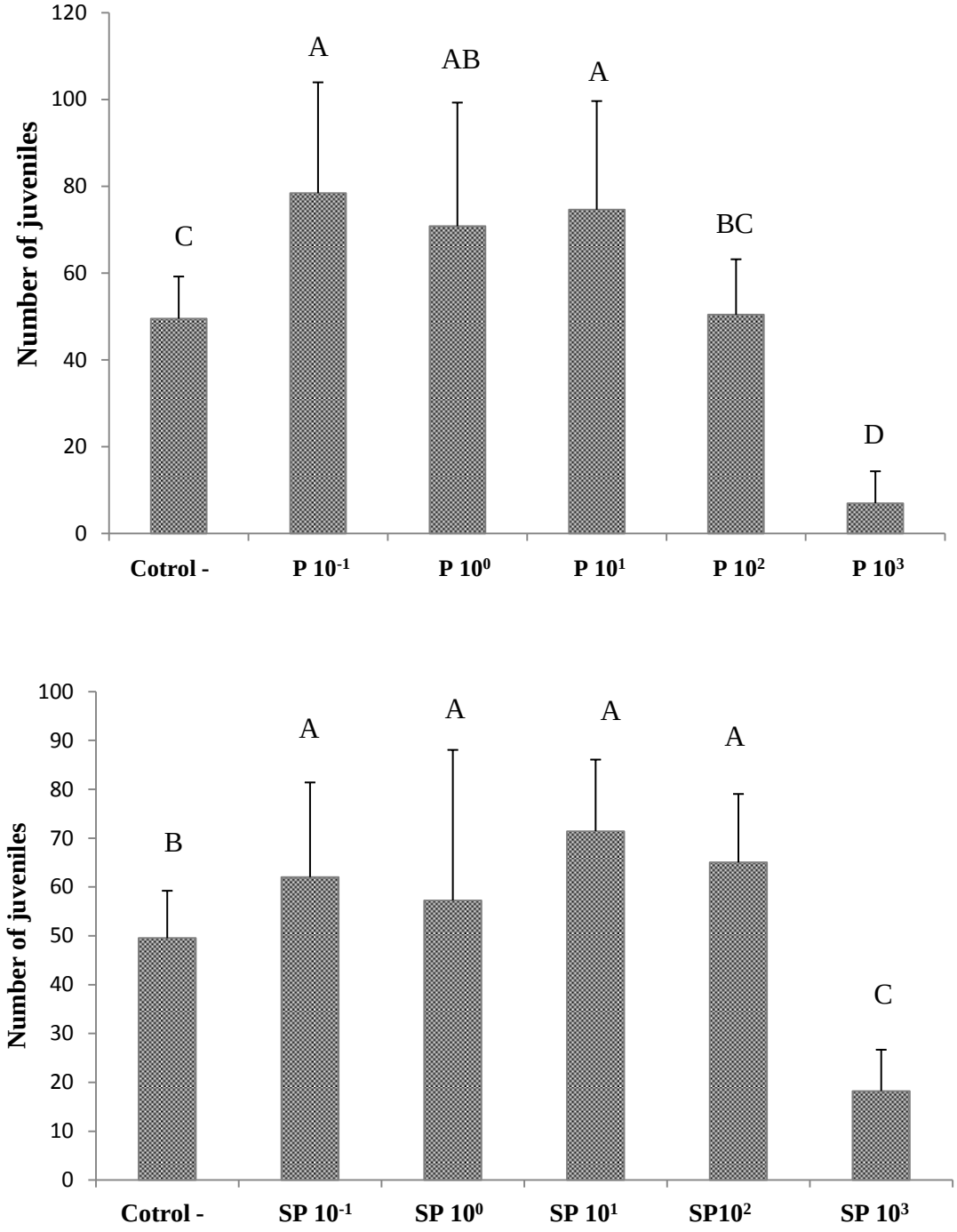

Fig. 1. Number of *E. fetida* juveniles per test vessel on day 56 in the presence increasing doses of Prosaro (P; above) and Swing Plus (SP, below). See table 2 for specifications on dose. Error bars represent standard deviation. Means with different letters are significantly different ($p < 0.05$).

5.2.3.2. Toxicity test 2: Acute effects – lethality

The results for *E. fetida* in this test were considered valid according to ISO 11268-1, since mortality of adults in the negative control was only 4 %. *E. fetida* at 14-day LD_{50} values >1000 L/ha (Fig. 2). The mean mortality of *G. rione* in the negative control was 52 %, and there were no survivors in the two highest doses. *Glossoscolex rione* presented 14-day LD_{50} values of 174 L/ha (with 136 L/ha and 210 L/ha as 95% confidence limits) (Fig. 2).

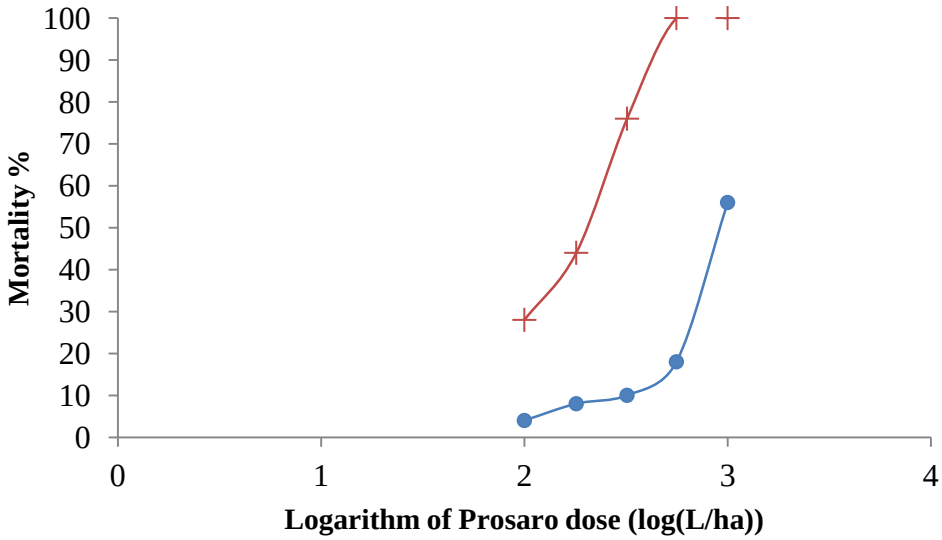

Fig. 2. Mortality at day 14 for *E. fetida* (circle) and *G. rione* (cross) with increasing doses of Prosaro, from 1 x 10^2 to 1 x 10^3 L/ha, which were the two highest doses in the sublethal experiment. See table 3 for specifications on doses.

