Universidad de Concepción
Dirección de Postgrado
Facultad de Ciencias Ambientales, Centro EULA-Chile
Programa de Doctorado en Ciencias Ambientales mención Sistemas Acuáticos Continentales

USO DE LA LOMBRIZ DE TIERRA COMO ORGANISMO INDICADOR DEL IMPACTO DE PESTICIDAS EN EL AGROSISTEMA

Tesis para optar al grado de Doctor en Ciencias Ambientales con Mención en Sistemas Acuáticos Continentales

ANA DELIA ARANEDA GÓMEZ
CONCEPCIÓN – CHILE
2016

Profesor Guía: **Dr. Ricardo Barra Ríos**
Facultad de Ciencias Ambientales-Centro EULA-Chile,
Universidad de Concepción, Chile

Esta Tesis ha sido realizada en el Laboratorio de Biomarcadores, Departamento de Sistemas Acuáticos de la Facultad de Ciencias Ambientales- Centro EULA-Chile, de la Universidad de Concepción.

Profesores Integrantes Comisión Evaluadora:

Dr. Ricardo Barra Ríos
Profesor guía
Facultad de Ciencias Ambientales EULA-Chile
Universidad de Concepción, Chile

Dr. Marco Sandoval
Evaluador interno
Facultad de Agronomía
Universidad de Concepción, Chile

Dr. Gustavo Chiang
Evaluador interno
Facultad de Ciencias Ambientales EULA-Chile
Universidad de Concepción, Chile

AGRADECIMIENTOS

Esta tesis doctoral pudo ser realizada gracias al apoyo de la Universidad de Concepción, específicamente la Facultad de Ciencias Ambientales, que me forjó en su programa y en sus instalaciones durante los 5 años del doctorado. Asimismo, quisiera agradecer al Grupo de Biomarcadores, que mediante la diversidad de formación nos permite lograr avances considerables en la investigación científica. Quiero hacer especial mención al Dr. Ricardo Barra R., por haberme dado la oportunidad de realizar esta tesis doctoral.

Mis reconocimientos a todos los profesores del programa de Doctorado en Ciencias Ambientales, quienes contribuyeron en mi formación profesional. Especialmente, a la Directora del programa de doctorado, Dra. Evelyn Habit, que en los últimos años ha demostrado gran interés y preocupación por cada uno de los alumnos del programa.

En especial quisiera agradecer a Nicholas Simian por permitir el ingreso a sus predios cada vez que lo necesite y que apoya fehacientemente la transferencia de conocimientos entre la Universidad y Comunidad.

No puedo dejar de mencionar a quienes me ayudaron, tendieron un hombro en momentos complejos del programa y se alegraron por mis logros, a mis queridos: Daniela Lopez, Rafaela Retamal, Daniela Pedraza, Rúben De la Hera y Melisa Villegas.

Finalmente dar las gracias al Dios de todas las formas y energías por moldearme.

SI NO ERES FELIZ, FRACASASTE COMO LOMBRIZ

ESTA TESIS FUE REALIZADA CON FINANCIAMIENTO OTORGADO POR:

PROYECTO AT- 21110229
Programa de Becas de Apoyo a la realización de la tesis Doctoral
Becas para Estudios de Doctorado en Chile

PROGRAMA APOYO ASISTENCIA A EVENTOS
Programa Becas de Estipendio
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PROYECTO CONICYT/FONDAP N°15130015
Beca Doctorado
Centro de Recursos Hídricos para la Agricultura y la Minería (CRHIAM)
Universidad de Concepción, Chile

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RESUMEN

El control químico de las plagas continúa siendo una estrategia necesaria en la agricultura. El uso intensivo de pesticidas genera una serie de riesgos ambientales en organismos no objetivos de importancia ecológica como las lombrices de tierra. En Chile, el sector agrícola ocupa un importante lugar en la economía del país, siendo necesario el uso de agroquímicos, y al mismo tiempo la protección del agrosistema.

Esta tesis abordo el estudio de los efectos biológicos de los pesticidas que se aplican en la agricultura convencional, desarrollada en la cuenca hidrográfica de Chillán. Para tal fin, se utilizó la lombriz de tierra, *Lumbricus terrestris*, especie conocida por sus características edáficas, agronómicas y toxicológicas como indicador de efectos adversos a la exposición de los pesticidas.

La metodología se centró en el estado actual de la agricultura en la ciudad de Chillán, y el registro de los agricultores orgánicos de huertos de manzano, para la comprensión de la adopción a la agricultura orgánica, este primer escenario, permitió posteriormente realizar comparaciones entre huertos de manzano con manejo convencional y orgánico a través de una serie de respuestas biológicas tanto a nivel bioquímico y molecular como los biomarcadores, para ello se utilizaron enzimas esterasas como las acetilcolinesterasas cuya función principal es hidrolizar el neurotransmisor acetilcolina y las carboxilesterasas, que están involucradas en la detoxificación de xenobióticos, además se complementó con respuestas a nivel del individuo (conductual) que consideraron ensayos en campo y laboratorio por un periodo de un año.

Los resultados de esta investigación indicaron que las principales motivaciones para la adopción de la agricultura orgánica es el factor económico, seguido por el cuidado del medio ambiente y la salud de las personas e identificaron barreras como la producción y la escasa relación con el sector público y académico como limitaciones para la adopción de la agricultura orgánica.

Las comparaciones realizadas entre los huertos de manzano tanto de manejo convencional como orgánicos evidenciaron que el uso de la enzima carboxilesterasa y acetilcolinesterasa son buenos biomarcadores de exposición de pesticidas organofosforados en *Lumbricus terrestris* que evidencio una diferencia estacional siendo mayor en invierno con 14.22 µmol min^{-1} mg^{-1} y en otoño con 13.93 µmol min^{-1} mg^{-1} y menor actividad en verano con 6.15 µmol min^{-1} mg^{-1} coincidiendo con la mayor aplicación de pesticidas en el huerto con manejo convencional. Esta actividad enzimática fue más contrastante en el tejido buche-molleja que en el

intestino anterior e intestino medio que no presentaron diferencias de actividad con el manejo convencional y orgánico de los suelos. Similar es lo que se evidencio en los ensayos de comportamiento, las lombrices de tierra evitaban los suelos de la época de primavera y verano.

Las esterasas de lombrices de tierra *Lumbricus terrestris* pueden ser una herramienta adecuada para efectos regulatorios y ambientales. En esta investigación, se sugiere el uso de esterasas de lombrices como biomarcadores para ser incluidos en una prueba de toxicidad de campo. Por otra parte, se postula que la secreción de las CbEs en el intestino lombriz de tierra podría ser una metodología respetuosa con el medio ambiente en la biorremediación enzimática de los suelos contaminados con pesticidas.

ABSTRACT

Chemical pest control continues to be a necessary strategy in agriculture. The intensive use of pesticides cause a series of environmental risks on non-target organisms ecologically important as earthworms. In Chile, the agricultural sector occupies an important place in the economy, requiring the use of agrochemicals, while also agrosystem protection.

This thesis approached the study of the biological effects of pesticides applied in conventional agriculture, developed in the watershed of Chillán. For this purpose the earthworm Lumbricus terrestris, a species known for their ecological, agronomic and toxicological characteristics, as an indicator of exposure adverse effects of pesticides was used.

The methodology focused on the current state of agriculture in the city of Chillan, and registration of organic farmers in apple orchards, for understanding the adoption of organic agriculture, this first stage, then allowed comparisons between orchards apple with conventional and organic management through a series of biological responses both biochemical and molecular level as biomarkers, for this purpose enzymes esterases as acetylcholinesterases whose main function is to hydrolyze the neurotransmitter acetylcholine and carboxylesterases were used, which are involved in detoxification of xenobiotics, also supplemented with responses at the individual level (behavioral) that considered in field and laboratory tests for a period of one year.

The results of this investigation indicated that the main reasons for the adoption of organic agriculture is the economic factor, followed by caring for the environment and health of people, and identified barriers such as production and scant relation to the public sector and academic, and limitations for the adoption of organic agriculture.

Comparisons between the apple orchards both from conventional and organic management showed that the use of carboxylesterase and acetylcholinesterase enzymes are good biomarkers of exposure to organophosphate pesticides in Lumbricus terrestris that showed a seasonal difference being greater in winter with 14.22 µmol min -1 mg -1 and autumn with 13.93 µmol min -1 mg -1 and less activity in summer with 6.15 µmol mg -1 min -1 coinciding with the increased application of pesticides in the conventional orchard management. This enzymatic activity was more contrasting in the crop-gizzard tissue than in the foregut and

midgut did not show differences in activity with conventional and organic soil management. Similar was evident in the behavioral assays, earthworms avoided soil of spring and summer season.