Surviving *E. fetida* decreased in mean individual weight in all doses except for the highest, which was significantly different from the other (p=0.012). On the other hand, *G. rione* individual mean weight increased among the surviving individuals, although weight did not change overtime in the negative control. However, due to high variation, no significant differences in weight could be determined for this species (p=0.167; table 4). Positive control did not show significant differences with the negative control in any of the measured variables.

Table 4. Mean individual biomass (increase or decrease relative to biomass at start) (±StDv) after 14-day exposure to Prosaro in different doses and negative control (0 L/ha). Different letters represent significant differences ($p<0.05$).

Dose (L/ha)	Mean biomass variation (%±St Dv)
	Eisenia fetida
0	-0.37 ± 1.48 a
100	-0.90 ± 1.55 a
180	-1.32 ± 2.77 a
320	-0.59 ± 1.08 a
560	-0.33 ± 3.19 ab
1000	0.98 ± 2.83 b
	Glossoscolex rione
0	-1.23 ± 3.57
100	5.35 ± 2.98
180	6.36 ± 11.24
320	4.78 ± 15.47

5.2.4. Discussion

For both tested fungicides, 25 % inhibiting dose (ID_{25}) was two orders of magnitude higher than recommended field dose. To our knowledge, this is the first report of ID_{25} for earthworm reproduction. This sub lethal effect estimator, lately recommended as superior to NOEC and LOEC (no- and lowest- observed-effect-concentration, respectively) (Environment Canada, 2005), have mostly been used as inhibition concentration (IC_{25}) for aquatic toxicity tests (Wang et al., 2007; Bailer et al., 2000).

The sublethal effects test covered a very broad dose range (1000-fold), which was used as a first screening. Hence, 28-day mortality results lacked intermediate values, leaping in the last two doses from almost 0 % to close to 100%. However, in terms of reproduction, the chosen doses allowed to observe the hormetic effect on reproduction caused by both fungicides after 56 days' exposure. Hormesis, *i.e.* the fact that a variable which is inhibited by high doses of fungicide is also stimulated by low doses, should not be regarded as a flaw, but merely as a natural phenomenon discovered already in the 19th century (Calabrese & Baldwin, 1997). The hormesis observed for both fungicides, fell within the range of maximum stimulation averages (+30 % to +60 %, compared to control) reported by Calabrese and Baldwin's review (1997).

Domínguez et al. (2016) found that mean juvenile weight was significantly lower in their highest AMPA dose, compared to the control. They argued that this could represent a long term negative effect as descendant earthworms would be weaker and less able to provide their ecosystem services. In the present tests no significant difference in weight was found after 14-day or 28-day of exposed adults or on surviving juveniles after 56 days of incubation. Although, high variation played a role in hindering the differentiation between groups, biomass is not such an independent variable in the presence of certain percentage of mortality. Either all the earthworms die and no final biomass data can be registered, or part of the earthworms die, and it is very likely that the weaker would die first, skewing the biomass average in favor of the more robust ones. Factor as food and amount of toxicant per earthworm can also be influencing the mean individual biomass value either favoring or disfavoring a reduced amount of earthworms (Environment Canada, 2005). In the present experiment food rests were observed in most of the vessels, hence that could be discarded as a limiting factor.

Mortality results of this first screening served as criteria to select the logarithmic series of doses for the acute lethality test, as it was found that they should range

between the two highest doses of the previous test (100 - 1000 L/ha). Although, almost 100 % mortality was reached for *E. fetida* in the first test after 28 days, the second test, as intended to be acute, measured mortality after a shorter period (14 days) and mortality for the same concentration was lower, slightly above 50%, hence DL50 was reported as >1000 L/ha.

Evidently, commercial fungicide toxicological effects are sub estimated if assessed by the toxicity of their active substances when it comes to acute effects. If these effects were additive, neither of the commercial fungicides would have reached a dose in this test containing LD_{50}, according to the active substances concentration in the commercial fungicide, and their reported individual acute lethal toxicity (table 1). However, almost 100% 28-day lethality was observed in the first test for both fungicides at the highest dose. In the second test, 14-day LD_{50} for P was reached at the highest dose, in the case of *E. fetida*, which is the standard toxicity test earthworm. Particularly, the highest dose of P contained the amount of each active substance equivalent to one fifth of the reported LD_{50}. Had the effects been additive, we would have expected this highest dose to be two fifths of the LD_{50}. Yet, more than 50% of the earthworms died. Therefore, either the effects of the two active substances mixed in P have a synergistic deleterious effect on earthworms, or other components of this commercial fungicide are also affecting earthworm survival.

Conversely, based on active substance sub lethal toxicity for reproduction (table 1), we expected to observe negative effects from the third dose ($SP10^1$ and $P10^2$), but in the results of this experiment only the highest dose presented an observable negative effect. Hence, ID_{25} were higher than expected. Mismatches can also be attributed to the fact that two different estimators are being compared (NOEC and ID_{25}).

As far as we know, this is the first report of a toxicity test performed on South American native earthworms of the Glossoscolecidae family. Due to the high mortality of *Glossoscolex rione* obtained in the control treatment, the lethality results of P for this species must be considered preliminary, and further studies should be carried out for corroboration. High mortality in the control could be attributed to the

fact that these individuals were collected from the field, from different sites, and not bred in the laboratory. Hence, future efforts should be directed to adjust laboratory breeding and survival conditions for this species, and assess levels of acceptance of the artificial soil. The previous study which reported high survival of *G. rione* in experimental conditions for several weeks had used natural soil in the experiment (chapter 4). The present preliminary results suggest that this native endogeic earthworm may be more sensitive to the tested fungicide than the standard test earthworm, with its DL_{50} being one order of magnitude lower than that found for *E. fetida*. Although not confirmed yet, this is an issue to highlight, especially concerning native fauna conservation. Aware of the fact that different species may differ in their tolerance to toxicants, Kuperman et al. (2009) suggests that in order to gain ecological relevance, ecotoxicological research should "include species that are geographically and ecologically representative of the location and conditions at the site". Testing alternative earthworms, it has been found that *Ponthoscolex corethrurus* (Pontoscolecidae family) was equally sensitive, and *Perionyx excavatus* (Megascolecidae family) less sentitive, than *E. andrei* in avoindance and/or lethality tests (Buch et al., 2013; De Silva & Gestel, 2009), whereas *L. terrestris* and *A. caliginosa* were more sensitive than the standard species.

No effect was observed in the "positive control" in the sub lethal test, in which a field dose was used; or in the lethal test, where a dose within the range suggested by ISO standards was used. Although this second dose was 6 times greater than that used in the previous test, it was still below the IUPAC 14-day LD_{50} for carbendazim (5.4 mg/kg). Besides, it is important to note that in these experiments carbendazim was applied as Cibencarb (Ci), a commercial mix with 500 mg/L of carbendazim. Possibly, there might be a cocktail effect which could either emphasize or mitigate the effect of the isolated toxic substance (Relyea, 2009).

In these experiments, field doses of SP, P and Ci had no negative effect on earthworm survival, growth or reproduction. Assessment was restricted to a limited time (14, 28, or 56 days) and to one single application of one single fungicide.

However, in the field during a crop cycle more than one application of the same fungicide occurs, and fungicides have proved to increase in toxicity to earthworms when frequency of application increases (Tu et al., 2011). In addition, these will be combined with a whole set of other pesticides (insecticide and herbicides). Interactions of these agrochemical applications have proved to decrease earthworm activity (Van Hoesel et al., 2017), not to mention long-term residual contamination of agricultural soils. The cocktail effect on earthworms, produced by all the pesticides applied for crop production needs to be addressed in long term-studies, so as to arrive to a more accurate estimation of the effect on earthworms of chemical management as a whole.

From the results of the tests with *E. fetida,* we conclude that the toxicity to earthworms of commercial fungicides may be far higher than the sum of the toxicities of their active substances assessed individually. Regarding the toxicity effect of the tested fungicide on *G. rione,* although there is still high uncertainty, the preliminary results suggest that this native species might have a higher sensitivity compared to the standard test organism. Further research should be done with *G. rione* to obtain consistent results and confirm the bioassay application. These results are promising and go in the same sense of raising awareness of how toxicity of agrochemicals is currently underestimated, despite all the regulation and standardizing efforts. This study has given a new insight to the estimation of commercial products, which is important to study beyond the toxicity of active ingredients solely.

5.2.5. References

Bailer AJ, Hughes MR, Denton DL, Oris JT. 2000. Annual Review. An empirical comparison of effective concentration estimators for evaluating aquatic toxicity test responses. Environmental Toxicology and Chemistry. 19(1): 141–150.

Balzarini MG, Gonzalez L, Tablada M, Casanoves F, Di Rienzo JA, Robledo CW. 2008. Infostat: Manual del Usuario. Córdoba, Argentina. Editorial Brujas. 318p.

Blouin M, Hodson ME, Delgado EA, Baker G, Brussaard L, Buttf KR, Dai J, Dendooven L, Peres G, Tondoh JE, Cluzeau D, Bru NJ-J. 2013. A review of earthworm impact on soil function and ecosystem services. European Journal of Soil Science. 64(2):161–182.

Brown G, Pashanasi B, Villenave C, Patron JC, Senapati BK, Giri S, Barois I, Lavelle P, Blanchart E, Blakemore RJ, Spain AV, Boyer J. 1999. Effects of earthworms on plant production in the tropics. In: Lavelle P, Brussaard L, Hendrix P. (Eds.). Earthworm Management in Tropical Agroecosystems. Wallingford: CABI Publishing. 87-137.

Buch AC, Brown GG, Niva CC, Sautter KD, Sousa JP. 2013. Toxicity of three pesticides commonly used in Brazil to Pontoscolex Corethrurus. (Müller, 1857) and *Eisenia andrei* (Bouché 1972). Applied Soil Ecology. 69: 32–38.

Calabrese EJ, Baldwin LA. 1997. The dose determines the stimulation (and poison): development of a chemical hormesis database. International Journal of Toxicology. 16: 545–559.

Cordero EH. 1943. Oligoquetos sudamericanos de la familia Glossoscolecidae, I. El género *Glossoscolex* en el Uruguay, con una sinopsis de las especies del grupo truncatus. Comunicaciones Zoológicas del Museo de Historia Natural de Montevideo. 20:1-9.

De Silva PMCS, Van Gestel CAM. 2009. Comparative sensitivity of *Eisenia andrei* and *Perionyx excavatus* in earthworm avoidance tests using two soil types in the tropics. Chemosphere. 77:1609–1613.

Domínguez A, Brown GG, Sautter KD, Ribas De Oliveira CM, De Vasconcelos EC, Niva CC, Bartz MLC, Bedano JC. 2016. Toxicity of ampa to the earthworm *Eisenia andrei* Bouché, 1972 in tropical artificial soil. Scientific Reports. 6: 1-9.

ECHA (European Chemicals Agency) . 2018. Legislation. [On line]. 3 April 2018. http://ec.europa.eu/environment/chemicals/reach/legislation_en.htm#legal

Environment Canada. 2005. Guidance Document on statistical methods for environmental toxicity tests. Method Development and Applications Section. Environmental Technology Centre. Ottawa, Ontario.

Environment Canada. 2004. Biological Test Method: Tests for Toxicity of Contaminated Soil to Earthworms (Eisenia andrei, Eisenia fetida, or Lumbricus terrestris). Method Development and Applications Section. Environmental Technology Centre. Ottawa, Ontario. 184p.