The esterases earthworm Lumbricus terrestris can be a suitable tool for regulatory and environmental issues. This study suggests the use of esterases of earthworms as biomarkers to be included in a toxicity test field. Moreover, it indicates that CBEs secretion in the intestine earthworm methodology could be respectful with the environment in the enzymatic bioremediation of soils contaminated with pesticides

CAPITULO I

INTRODUCCION

1. USO DE AGROQUÍMICOS EN LA AGRICULTURA: UNA NECESIDAD QUE REQUIERE CONTROL

El uso de pesticidas en la agricultura evita una perdida cercana al 40% de los cultivos en el planeta por acción de plagas, malezas y enfermedades, incidiendo en el abastecimiento alimenticio de la población mundial (Diersmeier 2001; Berenbaum 2016). A pesar del indudable beneficio de los pesticidas en la agricultura, durante la década de los 70s la comunidad científica atribuyó el uso intensivo de pesticidas orgánicos persistentes (ej. DDT) a episodios de contaminación y toxicidad en los sistemas edáficos y acuáticos (Kookana *et al*., 1998; PNUMA, 2002; Palma *et al*., 2004). Con la prohibición del uso de estas últimas sustancias, el mercado se diversificó hacia el uso de formulaciones menos persistentes aunque con mayor toxicidad aguda. Surgieron así grupos de pesticidas como los organofosforados (OFs), carbamatos (CBs), piretroides (PTs) o neonicotinoides (NTs), entre otros, cuya reactividad en el ambiente auguraba un impacto despreciable (Fest and Schimidt, 1982). Sin embargo, la intensificación de la producción agrícola convencional y el surgimiento de plagas cada vez más resistentes propiciaron el incremento del uso de estas sustancias en el planeta (Diersmeier, 2001). Un hecho que, sumado a las malas prácticas en el uso de estas sustancias, han desencadenado procesos de contaminación en los suelos y aguas (Barra *et al*., 1995; Barra *et al*., 1999; Barra *et al*., 2000; Donal *et al*., 2004; Bonzini *et al*., 2006; Finizio *et al*., 2005; Sala and Vighi, 2008; Ondarza *et al*., 2011). En el año 2005, la Organización de Cooperación y Desarrollo Económico (OCDE) en conjunto con la Comisión Económica para América Latina y el Caribe (CEPAL), entregaron un informe sobre la evaluación del desempeño ambiental de Chile en cada una de las áreas de la gestión ambiental institucional. En relación a la gestión de los recursos hídricos, el informe calificó a las fuentes difusas de contaminación de las aguas como un problema incipiente, destacando la incidencia de la agricultura intensiva en la contaminación de los cuerpos de aguas, en especial, debido al uso excesivo de plaguicidas y fertilizantes en los sectores más productivos (OCDE, 2005).

La agricultura en Chile es un sector enormemente desarrollado y de gran valor económico. Aunque escasos, resultan significativos los estudios que muestran un consumo importante de fitosanitarios en el sector agrario del país. A modo de ejemplo, la Tabla 1, recoge el consumo durante el año 2004 de plaguicidas anticolinesterásicos (Pancetti *et al*., 2011). Como ocurre en otros países o territorios, los pesticidas organofosforados como el clorpirifós encabezan las listas de consumo de agroquímicos. Un dato que justifica inclusión de esta clase de agroquímicos en esta investigación.

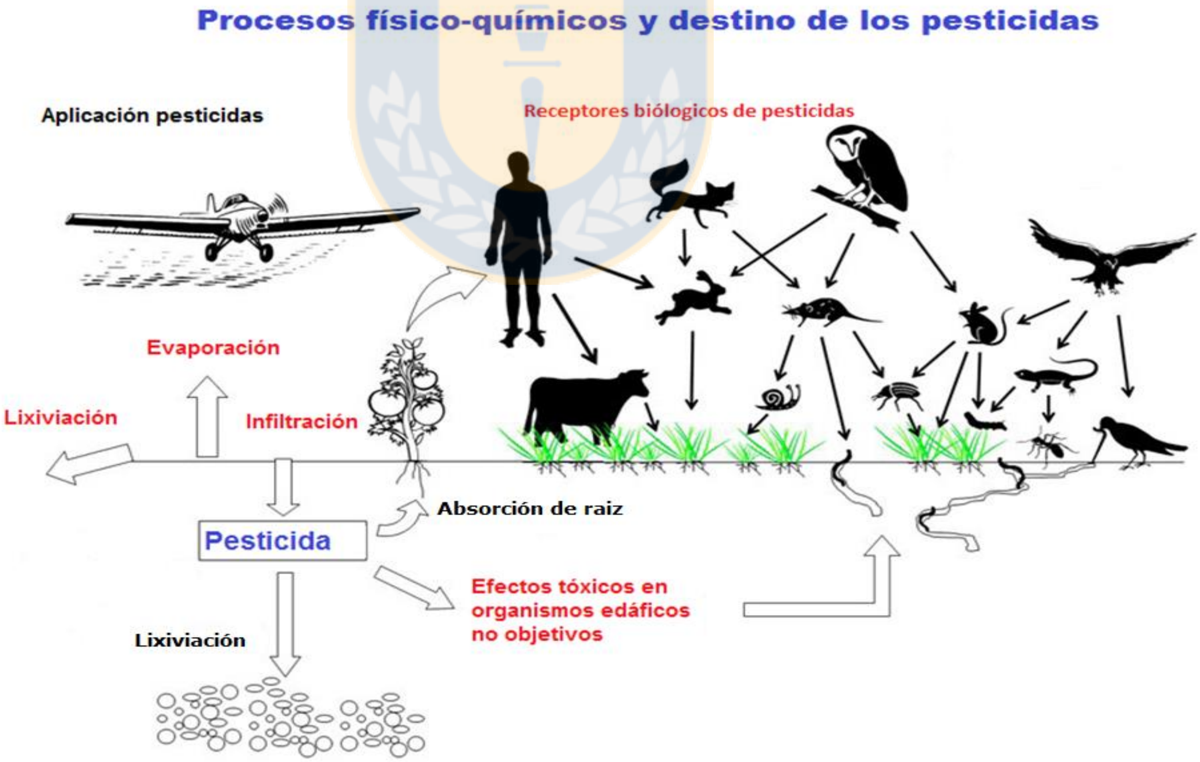
Tabla 1. Consumo de organofosforados y carbamatos en Chile durante el 2004 (datos tomados de Pancetti et al., 2011).

Ingrediente Activo	**Cantidad Kg/L**	**Clase**
Clorpirifós	1.283.729	Organofosforado
Metamidofos	425.941	Organofosforado
Azinfosmetil	396.699	Organofosforado
Diazinon	274.367	Organofosforado
Carbarilo	169.561	Carbamato
Carbofurano	147.616	Carbamato
Dimetoato	112.393	Organofosforado
Metomilo	91.718	Carbamato
Metidatión	69.700	Organofosforado
Fenamifos	48.824	Organofosforado
Etoprofos	27.680	Organofosforado
Aldicarb	25.118	Carbamato
Profenofos	18.909	Organofosforado
Malatión	17,905	Organofosforado
Cadusafós	16.740	Organofosforado
Fosmet	14.703	Organofosforado
Oxamilo	8.936	Carbamato
Metiocarb	8.571	Carbamato
Diclorvos	5.902	Organofosforado
Formetanato	5.089	Carbamato
Pirimifós metil	3.540	Organofosforado
Fenoxicarb	191	Carbamato
Clorfenvinfos	36	Organofosforado

No obstante, no existe un seguimiento de la presencia de residuos de pesticidas ni de sus efectos tóxicos en el ecosistema agrícola. Un aspecto de la política medioambiental del país que necesita ser abordada para una gestión racional en la aplicación de agroquímicos y protección de la biodiversidad.

En el control químico de las plagas, el suelo es el compartimento ambiental donde se acumulan la mayoría de los pesticidas aplicados en la agricultura (Cáceres *et al*., 2010). Una vez allí, numerosos procesos fisicoquímicos y biológicos actúan sobre estas sustancias alterando su estructura original o modificando su reactividad, lo que en último término condiciona su distribución ambiental. La Figura 1 muestra los principales mecanismos y procesos que intervienen en la distribución ambiental de los pesticidas y su impacto en organismos no diana. Como se deduce de la Figura, los pesticidas pueden tener efectos tóxicos directos en los organismos o bien indirectos, éstos últimos como consecuencia de la acción de los pesticidas sobre las especies que son presas o competencia. Por lo tanto, existe preocupación por los efectos potencialmente insidiosos de los pesticidas que operan a través de la cadena alimenticia (Vale *et al*., 2004; Morris *et al*., 2015).

Figura 1. Principales procesos fisicoquímicos y biológicos que determinan el destino ambiental de los agroquímicos en el agrosistema. Esquema elaborado por Köhne *et al*. (2009).



A este punto cabría cuestionarse si la acumulación de los pesticidas en el suelo resulta un mecanismo de atenuación natural de sus efectos tóxicos potenciales o si, por el contrario, generan un riesgo ambiental como fuente secundaria de contaminación. Un examen general de la literatura científica nos aporta la siguiente información:

En primer lugar, el uso masivo de agroquímicos particularmente en sistemas terrestres aumenta el riesgo de absorción por la planta pudiendo alcanzar concentraciones altas en las partes aéreas de estas (Juraske *et al*., 2009). En segundo lugar, el uso de pesticidas puede obstaculizar el control biológico de las plagas, de hecho, los predadores naturales de las especies plaga manifiestan una reducida capacidad de desarrollar resistencia a los pesticidas (Devotto *et al*., 2007), y ello contribuye a un mayor impacto de los pesticidas en las poblaciones de predadores que en la misma plaga (Mazzia *et al*., 2011). En tercer lugar, una elevada persistencia de los pesticidas en el suelo puede originar efectos a largo plazo sobre los organismos no diana. Los efectos indirectos de la contaminación por pesticidas, tales como la reducción de las poblaciones de presas tienen graves consecuencias en los principales depredadores (Geiser *et al*., 2003; Fleeger *et al*., 2010). En cuarto lugar, los suelos que sufren el aporte contínuo de pesticidas pueden comportarse como una fuente de contaminación secundaria. Se ha mostrado que la lixiviación y escorrentía superficial de los pesticidas y sus metabolitos, llegan a contaminar otros compartimentos ambientales de interés fundamental para la salud pública (aguas superficiales y subterráneas) (Arias *et al*., 2008). Por último, los plaguicidas pueden inhibir la actividad de las enzimas del suelo involucradas en los ciclos de los nutrientes acarreando una pérdida de la calidad del suelo desde el punto de vista agrícola (Sinsabaugh *et al*., 2008). Ante este escenario, parece evidente que los efectos adversos de los pesticidas en el agrosistema deberían ser evaluados no solo antes de la autorización del ingrediente activo sino en su etapa de post-autorización.