García M, Römbke J, De Brito MT, Scheffczyk A. 2008. Effects of three pesticides on the avoidance behaviour of earthworms in laboratory test performed under temperate and tropical conditions. Environmental Pollution. 153: 450–456.

Grosso EG, Brown GG. 2007. Biodiversidad y ecología de las lombrices de tierra en el Uruguay. In: Brown GG, Fragoso C. (Eds.). Minhocas na América Latina: Biodiversidade e ecologia. Londrina: EMBRAPA. 287-296.

Grubbs FE. 1969. Procedures for detecting outlying observations in samples. Technometrics. 11(1): 1-21.

ISO (International Organization for Standardization). 2012. Soil quality – Effects of pollutants on earthworms – Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia Andrei and Part 2: Determination of effects on reproduction of Eisenia fetida/Eisenia andrei. ISO 11268-1 & ISO 11268-2, Geneva, Switzerland.

ISO (International Organization for Standardization). 2008. Soil quality – Avoidance test for determining the quality of soils and effects of chemicals on behaviour – Part 1: Test with earthworms (Eisenia fetida and Eisenia andrei). ISO 17512-1. Geneva, Switzerland.

Kuperman RG, Checkai RT, Bastos García MB, Römbke J, Stephenson GL, Sousa JP. 2009. State of the science and the way forward for the ecotoxicological assessment of contaminated land. Pesquisa agropecuaria brasileira. 44(8): 811-824.

Ljungström PO. 1972. Biology of *Glossoscolex uruguayensis* (Glossoscolecidae, Oligochaeta). A new species for Argentina. Studies on Neotropical Fauna & Environment. 7: 195-205.

Machado FJ, Del Ponte EM. 2016. Fungicide efficacy for managing Fusarium head blight of wheat in Brazil: systematic review and meta-analysis. In: Proceedings P47, Fifth ISFHB: Session 4 – Epidemiology and Management. Florianopolis. 94 - 94.

MGAP (Ministrerio de Ganadería Agricultura y Pesca). 2017. Autorización de venta de productos fitosanitarios. [On line]. 3 April 2018. http://www.mgap.gub.uy/unidad-ejecutora/direccion-general-de-servicios-agricolas/tramites-y-servicios/servicios/productos-fitosanitarios/autorizacion-de-venta

OECD (Organization for Economic Cooperation and Development). 1984. Guideline for the Testing of Chemicals No. 207. Earthworm Acute Toxicity Tests. Paris, France. 9 p.

OSHA (Occupational Safety and Health Administration). 2017.Comparison of Hazard Communication Requirements. OSHA Hazard Communication Standard 29 CFR 1910.1200 (HCS) Globally Harmonized System (GHS.) [On line]. 3 April 2018. https://www.osha.gov/dsg/hazcom/ghoshacomparison.html

Pelosi C, Joimel S, Makowski D. 2013. Searching for a more sensitive earthworm species to be used in pesticide homologation tests – A meta-analysis. Chemosphere. 90: 895–900.

Relyea RA. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia. 159: 363–376.

Santadino M, Coviella C, Momo F. 2014. Glyphosate sublethal effects on the populations dynamics of the earthworm *Eisenia fetida* (Savigny, 1826). Water Air Soil Pollution. 225(2207):1-8.

Scheu S. 2003. Effects of earthworms on plant growth: patterns and perspectives. Pedobiologia. 47(5-6): 846-856.

Tu C, Wang Y. Duan W, Hertl P, Tradway L, Brandenburg R, Lee D, Snell M, Hu S. 2011. Effects of fungicides and insecticides on feeding behavior and community dynamics of earthworms: Implications for casting control in turfgrass systems. Applied Soil Ecology. 47: 31–36.

Van Hoesel W, Tiefenbacher A, König N, Dorn VM, Hagenguth JF, Prah U, Widhalm T, Wiklicky V, Koller R, Bonkowski M, Lagerlöf J, Ratzenböck A and Zaller JG. 2017. Single and combined effects of pesticide seed dressings and herbicides on earthworms, soil microorganisms, and litter decomposition. Frontiers in Plant Science. 8:215. doi: 10.3389/fpls.2017.00215.

Wang C, Sun Z, Liu Y, Zhang X, Xu G. 2007. A novel antimicrobial vermipeptide family from earthworm Eisenia fetida. European Journal of Soil Biology. 43: 127-134.

6: DISCUSIÓN GENERAL

6.1. PRINCIPAL APORTE DEL PRESENTE TRABAJO

El presente estudio permitió identificar las especies de lombrices presentes en suelos que incluían trigo en su rotación, en los departamentos de Montevideo y Paysandú, así como comprobar que los factores que determinan la composición de la comunidad son diversos, entre los cuales C, N y humedad del suelo, se confirman como los factores más influyentes y correlacionados con la diversidad, riqueza, biomasa y densidad. Se pudo cuantificar el impacto de especies exóticas, de la familia Lumbricidae, sobre la presión de inóculo de *Fusarium*, considerando su potencialidad para contribuir en el control biológico del mismo. Se determinó además, según estándares internacionales, el efecto toxicológico agudo y crónico sobre dos especies de lombrices de los fungicidas utilizados para controlar este fitopatógeno.

El concepto de control biológico mediante el uso de lombrices, resulta novedoso por la escasa literatura escrita en español y es de gran aplicabilidad en el desarrollo de sistemas de agricultura sustentable y agroecológica. Al publicar la revisión bibliográfica realizada para esta Tesis, en español y en una revista reconocida a nivel regional, se acercó al mundo hispano-parlante una compilación de resultados de estudios que demuestran cómo las lombrices pueden tener un efecto positivo significativo sobre la sanidad vegetal, al suprimir ciertas plagas y enfermedades, o influir directamente sobre el sistema de defensa de las plantas (Jorge-Escudero, Lagerlöf & Pérez, *en prensa*).

El estudio de las lombrices aplicado a la agricultura, con un enfoque holístico que integra la interacción de éstas con los cultivos, con los microorganismos (benéficos o patógenos) y con los manejos químicos realizados representa una línea de investigación innovadora para el país y podría aportar una nueva herramienta a integrar en el manejo de la presión del inóculo de este fitopatógeno y probablemente otros de gran importancia a nivel nacional y mundial, pudiendo a su vez ser modelo para otros patosistemas.

6.2. APORTES AL CONOCIMIENTO DE LA DIVERSIDAD NACIONAL DE LOMBRICES

Trece de las 19 especies de lombrices descritas para Uruguay se encontraron en suelos agrícolas y áreas circundantes menos perturbadas de Montevideo y Paysandú. Además, se encontraron cuatro especies aún no registradas para Uruguay, las cuales son reportadas en el artículo a publicarse correspondiente a la sección 4.2. Este primer registro corresponde a las especies nativas *Microscolex phosphoreus* y *Glossodrilus parecis*, y a las exóticas *Aporrectodea tuberculata* y *Murchieona minuscula*. El hallazgo de especies aún no registradas a nivel nacional en un número reducido de sitios muestreados, evidencia la escasa cobertura de muestreo que presentan los paisajes antropizados y naturales del país. El presente estudio registra, además, por primera vez lombrices exóticas en el departamento de Paysandú, ya que allí se encontraron varios especímenes de *Ap. caliginosa y de M. murchieona*, ambas de la familia Lumbricidae, siendo la primera, la especie más ubicua en ambos departamentos.

La relación exóticas:nativas de las lombrices varió en función de la ubicación y del grado de perturbación de los sitios muestreados, al comparar parcelas agrícolas y áreas circundantes menos perturbadas en Montevideo y Paysandú. Las exóticas dominaron tanto en la agricultura como en las parcelas de control en el departamento de Montevideo, ubicado más cercano al puerto natural, puerta de entrada a la mayor inmigración al país. No obstante, en Paysandú los sitios muestreados estaban dominados por nativas y, curiosamente, dominaban más en las parcelas agrícolas que en las parcelas de control. Sin dejar de reconocer la dificultad que presentó la selección de parcelas control en estado prístino y que a la vez mantuvieran las otras variables de suelo comparables a las de las rotaciones, el hecho que las especies exóticas no dominen en los campos agrícolas en Paysandú deja abierta la pregunta si el éxito de la invasión de exóticas en esta localidad ha sido limitado por el bajo ingreso de especies exóticas, por el tiempo insuficiente para alcanzar el dominio, por

condiciones climáticas, o por competencia, dado que se ha comprobado que las especies nativas presentes en Paysandú toleran las condiciones de la agricultura y, por lo tanto, pueden convivir con las exóticas sin ser desplazadas por competencia.

Elucidar si las especies nativas han sido desplazadas por exóticas y/o perturbaciones, representa un desafío ya que pocos investigadores han estudiado los oligoquetos en este país (Grosso, Jorge & Brown, 2006). Se carece de un estudio sistemático de las lombrices nativas presentes en diferentes ecosistemas no perturbados, los cuales pudieran generar una línea de base. Probablemente, con un mayor esfuerzo de muestreo e investigación, el reducido inventario nacional lombrices actual se podría extender, particularmente dentro del grupo de nativas, ya que la mayoría de los estudios se han llevado a cabo en agroecosistemas, donde se ha visto que las exóticas son más competitivas (Zerbino et al., 2008; Grosso, Jorge & Brown, 2006); Zerbino Rodríguez & Altier, 2006).

6.3. APORTES A LA METODOLOGÍA DE IDENTIFICACIÓN DE ESPECIES POR MÉTODOS MOLECULARES

Las técnicas moleculares junto con la morfología permitieron una identificación completa de varias especies de lombrices encontradas en los dos departamentos muestreados (Jorge-Escudero et al., *en prensa*). La posibilidad de identificar especies mediante el "código de barras de ADN" (DNA *Barcoding*) se ha hecho más accesible últimamente (Chang, Rougerie, & Chen, 2009; Pop, Wink, & Pop, 2003), con un repositorio importante de secuencias disponibles en internet (por ejemplo, GenBank). Sin embargo, la identificación de especies por *Barcoding* se basa en el hecho de que las secuencias de las especies estudiadas hayan sido previamente cargadas y publicadas. La mayor dificultad encontrada fue el número limitado de secuencias de especies nativas uruguayas disponibles en GenBank, además de haber sólo una especie de lombriz a nivel mundial cuya secuencia COI ha sido anotada en RefSeq (banco de secuencias más confiable que GenBank, ya que las secuencias de RefSeq las han sido curadas por el staff del Centro de Información de Biotecnología de los

Estados Unidos (NCBI)). Superar la limitación que representa el reducido número de secuencias especies de lombrices nativas en GenBank constituye un primer paso y requerirá un estudio morfológico detallado con una identificación precisa de las morfoespecies, la generación de las secuencias correspondientes y la consiguiente expansión de esa base de datos para las especies nativas.

6.4. APORTES A LA EVALUACIÓN DE LAS LOMBRICES COMO POTENCIALES AGENTES DE CONTROL BIOLÓGICO

Las especies seleccionadas en Suecia para evaluar su efecto sobre *Fusarium* fueron: *Lumbricus rubellus, L. terrestris* y *Aporrectodea longa*, las tres de la Familia Lumbricidae, de origen Europeo; la primera epígea y las dos siguientes del grupo ecológico anécico. Su crecimiento o actividad no se vieron afectados por *Fusarium graminearum* (incluidas sus toxinas) durante la experimentación, y las tres contribuyeron a la reducción de *F. graminearum* en los residuos del cultivo de trigo al disminuir la cantidad de rastrojo de trigo en la superficie en condiciones de humedad y alimento óptimos, por lo que redujeron el sustrato para la supervivencia del patógeno. Asimismo, *L. rubellus* también tuvo la capacidad de reducir la cobertura de rastrojo de trigo en la superficie del suelo cuando tales condiciones eran subóptimas. Además, *L. rubellus* y *A. longa* redujeron el inóculo del patógeno en el rastrojo, muy probablemente a través de la ingesta del hongo, cuando no se ofrecía ninguna otra fuente de alimento.