2. BIOMARCADORES Y LOMBRICES DE TIERRA

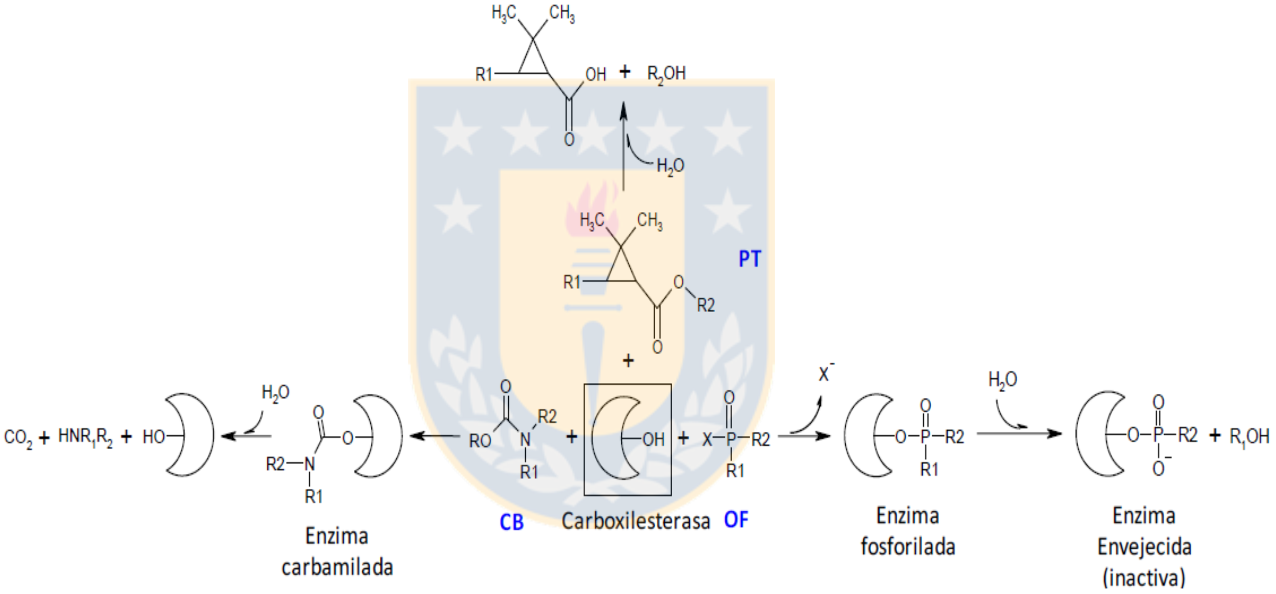
El proceso de evaluación de riesgo ambiental de una sustancia contaminante involucra una amplia gama de ensayos de laboratorio estandarizados a objeto de estimar su potencial de bioacumulación, su destino ambiental (reactividad y persistencia) y la manifestación de efectos tóxicos. Los ensayos de toxicidad se acompañan generalmente de biomarcadores con la finalidad de elaborar un cuadro de líneas de evidencias que se integran mecanísticamente para definir la toxicidad o toxicidades potenciales del pesticida. Se entiende por biomarcador toda respuesta biológica a nivel molecular, bioquímico, fisiológico o histológico que ocurre en un organismo en respuesta a la exposición a una o más sustancias tóxicas (Chapman *et al*., 2001).

De los múltiples biomarcadores que se utilizan comúnmente en toxicología ambiental, esta investigación se centra en aquellos relacionados directamente con los mecanismos de acción tóxica de los plaguicidas de interés y con sus vías de detoxificación. Así, el análisis de la exposición y toxicidad a los pesticidas OFs, CBs y PTs involucrará la determinación de la actividad hidrolítica de dos esterasas carboxilesterasas y acetilcolinesterasa.

Tradicionalmente, la inhibición de la actividad acetilcolinesterasa (AChE) ha sido el método más rápido y simple para evaluar la exposición de los organismos a los insecticidas OFs y CBs (Reinecke and Reinecke, 2007a; Jemec *et al*., 2009), puesto que, se sabe que los pesticidas organofosforados y carbamatos o sus metabolitos activos son inhibidores de este grupo de enzimas, ya que reaccionan con la enzima de manera similar a la acetilcolina, es decir, inhiben competitivamente la actividad colinesterásica comportándose como sustancias anticolinesterásicas (permitiendo así que la acetilcolina siga ejerciendo su actividad). La enzima acetilcolinesterasa es la responsable de la destrucción y terminación de la actividad biológica del neurotransmisor acetilcolina, al estar esta enzima inhibida se acumula acetilcolina en el espacio sináptico alterando el funcionamiento normal del impulso nervioso. Causando hiperactividad dentro de las vías neuromusculares, lo que finalmente interrumpe la transmisión de los impulsos nerviosos (O’Brien, 1967; Savolainen, 2001).

Otras enzimas relevantes en el control de la toxicidad de los OFs, CBs y PTs son las carboxilesterasas. La principal característica de las carboxilesterasas (CbEs) en el campo de las ciencias ambientales es su capacidad de detoxificar pesticidas OFs, CBs y PTs. Los dos primeros grupos de pesticidas inhiben las CbEs (Figura 2). El grupo carbonilo de los CbEs reacciona con el grupo hidroxilo del residuo de serina (sitio activo) de la enzima. La enzima carbamilada es inestable y en presencia de agua el complejo enzima-inhibidor se descompone rápidamente, quedando la CbE nuevamente activa, pero el carbamato es químicamente descompuesto en CO_2 y una amina (Sogorb and Vilanova, 2002; Thompson and Richardosn, 2004). En un mecanismo similar, el grupo fosforilo de los pesticidas organofosforados reacciona con el grupo hidroxilo del sitio activo de la CbE para formar un complejo (enzima fosforilada) muy estable. La inhibición de las CbEs por compuestos organofosforados es considerada irreversible y la actividad enzimática se recupera generalmente por síntesis de nueva enzima (Thompson and Richardosn, 2004). En el caso de los insecticidas piretroides, éstos son hidrolizados por acción de las CbEs para originar el alcohol y ácido carboxílico correspondientes (Figura 2). En resumen, la acción detoxificante de las CbEs con los pesticidas CBs y OFs es una reacción estequiométrica en la cual la sensibilidad de la enzima a estos grupos de pesticidas así como el número de moléculas de enzima marcan la capacidad detoxificante de estas esterasas. Por su parte, la afinidad de la enzima por los piretroides define su capacidad de metabolizar pesticidas PTs (Ross *et al*., 2006). Los pesticidas piretroides, sin embargo, son hidrolizados a sus correspondientes alcoholes y ácidos carboxílicos.

Figura 2. Interacción de las carboxilesterasas con pesticidas carbamatos (CBs), organofosforados (OFs) y piretroides (PTs).



Esquema elaborado a partir de Sogorb and Vilanova (2002) y Thompson and Richardson (2004)

Los compuestos CBs y OFs se unen al residuo de serina (-OH) ubicado en el sitio activo de la enzima de manera reversible e irreversible, respectivamente.

Este tipo de interacciones generan una serie de implicaciones toxicológicas e incluso biotecnológicas. Así, una inducción de la actividad hidrolítica de las CbEs es responsable, en parte, de la resistencia de las plagas a los insecticidas piretroides (Hemingway *et al*., 2004). Los niveles de actividad CbEs, los tejidos donde se expresa esta enzima, y la abundancia de isoenzimas parecen contribuir a la diferente sensibilidad de los organismos a los insecticidas organofosforados, carbamatos y piretroides (Wheelock *et al*., 2008).

Tan importante es seleccionar los biomarcadores en el proceso de evaluación de la exposición y toxicidad de los plaguicidas en el suelo como la elección de uno o más organismos indicadores. En esta investigación, se ha seleccionado la lombriz de tierra como organismo diana del análisis de biomarcadores. Las razones obedecen a criterios ecológicos, agronómicos y toxicológicos fundamentalmente.

Características agronómicas: Las lombrices son componentes clave del sistema suelo. Su actividad excavadora y alimentaria, en especial de aquellas especies anécicas como *Lumbricus terrestris*, contribuye a mejorar la función y estructura del suelo, resultando en último término beneficioso para la salud de las plantas (Jongmans, 2013). Las lombrices favorecen el crecimiento de las raíces de las plantas, contribuyen a la dispersión de semillas o reducen las infecciones de las raíces por parásitos (Scheu, 2003). Asimismo, estos anélidos son considerados verdaderos ingenieros del suelo (Jouquet *et al*., 2006), su actividad excavadora aumenta la aireación del suelo, porosidad, drenaje y fertilidad. Por otro lado, los excrementos de las lombrices incrementan la capacidad de retención de agua del suelo, la disponibilidad de nutrientes y estimula la biomasa y diversidad de microorganismos (Potter *et al*., 2009; Raja and Karmegam, 2010).

Características ecológicas: Las lombrices son un elemento importante en la red trófica del ecosistema terrestre. Constituyen las presas favoritas de muchas especies de invertebrados y vertebrados (Curry, 2014). Pero quizás la característica ecológica más relevante es su función en la dinámica de las poblaciones de microorganismos del suelo, contribuyendo así indirectamente a la descomposición de la materia orgánica y ciclos de nutrientes (Domínguez *et al*., 2009; Lavelle, 2004).