El estudio de la respuesta de las lombrices en condiciones subóptimas de humedad y disponibilidad de alimentos, como se hizo en el Experimento 1 en SLU, simula las condiciones de campo, en suelos sin enmiendas orgánicas, en períodos de precipitación escasos. Estas variables son importantes para incluir en estudios de laboratorio, particularmente en el caso de *F. graminearum*, ya que es capaz de sobrevivir en una amplia gama de condiciones ambientales (Ramirez et al., 2006; Burgess y Griffin, 1968). Este estudio representa el primer trabajo que investiga la

contribución de lombrices al control biológico de *F. graminearum* en condiciones ambientales contrastantes y brinda información importante para la comprensión aplicada de este nuevo enfoque.

Concluimos que las lombrices anécicas y las epígeas testeadas tuvieron potencial para contribuir a la reducción del inóculo de *F. graminearum* en el rastrojo de trigo, tanto en condiciones óptimas como subóptimas. Sus mecanismos de acción o la magnitud de esta contribución dependerán de las especies de lombrices y su respuesta a las condiciones ambientales. El efecto de las lombrices sobre *F. graminearum* puede maximizarse aún con un manejo del suelo respetuoso con el ambiente que favorezca las poblaciones de lombrices, así como incorporación de manejos prácticos como triturar los residuos de los cultivos a la hora de la cosecha, para facilitar que las lombrices se alimenten de ellos y otros hongos saprófitos puedan colonizarlos desplazando por competencia a *F. graminearum* y otros patógenos de plantas.

Las especies seleccionadas en Uruguay para evaluar su efecto sobre *Fusarium* fueron: *Lumbricus friendi,* anécica, exótica, similar a *L. terrestris*, pero más pequeña; y *Glossoscolex rione*, endógea, nativa, de tamaño similar a *Aporrectodea caliginosa.* La actividad notoria de *L. friendi* en la superficie, a pesar de su alta mortalidad, redujo más del 30% de la cobertura por rastrojo en la superficie del suelo, por lo que mostró potencial para contribuir al control biológico de *F. graminearum* en el rastrojo de trigo. Sin embargo, el tratamiento con *G. rione* no difirió del control, probablemente debido al hecho de que esta lombriz es de hábito endógeo y no suele subir a la superfice. Siendo éste el primer experimento reportado con *G. rione*, su alta supervivencia se registró como un éxito. Las lombrices en el experimento en Uruguay tampoco se vieron negativamente afectadas por *Fusarium* o sus toxinas. Dado que las cantidades del ADN del patógeno estaban por debajo de los niveles de detección en el presente estudio, probablemente debido a la degradación causada por una falla en la conservación de las muestras, será necesaria una repetición del experimento para confirmar si este efecto de control del patógeno también se

extiende a la reducción del inóculo de *Fusarium* en el rastrojo remanente en superficie.

6.5. APORTES AL CONOCIMENTO DE LOS IMPACTOS TOXICOLÓGICOS SOBRE LAS LOMRBICES DE LOS FUNGICIDAS COMERCIALES

Un aumento en el uso de fungicidas, debido a un área creciente con sistemas de siembra directa, donde el rastrojo en la superficie favorece la incidencia de la Fusariosis de la espiga, podría contrarrestar la minimización de los impactos ambientales que este manejo de conservación del suelo ofrece. Las lombrices, que se benefician con la reducción del laboreo, tienen el potencial de contribuir al control biológico de la Fusariosis. Por lo tanto, es interesante saber cómo este grupo se ve afectado por los fungicidas utilizados para controlar esta patología. Aunque las regulaciones de comercialización requieren estudios de toxicidad sobre lombrices de los principios activos de los agroquímicos, el efecto de cóctel de las formulaciones comerciales que combinan varios principios activos, con otros componentes (como solventes y adyuvantes), no suele estudiarse para este grupo. En el presente trabajo se seleccionaron los dos fungicidas comerciales, más comúnmente usados para el control de la Fusariosis en Uruguay (Prosaro y Swing Plus) a fin de evaluar su excotoxicidad sobre lombrices según los estándares internacionales determinados por las normas ISO 11268. La concentración de inhibición del 25% de la progenie de *E. fetida* se estimó en 212 L / ha para Swing Plus y en 700 L / ha para Prosaro, y para este último, la dosis letal a los 14 días fue > 1000 L / ha. Además se realizó un ensayo preliminar de toxicidad con la especie nativa *G. rione.* Si bien se debe repetir el ensayo con la especie nativa, ajustando la aclimatación al sustrato experimental, con el fin de obtener una mayor sobrevivencia en el tratamiento control, se encontró que probablemente la lombriz nativa sea mucho más sensible al fungicida Prosaro que la estándar, *E. fetida*, con valores preliminares de dosis letal a los 14 días de 174 L / ha.

Se observó que los efectos toxicológicos de las formulaciones comerciales de los fungicidas se subestiman si se evalúan por la sumatoria de la toxicidad individual de sus principios activos. Sorprendentemente, se obtuvieron efectos letales para concentraciones que, según los principios activos, sumarían un quinto de la concentración requerida para ser la dosis letal del 50 %. Esto puede deberse a que sus efectos no son aditivos, sino que se potencian en combinación (ya que las formulaciones estudiadas combinaban dos principios activos) o que otros componentes del formulado pudieran potenciar el efecto letal de los principios activos o tener un efecto letal en sí mismo, no estudiado.

6.6. CONCLUSIÓN Y PERSPECTIVAS

Se concluye que hay una mayor riqueza de lombrices presentes en los agroecosistemas que la esperada, representando éste el primer estudio en identificar las especies combinando caracteres morfológicos con técnicas moleculares. Los resultados experimentales indican que especies con hábitos de vida anécico y epígeo presentan interacción con el rastrojo en superficie pudiendo afectar la cantidad del mismo y del inóculo de patógenos allí presentes. Por último, los resultados toxicológicos indican que los fungicidas utilizados podrían afectar la sobrevivencia, el crecimiento y la reproducción de las lombrices. No obstante, a la dosis normalmente utilizada a campo, con una única aplicación y en ausencia de otros agroquímicos, los fungicidas no tuvieron un efecto medible sobre las lombrices en el tiempo que duraron los ensayos. Resta estudiar el impacto acumulativo de sucesivas aplicaciones ya sea de fungicidas utilizados para esta enfermedad, como de otros agroquímicos que pudieran afectar el normal desarrollo de las lombrices.

6.7. PROYECCIONES A FUTURO

Los resultados actuales muestran un desafío futuro para continuar descubriendo la riqueza local de lombrices, lo que implicará no sólo un mayor esfuerzo de muestreo, sino también expandir la diversidad de ambientes y ecosistemas muestreados, en particular los ambientes prístinos, buscando poder recomponer una línea de base de especies nativas que permita estudiar el fenómeno de las invasivas con un marco de referencia. Por otro lado, realizar el análisis filogenético de las especies secuenciadas, incluyendo secuencias de las mismas especies encontradas en otros países, permitirá introducirse en el estudio de la existencia de posibles especies crípticas (Martinsson & Erseus, 2017; Spurgeon et al., 2016). Agregar más especies nativas a la base de datos en línea (GenBank) representa otro desafío futuro. Por lo tanto, es esencial fomentar la capacitación en taxonomía de lombrices, ya que la adopción de técnicas moleculares no implica que se pueda prescindir de la taxonomía tradicional basada en características morfológicas. Los escasos registros de muestreos de lombrices en Uruguay, y la ausencia de estudios nacionales que hayan identificado especies por secuencias de ADN, hacen que este estudio preliminar sea un puntapié inicial para un programa de investigación local innovador (Jorge-Escudero et al., *en prensa*).

La experimentación con especies nativas, ya sea estudiando su efecto sobre el control de patógenos, o en la evaluación de cómo son afectadas por los agroquímicos deberá seguir perfeccionándose a fin de confirmar los resultados preliminares obtenidos en esta investigación. Profundizar particularmente en la investigación del efecto de las lombrices locales sobre el inóculo de *Fusarium*, así como su interacción con otros agentes propuestos para control microbiológico (Pérez et al., 2008), permitirá contar con una tecnología que aporte al manejo integrado y/o agroecológico, sumando una alternativa más, tendiente a disminuir el inóculo y por consiguiente disminuir el riesgo de epidemias, lo cual eventualmente podría reducir la necesidad del uso de fungicidas, contribuyendo a la producción sustentable (con minimización de impactos económicos, ecológicos, y sociales), y al sello “Uruguay Natural”. Además

se podrá profundizar el estudio de los efectos de los fungicidas sobre las lombrices, analizando otros aspectos, no considerados aquí, como ser daños a nivel histológico, daños en el ADN o cambios a nivel de marcadores biológicos (Gong & Perkins, 2016; Schwarzbacherová et al., 2015).

Todos estos estudios se deberán llevar a cabo en interacción con equipos interdisciplinarios, fomentando el intercambio de saberes y afianzando los vínculos inter-institucionales generados a partir de este trabajo (Departamentos de Sistemas Ambientales, de Suelos y Aguas, y de Protección Vegetal, dentro de la Facultad de Agronomía, y a su vez, los vínculos de esta Facultad con la Facultad de Ciencias, la Facultad de Química, y la fundación Latitud-LATU). Finalmente, una vez comprobado el efecto de las lombrices locales en la reducción del inóculo de *Fusarium* en condiciones de laboratorio, deberá llevarse la experiencia a escala de campo, enfrentando la complejidad presentada por esta transición y evaluando la aplicabilidad de este control biológico como una alternativa aplicable al manejo de los cultivos.

Por lo antedicho, el presente proyecto buscó generar conocimiento dirigido a un mejor conocimiento de las lombrices locales, a fin de lograr su mejor aprovechamiento por los servicios ecosistémicos que nos brindan, en particular en la regulación de enfermedades, reduciendo potencialmente el riesgo sanitario en uno de los principales cultivos agrícolas de Uruguay, como una forma de explorar alternativas ambientalmente amigables, utilizables en la producción integrada y agreocológica.

BIBLIOGRFÍA

Ayuke FO, Lagerlöf J, Jorge G, Söderlund S, John Muturi J, Sarosh BR, Meijer J. 2017. Effects of biocontrol bacteria and earthworms on the severity of *Alternaria brassica*e disease and the growth of oilseed rape plants (*Brassica napus*). Applied Soil Ecology. 117:63 – 69.

Burgess LW, Griffin DM. 1968. The recovery of *Gibberella zeae* from wheat straws. Australian Journal of Experimental Agriculture & Animal Husbandry. 8: 364-370.

Chang C-H, Rougerie R, Chen J-H. 2009. Identifying earthworms through DNA barcodes: Pitfalls and promise. Pedobiologia. 52:171–180.

DIEA (Dirección de Estadísticas Agropecuarias). 2016. Producción [En línea]. En: Anuario estadístico agropecuario 2006. Montevideo: MGAP (Ministerio de Ganadería, Agricultura y Pesca). Consultado 28 setiembre 2017. Disponible en: Anuario Estadístico Agropecuario 2016. MGAP. http://www.mgap.gub.uy/unidad-ejecutora/oficina-de-programacion-y-politicas-agropecuarias/publicaciones/anuarios-diea/anuario2016.

Gong P, Perkins EP. 2016. Earthworm toxicogenomics: A renewed genome-wide quest for novel biomarkers and mechanistic insights. Applied Soil Ecology. 104: 12–24.

Grosso EG, Brown GG. 2007. Biodiversidad y ecología de las lombrices de tierra en el Uruguay. En: Brown GG, Fragoso C. (Eds.). Minhocas na América Latina: Biodiversidade e ecologia. Londrina: EMBRAPA. 287-296.

Grosso EG, Jorge G, Brown GG. 2006. Exotic and Native Earthworms in Various Land Use Systems of Central, Southern and Eastern Uruguay. Caribbean Journal of Science. 42(3): 294-300.

IUPAC (International Union of Pure Applied Chemistry). 2017. [En línea]. En: Acgrochemicals: General Information Propoerties. Consultado 28 setiembre 2017. Disponible en: http://agrochemicals.iupac.org/

Jorge-Escudero G, Lagerlöf J, Pérez CA. 2018. Control biológico de enfermedades y plagas promovido por lombrices. Revista Brasileria de Agroecologia. 13 (4): e*n prensa*.