Características toxicológicas: La interacción de la lombriz con el suelo se traduce en una exposición continua a los contaminantes presentes en este compartimento ambiental. El tegumento del animal y su tracto gastrointestinal son las principales rutas de ingreso de los contaminantes al medio interno de la lombriz (Vijver *et al.,* 2005). Ello ha llevado a utilizar este anélido (particularmente las especies *Eisenia fetida y E. andrei*) en los ensayos de toxicidad diseñados para evaluar el potencial tóxicos de nuevos fitosanitarios o para determinar la toxicidad de un suelo históricamente contaminado (ISO, 1993; ISO, 1998). Este organismo es igualmente utilizado como bioindicador de la contaminación del suelo. Esto quiere decir que cambios en la abundancia y diversidad de las poblaciones de lombrices han sido positivamente correlacionados con el nivel de contaminación del suelo (Freddy and Reddy, 1992; Sanchez-Hernandez, 2006). Por otro lado, la alta capacidad de acumular metales en sus tejidos y, a su vez, su tolerancia a éstos, han permitido usarlas como organismos bioacumuladores (Lanno *et al*., 2004). Por último y no menos importante, las lombrices son utilizadas en la remediación de suelos contaminados (Sanchez-Hernandez, 2006; Hickman and Reid, 2008) y en el seguimiento de la eficacia de los procesos de remediación (Lukkari, 2004).

3. CAMBIOS EN LOS AGROSISTEMAS CONVENCIONALES: ADOPCIÓN A LA AGRICULTURA ORGÁNICA

Toda la presión que se ha ejercido en el uso intensivo del suelo desde la revolución verde hasta la actualidad por la aplicación intensiva de agroquímicos, que ha tenido consecuencias e impactos negativos en la salud de las personas en forma directa e indirecta y al medio ambiente, esto ha llevado a muchos agricultores en todo el mundo a adoptar nuevas prácticas agrícolas, como es la agroecología, disciplina científica que enfoca el estudio de la agricultura desde una perspectiva ecológica con el fin de analizar los procesos agrícolas de manera más amplia (Altieri and Nicolls, 2000). El enfoque agroecológico considera a los ecosistemas agrícolas como las unidades fundamentales de estudio; y en estos sistemas, los ciclos minerales, las transformaciones de la energía, los procesos biológicos y las relaciones socioeconómicas son investigadas y analizadas como un todo. Esta disciplina no solo se interesa en la maximización de la producción de un componente particular, sino la optimización del agrosistema total. Esto tiende a reenfocar el énfasis en la investigación agrícola más allá de las consideraciones disciplinarias hacia interacciones complejas entre personas, cultivos, suelo, animales. Dentro de la agroecología se encuentra la agricultura orgánica, que mantiene monocultivos dependientes de insumos externos biológicos y/o botánicos (Altieri and Toledo, 2011).

3.1 Caracterización del sistema agrario orgánico en Chile

Chile es un país que posee ventajas para la producción orgánica, debido a:

- Características relativas a sus variadas condiciones climáticas; que permiten producir diversidad de productos durante el período invernal para el Hemisferio Norte.

- Patrimonio fito y zoosanitario privilegiado por tratarse de un cuerpo subcontinental totalmente aislado y protegido por el desierto, por la cordillera de los Andes, el Océano Pacifico y la Antártica.

- Condiciones institucionales de protección y vigilancia vegetal y animal igualmente excepcionales a través del Servicio Agrícola y Ganadero (SAG), órgano dependiente del Ministerio de Agricultura, ya que cuenta con altos estándares de control para evitar el ingreso de plagas.

- Contar con la existencia la Ley Nº 20.089 que crea el Sistema Nacional de Certificación de Productos Orgánicos Agrícolas; publicado el 17 de enero del año 2006.

Todos estos elementos han configurado que la producción orgánica en Chile haya tenido un crecimiento sostenido en los últimos años, generando a la fecha un total de 100.986 ha con certificación orgánica, de las cuales 81.054 ha son de recolección de especies silvestres certificadas y el restante 19.932 ha son de agricultura orgánica. De esta última cifra, la uva vinífera es el cultivo más importante en superficie (3.735 ha). Luego le siguen los frutales menores con 3.600 ha y los frutales mayores con 2.455 ha (ODEPA, 2015).

Por lo tanto, la agricultura orgánica tiene un gran potencial de contribuir eficazmente a la seguridad alimentaria, la salud de los ciudadanos, el aumento de la salud familiar y las normas ambientales a un bajo costo, respecto a las prácticas agrícolas convencionales. Si bien, este marco de promoción de la agricultura orgánica es conocido en diferentes áreas de la sociedad chilena (academia, la política agraria, los extensionistas, una parte importante de la población, etc.) surge la necesidad de explicar las variables que explican las motivaciones a desarrollar o no este tipo de producción agrícola.

4. MOTIVACIÓN DEL TRABAJO DE INVESTIGACIÓN

Las preguntas que motivan y orientan esta tesis doctoral son las siguientes:

¿Las tasas de aplicación de agroquímicos en Chile ocasionan efectos adversos en organismos no diana como las lombrices?

¿Podemos utilizar las lombrices como organismos indicadores de la contaminación por pesticidas de manera que puedan ser recomendadas en futuros programas de monitoreo ambiental?

¿Resultan eficaces las determinaciones de biomarcadores de exposición y toxicidad de pesticidas en la lombriz de tierra?

Un uso racional de agroquímicos que cause el mínimo impacto en el medio ambiente y por supuesto, respete la legislación nacional e internacional en materia de límites máximo de residuos tendría una exportación con garantías. Por lo tanto este estudio evidenció el escenario actual de la agricultura orgánica de la ciudad de Chillán y se evaluó el análisis de respuestas de biomarcadores comparando lombrices de tierra de la especie *Lumbricus terrestris*, especie ecológicamente relevante en los agrosistemas, en cultivos hortofrutícolas convencionales y orgánicos, junto con ensayos de comportamiento y repulsión. Este estudio pretende aportar nuevos conocimientos y nuevas metodologías o mejorar las ya existentes, para la toma de decisiones.

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CAPITULO II

HIPOTESIS Y OBJETIVOS

1. HIPOTESIS

Las aplicaciones de agroquímicos en agrosistemas de la Región del Biobío dominadas por cultivos hortofrutícola causan efectos adversos en organismos no diana como la lombriz de tierra, cuya actividad genera importantes beneficios para la estructura y dinámica del suelo.

2. OBJETIVOS

2.1 Objetivo general

Determinar el impacto de los pesticidas organofosforados principalmente y carbamatos en la especie de lombriz de tierra *Lumbricus terrestris*. Tal valoración se realizará desde una perspectiva química (residuos de pesticidas en el suelo) y biológica (biomarcadores a nivel individual).

2.2 Objetivos específicos

1. Realizar una caracterización de la agricultura orgánica en relación con el sector público, la relación con el sector académico y la relación con la biota existente.

2. Determinar y comparar los efectos tóxicos de la carga de pesticidas presentes en el suelo con manejo convencional y manejo orgánico a través de ensayos de toxicidad con lombrices de tierra en condiciones de laboratorio.

3. Determinar los efectos tóxicos de la carga de pesticidas presentes en el suelo con manejo convencional a través de ensayos de toxicidad *In situ*.

CAPITULO III

USO DE BIOMARCADORES A TRAVÉS DE LA LOMBRIZ DE TIERRA COMO HERRAMIENTA DE MONITOREO DE AGROSISTEMAS

***Chilean Journal Agricultural Research* (2016) 76: 3**
***In press* Septiembre 2016**

Use of earthworms as a pesticide exposure indicator in soils under conventional and organic management

Ana D. Araneda[1], Pablo Undurraga[2], Daniela Lopez[1], Katia Saez[3], and Ricardo Barra[1*]

[1]Universidad de Concepción, Facultad de Ciencias Ambientales, Barrio Universitario s/n Concepción, Chile. *Corresponding author (ricbarra@udec.cl)
[2]Instituto de Investigaciones Agropecuarias, INIA Quilamapu, Av. Vicente Méndez 515, Chillán, Chile.
[3]Universidad de Concepción, Facultad de Ciencias Físicas y Matemáticas, Barrio Universitario s/n Concepción, Chile.

ABSTRACT

The intensive use of agricultural soils reveals the massive application of agrochemicals. There is no follow-up of the presence of pesticide residues in the soil or their toxic effects on organisms that are beneficial for agrosystems, such as earthworms (*Lumbricus terrestris*). The objective of this study was to evaluate the effect of pesticides used in horticultural orchards on earthworms, and the use of earthworms as an indicator through carboxylesterase (CbE) activity, which is an enzyme involved in the detoxification metabolism of organophosphorus, carbamates, and pyrethroids. Eight individuals were place in each polyethylene container and the container were buried under the soil surface in two apple orchards, one under organic management and the other under conventional management. The experiment was carried out in triplicate. A control treatment was conducted in the laboratory. The experiment was repeated in autumn, winter, spring, and summer of the 2014-2015 study period. Three internal gut tissues of the earthworm were measured for CbE activity in the laboratory. Results showed higher CbE activity in the crop-gizzard of the control treatment with 14.22 ± 1.00 μmol min^{-1} mg^{-1} in winter; the lowest activity was recorded in soil under conventional management in summer with 6.15 ± 2.77 μmol min^{-1} mg^{-1} ($p \leq 0.05$). There was a seasonal difference in enzymatic activity that was higher in winter and autumn with 14.22 µmol min^{-1} mg^{-1} and 13.93 µmol min^{-1} mg^{-}1, respectively, and lower in summer and spring with 6.15 µmol min^{-1} mg^{-1} and 6.31 µmol min^{-1} mg^{-1} ($p \leq 0.05$), respectively; enzymatic activity was associated with higher pesticide application. It can be concluded that CbE activity is sensitive to the inhibitory action of pesticides and can therefore be used as a biological indicator of agrochemicals.