Jorge-Escudero G, Pérez C, Martínez C, Lagerlöf J. 2018. Identification of earthworm species in Uruguay based on morphological and molecular methods. Agrociencia Uruguay. *en prensa.*

Lagerlöf J, Ayuke F, Bejai S, Jorge G, Lagerqvist E, Meijer J, Muturi John J. 2015. Potential side effects of biocontrol and plant-growth promoting *Bacillus amyloliquefaciens* bacteria on earthworms. Applied Soil Ecology. 96: 159 - 164.

Martinsson S, Erseus C. 2017. Cryptic speciation and limited hybridization within Lumbricus earthworms (Clitellata: Lumbricidae). Molecular Phylogenetics & Evolution. 106: 18–27.

Oldenburg E, Kramer S, Schrader S, Weinert J. 2008. Impact of the earthworm *Lumbricus terrestris* on the degradation of *Fusarium*-infected and deoxynivalenol-contaminated wheat straw. Soil Biology & Biochemistry. 40: 3049-3053.

Pereyra, S. 2013. Herramientas disponibles para el manejo de dos enfermedades relevantes de la pasada zafra: Fusariosis de la espiga en trigo y Ramularia en cebada. En: Jornada Cultivos de Invierno (2013, Young, UY). "Herramientas para un manejo inteligente en trigos y cebadas". La Estanzuela, INIA Serie Actividades de Difusión, Colonia, Uruguay. 720: 33-41.

Pérez C, Dill-Macky R, Kinkel LL. 2008. Management of soil microbial communities to enhance populations of *Fusarium graminearum*-antagonists in soil. Plant & Soil. 302 :53–69.

Pop AA, Wink M, PopVV. 2003. Use of 18S, 16S rDNA and cytochrome c oxidase sequences in earthworm taxonomy (Oligochaeta, Lumbricidae). Pedobiologia. 47: 428–433.

Ramirez ML, Chulze S, Magan N. 2006. Temperature and water activity effects on growth and temporal deoxynivalenol production by two Argentinean strains of

Fusarium graminearum on irradiated wheat grain. International Journal of Food Microbiology. 106(3): 291–296.

Schwarzbacherová V, Šiviková K, Drážovská M, Dianovský J. 2015. Evaluation of DNA damage and cytotoxicity induced by triazole fungicide in cultured bovine lymphocytes. Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics. 68(3): 233–238.

Spurgeon DJ, Liebeke M, Anderson C, Kille P, Lawlor A, Bundy JG, Lahive E. 2016. Ecological drivers influence the distributions of two cryptic lineages in an earthworm morphospecies. Applied Soil Ecology. 108: 8–15.

Stewart S, Pereyra S, Díaz M. 2004. El efecto de la intensificación agrícola en las enfermedades de los cultivos. En: Simposio: Sustentabilidad de la intensificación agrícola en Uruguay (2004, Mercedes, Uruguay). Resúmenes del Simposio. Mercedes, Uruguay. INIA. 19-24.

Umpiérrez M, Garmendia G, Cabrera M, Pereyra S, Vero S. 2013. Diversity of Pathogen Populations Causing Fusarium Head Blight of Wheat in Uruguay. En: Alconada Magliano MT, Chulze SN. (Eds.). Fusarium Head Blight in Latin America. Dordrecht. Springer. 32-44.

UPEP (Unidad de Posgrados y Educación Permanente, Facultad de Agronomía). 2017. Guía para la presentación de trabajos finales de Posgrado. Montevideo, Uruguay. 25p.

Villar HA, Ernst O, Cadenazzi M, Vero S, Altier N, Pereyra S, Langone F, Chouhy D, Pérez CA. 2012. Dinámica de la población nativa de Trichoderma spp. bajo distintas secuencias de cultivos, en sistemas de agricultura continua. En: Jornada Bianual de Fitopatología (2da, 2012, Montevideo, Uruguay). Impacto de la intensificación de los cultivos en el desarrollo de las enfermedades. Montevideo. SUFIT. p12.

Villar HA, Robaina F, Vero S, Altier N, Pereyra S, Pérez CA. 2014. Efecto de la aplicación a campo de *Trichoderma* sobre la densidad de inóculo de patógenos de trigo. Congreso Argentino de Fitopatología (3er, 2014, San Martín de Tucumán, Argentina). Libro de resumenes del III Congreso Argentino de Fitopatología. Tucuman, Argentina. Santamarina & Asociados. p. 430.

Wolfarth F, Schrader S, Oldenburg E, Weinert J, Bruttone J. 2011. Earthworms promote the reduction of *Fusarium* biomass and deoxynivalenol content in wheat straw under field conditions. Soil Biology & Biochemistry. 43: 1858-1865.

Yuen G Y, Schoneweis SD. 2007. Strategies for managing *Fusarium* head blight and deoxynivalenol accumulation in wheat. International Journal of Food Microbiology. 119: 126-130.

Zerbino MS. 2012. Efecto de rotaciones cultivo-pasturas en siembra directa, con pastoreo, sobre comunidades de Oligochaeta. Agrociencia Uruguay. 16(1): 15-23.

Zerbino MS. 2010. Evaluación de la macrofauna del suelo en rotaciones cultivo-pasturas con laboreo convencional. Acta Zoológica Mexicana. 26: 189-202.

Zerbino MS. 2005. Evaluación de la densidad, biomasa y diversidad de la macrofauna del suelo en diferentes sistemas de producción. Tesis Maestr. Cien. Amb. Montevideo, Uruguay. Facultad de Ciencias, Universidad de la República. 92p.

Zerbino MS, Altier N, Morón A, Rodríguez C. 2008. Evaluación de la macrofauna del suelo en sistemas de producción en siembra directa y con pastoreo. Agrociencia Uruguay. 1: 44-55.

Zerbino MS, Rodríguez C, Altier N. 2006. Oligochaeta communities in Uruguayan Agro-ecosystems. Caribbean Journal of Science. 42: 315 – 324.