Key words: Carboxylesterase, earthworm, organic management, conventional management, pesticide

INTRODUCTION

Agricultural soils in Chile are under intensive use and this is revealed by an elevated application of agrochemicals (OECD, 2008); there are scarce studies about the presence of pesticide residues in soil and their toxic effects in soil organisms, such as earthworms, which are beneficial because of their physical, chemical, and biological soil functions (Bottinelli et al., 2015; Blouin et al., 2013). Earthworm interaction with soil is continuous exposure to contaminants present in the integument and its gastrointestinal tract, both of which are the main routes of entry of contaminants inside the earthworm.

This has led to the use of the earthworm in toxicity assays to evaluate the toxic potential of new agrochemicals or determine toxicity in contaminated soils (ISO, 1993; ISO, 1998). At the same time, earthworms can accumulate and respond to contaminants at different levels of complexity ranging from molecular, cellular, to ecological changes (Dimitrova et al., 2010; Sforzini et al., 2012; Hayashi et al., 2013).

Numerous enzymatic mechanisms have been studied to evaluate the ecotoxicological effects in soil that play an important role in the metabolism and detoxification of pesticides belonging to the organophosphorus (OP), carbamate (CB), and pyrethroid (PT) groups (Sanchez-Hernandez and Wheelock 2009; Nigam et al., 2014), such as carboxylesterase (CbE) which is an enzyme of increasing interest (Wheelock et al., 2010). The higher sensitivity of CbE to the inhibitory action of pesticides is the reason why these enzymes are attracting attention in the environmental monitoring of the exposure of organisms to this group of pesticides, which are considered to be good exposure biomarkers (Sanchez-Hernandez et al., 2015). Few studies have examined the earthworm's response under natural conditions to evaluate the intensity of pesticide usage and consider it as an indicator of orchard management because it can modify the physical and chemical activity of organisms found in the soil. We hypothesize that CbE activity is affected by pesticide use in conventional agriculture.

Therefore, the objective of this study was to evaluate CbE activity in conventionally and organically managed horticultural soils in four different seasons using the earthworm as an indicator.

MATERIALS AND METHODS

Study sites characterization

Two apple orchards were selected as the two study sites, one with conventional management (historical use of agrochemicals) and the other with organic management (free of pesticide application). These sites are located 30 km one from the other in an agricultural area with intensive and heterogeneous crops in the Biobío Region, Chile. Both sites have soils belonging to the Andisol order and are classified as medial, thermic Humic Haploxerands (Stolpe, 2006). The climate is Mediterranean with marked seasons, high precipitation and low temperatures in winter, and little rainfall and high temperatures in summer. Seasonal mean precipitation and temperatures in the 2014-2015 study period were 150.8 mm and 13.1 °C in autumn, 205.8 mm and 7.9 °C in winter, 51.2 mm and 13.7 °C in spring, and 19.2 mm and 18.2 °C in summer (Agromet, 2015). Some physical and chemical properties were determined for both soils in each season, such as moisture content by gravimetry (Sandoval et al., 2012), organic matter content by the oxidation method with dichromate in an acidic medium and determined by colorimetry, electrical conductivity by conductivimetry, and pH in water with a soil:water ratio of 1:2.5 determined by potentiometry (Sadzawka et al., 2006). Organic matter was high and its values were normal for volcanic soil; it was slightly higher in the organically managed orchard because greater residue cover remained on the soil. The same situation occurred for water pH where values were moderately acidic and slightly higher in the orchard under organic management. Electrical conductivity was low and had similar values for both orchards (Table 1).

Table 1. Soil physical and chemical variables for apple orchard experimental sites under conventional and organic management in the four seasons.

	Conventional Orchard 36° 35.876' S 72° 49.10' W				**Organic Orchard** 36° 33.813' S 71° 49.560' W			
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
Dry soil base moisture, %	30.38 ± 1.16	32.28±1.26	36.61± 1.45	24.07± 1.24	32.10± 1.38	39.86 ± 1.56	32.63 ± 1.34	25.79 ± 1.28
Water pH (1:2.5)	6.05 ± 0.02	5.98 ± 0.03	5.88 ± 0.01	6.01 ± 0.02	6.42 ± 0.02	6.44 ± 0.03	6.98 ± 0.02	6.55 ± 0.07
Organic matter, %	10.7 ± 0.10	10.4 ± 0.07	10.9 ± 0.06	10.7 ± 0.10	12.7 ± 0.10	12.9 ± 0.01	12.6 ± 0.10	12.0 ± 0.02
EC, dS m^{-1}	1.33 ± 0.01	1.32 ± 0.01	1.33 ± 0.01	1.34 ± 0.02	1.26 ± 0.02	1.27 ± 0.01	1.30 ± 0.01	1.29 ± 0.02

EC: Electrical conductivity; ±: Standard deviation

Acquisition of earthworms and experiment in orchards under conventional and organic management

The *Lumbricus terrestris* species was obtained from a supplier in Concepción, Chile; specimens were taken to the laboratory where they were reproduced in polyethylene containers at 20 °C in clean soils free of agrochemicals (pH=7.58 ± 0.05, organic matter=2.03 ± 0.23%, and conductivity= 1.32± 0.03 dS m^{-1}) and fed contaminant-free horse manure. Earthworms were reproduced and adapted under controlled conditions to obtain a sufficient number of individuals grown under the same conditions for use in the assays. Before being taken to the experimental sites, earthworms underwent gut emptying, were placed in a clean container, and not fed for 24 h.

The assays in the apple orchards under organic and conventional management were repeated in four different seasons of the 2014-2015 study period. In each season, juvenile individuals with an undeveloped clitellum (maturity indicator) and weighing between 2.8 and 3.73 g after gut emptying were selected. They were taken to each site and placed in experimental polyethylene containers that were circular, 20 cm long x 15 cm diameter, and completely perforated (0.2 mm) for good soil water diffusion. Each container was filled with 3 kg of moist soil from the same site; soil was taken at the 0-20 cm depth and sieved with a 2 mm. The top of the containers was covered with a fine polyethylene mesh to prevent the earthworms from escaping, and containers were left at surface level. Three containers per site were randomly placed on an equidistant row between two trees and maintained for 30 d. This procedure was repeated in each season in the orchard under organic and conventional management; a control for each stage of the assay was maintained in the laboratory.

The 13 pesticide applications in the orchard under conventional management were recorded during the study period. An average of two applications per month was carried out in spring and summer while only one application per month in winter. The organophosphorus chemical group was the most frequently used and reached a total of seven applications. Only one application was at the soil (herbicide) and the rest were targeted to the foliage, that is, seven insecticide and five fungicide applications (Table 2). The second site was the apple orchard under organic management where no environmentally damaging pesticides were applied; cutting management was used to eliminate weeds while biological control of pests involved monitoring with pheromone traps. Refined sulfur and copper sulfate were applied once or twice to control diseases.

Table 2. Phytosanitary management of the conventionally managed apple orchard during the assay period.

Month of application	Commercial name	Chemical group	Active ingredient	Pesticide
January	Gusathion M 35%	Organophosphate	Azinphos-methyl	Insecticide
January	Lorsban 50 WP	Organophosphorus	Chlorpyrifos	Insecticide
February	Calypso 480 SC	Neonicotinoid	Thiacloprid	Insecticide
February	Glyphosate	Aminophosphonate	Glyphosate	Herbicide
April	Cuprodul	Copper compound	Cuprous oxide	Fungicide
May	Nordox	Copper compound	Cuprous oxide	Fungicide
July	Mineral-phosphate	Oleo phosphate	Fenitrothion	Acaricide
September	Mancozeb 80%	Dithiocarbamate	Alkylenbis	Fungicide
September	Mystic 520	Oximino acetate	Trifloxystrobin	Fungicide
October	Indar	Triazole	Fenbuconazole	Fungicide
November	Imidan	Organophosphorus	Phosmet	Insecticide
November	Lorsban	Organophosphorus	Chlorpyrifos	Insecticide
December	Lorsban/Gusathion	Organophosphorus	Azinphos-methyl/Chlorpyrifos	Insecticide

Collection of earthworm tissues and determination of carboxylesterase activity

After each 30-d period, containers were taken to the laboratory to retrieve the earthworms. Soil in the containers was spread onto a surface to collect the individuals; these were then washed with distilled water and frozen at -80 °C in an ultrafreezer (Biobase, BXC-86 HL-340, Jinan, China) for later analysis. Earthworms were thawed for the analyses and each one was dissected by a longitudinal incision in the dorsal midline of the clitellium toward the mouth; the crop-gizzard, foregut, and midgut were removed without scraping the epithelial tissue. Tissues were carefully washed to eliminate soil particles. Tissue samples were placed in 2.5 mL Eppendorf tubes and 2.0 mL of 10 mM Tris-HCl (pH 7.4) buffer solution were added; they were crushed in a glass micromortar and macerated to ensure membrane esterase extraction (Huang and Hammock, 1997). Tissue was centrifuged at 10000 rpm at 4 °C for 30 min (Hettich, Mikro 220 R, Tuttlingen, Germany). A supernatant fraction of post-mitochrondrial tissue was obtained and 1:50 dilutions were performed in the tissues that were stored in the ultrafreezer at -80 °C until further analysis.

Carboxylterase activity was performed by determining the absorbance of the Red ITR-naphthol complex at 530 nm in a reaction medium with 200 µL final volume (Gomori, 1953; Bunyan et al., 1968); enzymatic activity was then calculated based on the individual protein content of each sample. The procedure involved placing 10 µL of the sample on the flat-bottomed microplate of 96 dishes (TCL, Trueline, Santiago, Chile), adding 170 µL of a solution of 25 mM Tris-HCl with 1 mM CaCl2 (pH=7.6), and adding 20 µL of α-naphthyl acetate substrate after a 5-min incubation period at 25 °C. Naphthyl formation stopped after 15 min when 50 µL of a mixture of 2.5% Triton X-100 (25 mL), 25% SDS (25 mL), and 20 mg of Fast Red 0.1% was added. The solution was left to stand in the dark for 30 min at room temperature (20 °C). A spectrophotometric analysis was then performed in a microplate reader (Synergy HT, BioTek, Winooski, VT, USA) and specific enzymatic activity was expressed in µmol per minute per milligram of protein (µmol min^{-1} mg^{-1}.

The protein content of each earthworm tissue sample was quantified by the Biuret method (Gornall et al., 1949) using the bovine serum albumin standard. This value was used to calculate the enzymatic activity based on milligrams of proteins.

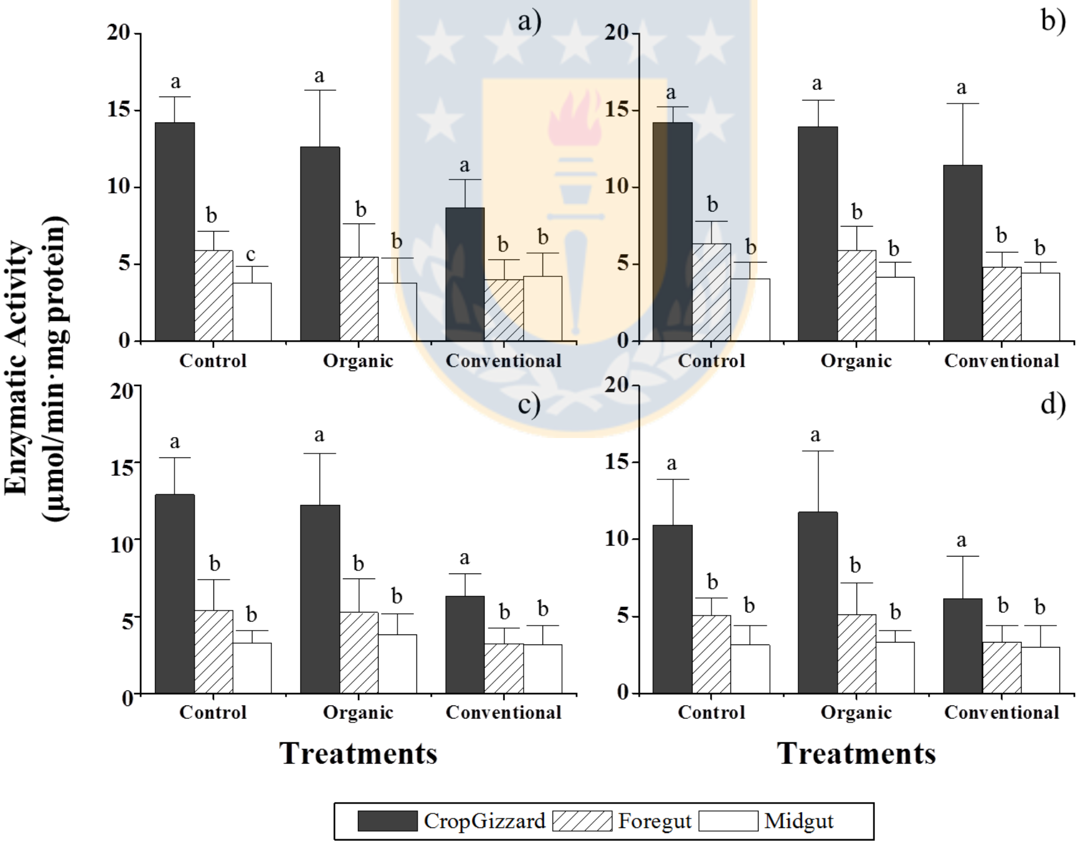
Statistical analyses

A three-way ANOVA was performed where soil type and season factors were in a factorial arrangement and the different gut tissue evaluations were considered as repeated measures because they were carried out on the same specimen (individual). Another factorial ANOVA was used to analyze the effects of the crop-gizzard tissue response. Tukey’s multiple comparison test was used a posteriori. The level of significance was 0.05. Results were analyzed with the statistical software SPSS v22.0. Evaluated variables were expressed as mean and standard deviation, and standard error was used in the graphs.

RESULTS

Enzymatic activity of CbE in autumn (Figure 1a) revealed a 31% difference in crop-gizzard tissue between conventional (8.69 ± 1.81 μmol min^{-1} mg^{-1}) and organic (12.61 ± 3.72 μmol min^{-1} mg^{-1}) management soil while the control (13.17 ± 3.26 μmol min^{-1} mg^{-1}) was 34% higher than under conventional management. When comparing the different tissues in autumn, the highest activity was in the crop-gizzard in the control (13.17 ± 3.26 μmol min^{-1} mg^{-1}) while the lowest activity was found in the foregut (3.77 ± 1.62 μmol min^{-1} mg^{-1}) in organically managed soil.

Figure 1. Carboxylesterase enzymatic activity in (a) autumn, (b) winter, (c) spring, and (d) summer for soil management treatments and in the crop-gizzard, foregut, and midgut tissues of *Lumbricus terrestris.*



Different letters over the bars indicate differences according to Tukey's test (P ≤ 0.05); vertical lines over the bars indicate standard error.

Enzymatic activity of CbE in winter (Figure 1b) showed a 22% difference in crop-gizzard tissue between conventional (11.43 ± 4.03 µmol min^{-1} mg^{-1}) and organic (13.93 ± 1.74 µmol min^{-1} mg^{-1}) management soil while the control (14.22 ± 1.00 µmol min^{-1} mg^{-1}) was 24% higher than under conventional management. When comparing these different tissues in winter, the highest crop-gizzard activity was in the control and the lowest activity was found in the midgut in soil under conventional management (3.10 ± 1.25 µmol min^{-1} mg^{-1}).

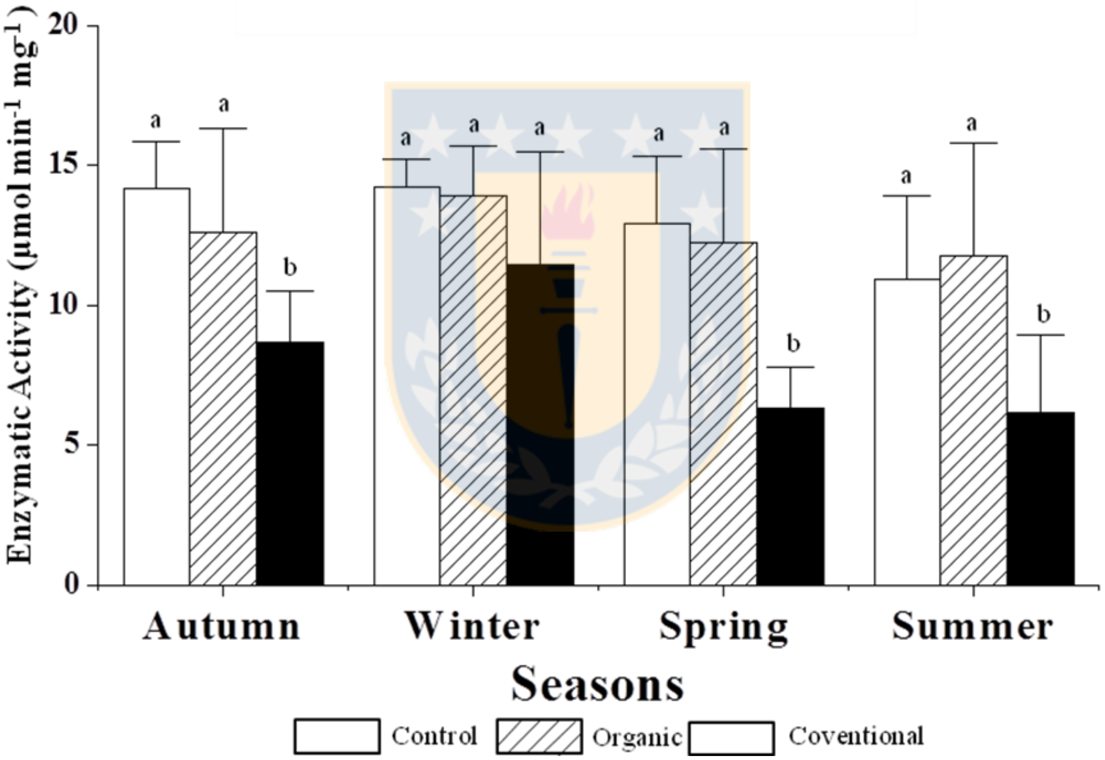
Enzymatic activity of CbE in spring (Figure 1c) showed the same behavior as in the other seasons where crop-gizzard tissue in soil under conventional management had lower activity (6.31± 1.48 µmol min^{-1} mg^{-1}) than soil under organic management (12.23 ± 3.34 µmol min^{-1} mg^{-1}); the difference between organic and conventional management was 94%. The CbE activity in the control was 87% higher than under conventional management and had a value of 11.77 ± 6.64 µmol min^{-1} mg^{-1}. When comparing tissues in spring, the highest activity was in the crop-gizzard with organic management soil, unlike winter and autumn where the maximum activity was found in the control and the lowest activity occurred in the midgut (3.21 ± 1.01 µmol min^{-1} mg^{-1}).

Enzymatic activity of CbE in summer (Figure 1d) was similar to autumn and winter in which crop-gizzard exhibited greater differences, difference of 92%, when comparing activity in conventional (6.15 ± 2.77 µmol min^{-1} mg^{-1}) and organic (11.78 ± 3.99 µmol min^{-1} mg^{-1}) management soil. The control treatment (10.93 ± 2.96 µmol min^{-1} mg^{-1}) was 78% higher than conventional management. The highest activity was in the crop-gizzard in organic management soil (11.78 ± 3.99 µmol min^{-1} mg^{-1}) when comparing the tissues in summer, which was similar to the spring observations; however, it was the lowest activity for this tissue among seasons. The lowest activity in the tissues was found in the midgut with values of 3.01 ± 1.40 µmol min^{-1} mg^{-1}.

These results allow us to establish that the crop-gizzard as the earthworm’s internal tissue that indicated a better trend with higher values in CbE activity; it also exhibited greater differences among treatments under different soil management systems regarding the foregut and midgut. The latter two tissues had lower enzymatic activity values that were similar between soil management systems. Therefore, crop-gizzard activity was used as an indicator tissue to study the effect of soil management on the enzymatic activity in the different seasons (Figure 2). The CbE activity in the crop-gizzard was only different in conventionally managed soil for all the seasons. The highest activity was in winter with values of 11.43 ± 4.03 µmol min^{-1} mg^{-1} and the lowest activity was recorded in summer with values of 6.15 ± 2.77 µmol min^{-1} mg^{-1}. The CbE activity in autumn (8.69 ± 1.82 µmol min^{-1} mg^{-1}) was higher than in spring (6.31 ± 1.48 µmol min^{-1} mg^{-1}. Organic management soil was not different among seasons and activity varied between 13.93 ± 1.74 and 11.78 ± 2.96

µmol min^{-1} mg^{-1} in winter and summer, respectively. These CbE activity crop-gizzard values are similar to those found in the control treatment, which varied between 14.22 ± 1.00 and 10.93 ± 2.96 µmol min^{-1} mg^{-1} in winter and summer, respectively.

Figure 2. Seasonal distribution of carboxylesterase enzyme activity in the crop-gizzard tissue of *Lumbricus terrestris* exposed to different soil management systems.



Different letters over the bars indicate differences according to Tukey's test (P ≤ 0.05); vertical lines over the bars indicate standard error.

DISCUSSION

Carboxylesterase activity in the crop-gizzard, foregut, and midgut tissues is consistent with findings by Sanchez-Hernandez and Wheelook (2009) in an i*n vitro* study where they indicate that the determination of CbE activity is well represented in the gastrointestinal tract of the *Lumbricus terrestris* species, and it is a good indicator to evaluate the effects of pesticides.

Results of the present study indicate that the crop-gizzard tissue had higher CbE activity values than the foregut and midgut tissues. It was also observed that CbE activity in the latter two tissues decreased as the ingested soil was processed through the gastrointestinal tract (Sanchez-Hernandez et al., 2009). This is why no differences in the foregut and midgut were found among soil management treatments with higher or lower pesticide application, which could be due to the gut's digestive function (Brown and Doube 2004). However, according to González et al. (2010), high enzymatic activity in the crop-gizzard would be explained because CbE activity in this organ could contribute to clorpyrifo retention and thus decrease the amount of pesticides passing to subsequent organs and reduce intestinal absorption; as a consequence, enzyme inhibition is lower.

The CbE activity found in the four seasons had a similar behavior among soil treatments, that is, the control treatment and the organic management soil had higher activity and the conventional management soil in which pesticides were applied always had lower activity.

The highest soil CbE activity was found in winter under organic management and the lowest CbE activity occurred in summer under conventional management, which was well identified in the crop-gizzard tissue. These results could be related to the greater use of organophosphorus pesticides and carbamates applied in spring and summer in the apple orchards under conventional management where pesticides were retained by soil organic matter at a rate varying between 10.2 and 10.7%; this is an important factor for pesticide retention in the soil (Boivin et al., 2005; Alvarez et al., 2013). There are also authors who maintain that pesticides in the soil can be biodegraded by organisms found in organic matter (Sorensen and Aamand 2001). These contrasting effects are not only related to pesticide properties but also to the nature of organic matter.

The CbE activity exhibited a slow recovery rate in autumn and winter. Authors, such as Aamodt et al. (2007); Rault et al. (2008), and Collange et al. (2010), have indicated that cholinesterase activity usually shows slow recovery rates until it reaches normal levels when exposed to organophosphates. This CbE activity in the intestinal tissue of *L. terrestris* remains inhibited for a long period of time, more than one month, after applying organophosphorus (Gonzalez et al., 2010), that is, enzymatic activity is not recovered either by the spontaneous reactivation of the phosphorylated enzyme by the organophosphorus pesticide (Rodríguez-Castellanos and Sanchez-Hernandez 2007) or the synthesis of the new enzyme during this inhibition period. This slow activity allows the stability of this response to organophosphorus pesticides and makes it a useful enzyme for biochemical assessment in monitoring exposure to agrochemicals.

This slow CbE recovery in the earthworm makes it a suitable biomarker for monitoring soils exposed to organophosphorus that can be detected several weeks after being applied. Some studies have concluded that CbE activity in other earthworm species, such as *Allolobophora caliginosa*, can be influenced by factors such as temperature and the nutritional and reproductive status (Lowe and Butt 2007). Furthermore, the hydrolytic activity can be altered by ingesting lipids because CbEs metabolize them. Enzymatic CbE activity can be affected by the availability of the food substrate for the earthworm; according to González et al., (2009), the *Allolobophora caliginosa* earthworm had a high variation during 35-d periods with no access to food. In the present study, earthworms had access to a sufficient food substrate despite being enclosed in polyethylene containers; they were under fruit trees so CbE activity was not affected by nutritional deficiency.

The toxic effects of pesticides on earthworms are different for dermal exposure or ingestion. Results of CbE in the gastrointestinal tract of *L. terrestris* offer an efficient chemical barrier against pesticide absorption because CbE intestinal activity is sensitive to pesticides when these are applied to soils. This has been demonstrated by Henson-Ramsey et al. (2007) when they exposed Malation through the skin and by ingestion in *L. terrestres*; they observed that dermal exposure is more tolerant than exposure by soil ingestion. In fact, Yu et al. (2006) indicated that high clorpyrifo absorption by the earthworm *Allolobophora caliginosa* was due to active ingestion of the pesticide found in the soil while clorpyrifo absorption through the skin surface was not significant. *Lumbricus terrestris* was a good exposure indicator in soils where pesticides were applied because they are resistant to the conditions of an agroecosystem with a high pesticide load; all the specimens (100%) in the present experiment survived during the four different seasons, which allowed us to collect them from the polyethylene container to determine enzymatic activity.

CONCLUSIONS

The use of carboxylesterase enzymes of *Lumbricus terrestris* provides evidence that they are good indicators of exposure to pesticides such as organophosphorus and carbamates; this was reflected by lower enzymatic activity in spring and summer, which coincided with the highest pesticide application in the orchard under conventional management. Enzymatic activity contrasted more in the crop-gizzard tissue than in the foregut and midgut, which showed no differences in activity in soils under conventional and organic management.

ACKNOWLEDGEMENTS

Financial support was provided by the FONDAP-CONICYT 15130015, Centro de Recursos Hídricos para la Agricultura y la Minería (CRHIAM).

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CAPITULO IV

COMPORTAMIENTO DE LAS LOMBRICES DE TIERRA Y BIOMARCADORES DE EXPOSICIÓN EN SUELOS CON MANEJOS CONVENCIONALES Y ORGÁNICOS

Seasonal response of avoidance behaviour and esterase inhibition (acetylcholinesterase and carboxylesterase) of the earthworm *Lumbricus terrestris* after exposure to pesticides in a conventional and organic apple orchard

Ana Araneda[1*], Katherine Muñoz [2] and Ricardo Barra[1]

[1] Faculty of Environmental Sciences, University of Concepción, Barrio Universitario s/n, 4070386 Concepción, Chile
[2] Institute for Environmental Sciences, University of Koblenz-Landau, Fortstraße 7, 76829
Landau in der Pfalz, Germany
*Corresponding author: A. Araneda (anaraned@udec.cl)

ABSTRACT

Organic residues in soil resulting from the application of pesticides in conventional agriculture are not free from risk and can affect beneficial soil organisms like earthworms. This study investigated the avoidance response (ABR test) of an agriculturally relevant species, *Lumbricus terrestris* after exposure to pesticides in a conventional and organic orchard. The study was conducted over the course of a year in a natural agricultural system. Levels of activity for acetylcholinesterase and carboxylesterase were used as indirect markers of the bioavailability of pesticides, in a modified version of the same ABR test, that is, without duality. For the first protocol the earthworms avoided the soils in spring and summer, which coincides with the highest pesticide applications in the field. Moreover, quantitative analysis of sterase activities was indicative of earthworms exposure to the pesticides used in conventional agriculture. Acetylcholinesterase activity was lower in spring (29.6 $nmolmin^{-1}mg^{-1}$) and summer (36.3 $nmolmin^{-1}mg^{-1}$) and recovered in autumn (91.8 $nmolmin^{-1} mg^{-1}$) and winter (108.4 $nmolmin^{-1}mg^{-1}$). Similar outputs were observed after measurements of carboxylesterase activity: A string inhibition was observed in spring and summer. In autumn and winter a recovery of 334.7 $nmolmin^{-1}mg^{-1}$ and 338.87 $nmolmin^{-1}mg^{-1}$ was observed, respectively. The enzymatic activity. This study indicates that avoidance behavior test of soils with pesticides-contaminated organic residues at a result of applying pesticides could be supported by specific biomarkers to facilitate a better understanding of exposure to pesticides during the test.

Keywords: Avoidance, *Lumbricus terrestris*, acetylcholinesterase, carboxylesterase, conventional orchard, pesticides.

INTRODUCTION

The growing demand for food worldwide is closely related to the intensification of agricultural practices and the use of diverse agrochemicals to facilitate crop production with imminent effects on soil quality and services (Power, 2010). In this context Chile has come to be termed an “agrofood power”. This has given rise to increased levels of pesticide residues in soils (Cerejeira et al., 2003; Wilson and Foos 2006; Tariq et al., 2007; Pereira et al., 2010). Soils host a wide range of beneficial flora and fauna that can be at risk owing to this practice. Among them non-target species, like Earthworms play a key role in agrosystems through its tunneling activity, responsible for soil function and structure (Jongmans et al., 2003; Scheu 2003; Jouquet et al., 2006). The interaction earthworms-soil is thus understood as a continuous exposure to contaminants in the soil. The tegument of this specie and its gastrointestinal tract are the main routes of entry of contaminants to the internal medium of the earthworm (Vijver et al., 2005). Soil contamination positively correlated with changes in the abundance and diversity of earthworms (Sánchez-Hernández 2006). Therefore this specie is frequently used as an indirect bioindicator of soil contamination (ISO 1993; ISO 1998), especially in short-term ecotoxicological assays, like avoidance behavior tests (Yeardley et al., 1996; Natal da Luz et al., 2004; Aldaya et al., 2006) and as a tool for the early detection in assessing low levels of ecological risk in contaminated sites (Loureiro et al., 2005; Natal da Luz et al., 2004).

In the 1990s artificial soils were recommended for testing ecotoxicity in laboratory conditions (Reinecke, 1992). However, ten years later in the synthesis of the Third International Ecotoxicity Workshop, Van Gestel and Weeks (2004) recommended the use of natural soils with earthworms given that the results with artificial soils cannot be translated directly to soils in the field. The soils should be characterized given that they can influence the fate of pesticides and therefore their bioavailability for different species habiting the soil.

The existing standard tests for worms are conducted mainly with *Eisenia fetida* and *Eisenia andrei* based on mortality, reproduction and avoidance behavior (OECD 1984, 2004; ISO 2008). However, *E. fetida* and *E. andrei* are epigeal species and are not relevant for pesticide tests given that they are not found in most agrosystems. Likewise, An example of this is that earthworms do not avoid soils contaminated with chlorpyrifos and that acetylcholinesterase activity (AChE) does not change. However, carboxylesterase activity (CbE) is inhibited at concentrations of 3 and 15 mg/kg (Martínez et al., 2013). Therefore behavior tests should be conducted in parallel using more sensitive biomarkers, namely the use of the esterase enzymes Acetylcholinesterase (AChE) and Carboxylesterase (CbE) for monitoring soil toxicity (Rodriguez-Castellanos and Sanchez-Hernandez, 2007). AChE is a biomarker of pesticide exposure and neurotoxic effects (Gambi et al., 2007; Venkateswara Rao et al., 2003; Rao and Kavitha, 2004), meanwhile. CbE represents a key enzyme in pesticide detoxification (Sanchez-Hernandez et al., 2009). Behavior tests may indicate repellence instead of being a true toxicological bioassay (Capowiez and Bérard 2006). Hodge et al. (2000) found evidence of this in the lack of response of worms in avoiding some chemicals like organophosphates, as Capowiez and Bérard (2006) found with neonicotinoid insecticides. This indicates that the avoidance tests in relation to neurotoxic pesticides may be biased if the cutoff points represent high concentrations given that the mechanisms of detoxification or the interruption of cholinergic functions can lead to the hyperactivity of the worm and does not necessarily imply an avoidance response (Pereira et al., 2010). An example of this is that earthworms do not avoid soils contaminated with chlorpyrifos and that acetylcholinesterase activity (AChE) does not change. However, carboxylesterase activity (CbE) is inhibited at concentrations of 3 and 15 mg/kg (Martínez et al., 2013).

The objective of our study is to examine whether the avoidance response of earthworms exposed to conventional agricultural soils with historical use of pesticides (organophosphates among others) is associated with inhibition of acetylcholinesterase, a key enzyme in the normal functioning of cholinergic synapses and neuromuscular joints, and determine whether the enzymatic activity of carboxylesterase exhibits more affinity for pesticides. These responses were examined over the four seasons of a year to obtain an annual characterization of pesticide use and behavior of earthworms and of the AChE and CbE enzymes, which are compared to organic chemical-free agricultural soils. The inhibition of activities of the two esterases was determined on the tissue of the gastrointestinal wall of the earthworm *Lumbricus terrestris*, an ecologically important species for agricultural soils.

2 Materials and methods

2.1 Field description and soil characterization

1) Soil samples were collected from an agricultural area located in Chillan, Biobio Region in Chile. In this are the soils are formed over recent volcanic ash (post-glacial) deposited on fluvial and fluvioglacial material, but not cemented, with predominantly silty and clayey soils. The climate of the region is Mediterranean, with annual precipitation of 1000 - 1300 mm and an average temperature of 13.5 - 14.0 °C (Del Pozo and Del Canto, 1999). Two sampling sites, 30 km apart, were selected, both apple orchards, one certified as organic and free from the use of agrochemicals (organic crop, location 36° 33.813'S and 71° 49.560'W) and other with a history of agrochemical use (conventional crop, location 36° 35.876'S and 72° 4.910'W). The annual agrochemical application is provided in table 1. Soils were collected every season for behavioural assays at a depth from 0-30 cm and sieved through a 2 mm mesh. Soil was gathered manually with a shovel and taken in insulated to the laboratory. The soil from organic orchard crops was used in bioassays as a reference soil. Soil characterization included water content via gravimetric method, pH and conductivity in water and % or organic matter. The last, using the wet combustion method (Page, 1982). Soil characterization is provided in table 2.

2.2 Experiment design

The experiments were conducted with soils collected four times over a year, which represent each climatic season: autumn, winter, spring and summer. Avoidance behaviour was examined using standardized avoidance behaviour response test (ABR) prescribed by the International Organizational for Standardization (ISO 2008). Simultaneously, the activity of the enzymes acetylcholinesterase (AChE) and carboxylesterase (CbE) in the earthworms after exposure was linked to the avoidance behaviour response. Soils from conventional crops were used as niche for incubation studies; soils from organic crop as control.

a) Avoidance behaviour response test*:* A rectangular box (265 x 162 x 100 mm) was divided into two equal sections using an extractable plastic device. One section of the box was filled with 0.5 kg of soil from conventional crops, in the other section; 0.5 kg of soil from organic orchard crops (control) was used. The pH level and the percentage of moisture in each side were adjusted at the beginning of the bioassays to mimic the field conditions (Table 2). The *L. terrestris* earthworms were adults and clitelated, purchased from a local vermiorganic supplier. The worms were acclimatized in plastic container under continuous darkness and at 15°C, fed weekly with moist horse dung that was applied on the soil surface. The tests were maintained as well at 15±2°C in darkness for 48 h. The divider separating the two soils was removed from the line of direct contact between the two soils and 10 earthworms (3-5 g fresh weight) were used for the experiment. Finally, the divider was placed again and the number of earthworms in each compartment was listed. The experiments were conducted in triplicate. Individuals found on the dividing line of the container were assigned based on the position of the head (De Silva and Van Gestel, 2008).

The avoidance response in each replicate was calculated using the equation 1, where NR is the net response (%), C is the number of earthworms in control soil, T is the number of individuals in the conventional agricultural soil and N is the total number of earthworms per replicate.

$$NR = \frac{(C - T)}{N} \times 100 \tag{1}$$

b) Acetylcholinesterase and carboxylesterase activity: This experiment used the same methodology of the first experiment in terms of temperature, humidity, pH and incubation time. The experiments were performed in triplicate with ten adult clitelated earthworms (3-5 g fresh weight) per replicate. After the 48-hours incubation period, the earthworms were removed from the containers, rinsed with distilled water. The excess of water from the surface of the earthworms was mechanically removed before storage at -80°C for subsequent analysis. For the study of the esterase activity the gastrointestinal wall were dissected from the clitellum to the anus. Samples of 0.5 g (fresh weight) of tissue were homogenized with a 10-Mm buffer of Tris-HCl (pH 7.4) containing 0.1% of Triton X-100 (Huang and Hammock, 1997). To avoid inhibiting enzymatic activity, the tissues were centrifuged at 9000 g for 20 min at 4°C, and a fraction of the post-mitochondrial supernatant was quantitatively obtained. Acetylcholinesterase activity was determined using the method of Ellman et al. (1961), which was adapted to the microplate format (Wheelock et al., 2005). The enzymatic reaction was done in flat-bottomed 96-well plates, mixing 235 µl of 0.1 M of Na phosphate buffer (pH=8.0) which contains 320 µM of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 5 µL of the sample. After 2 min of equilibrium, 10 µL of 60 mM iodized acetyltiocholine (AcSCh) was added to the mixture for the reaction and absorbance was read at 412 nm for 10 min (in one-minute intervals) at 22°C using a Synergy HT microplate reader (Synergy HT, Bio- tech®, Winooski, VT, USA). Esterase activity was calculated with a calibration curve produced with DTNB. Carboxylesterase activity used α-naphthyl acetate as a substrate (α-NA), (Thompson 1999). The hydrolytic reaction was initiated through the mixture of 170 µl of mM Tris–HCl (pH 7.6), 10 µl of 2mM (final concentration) α-NA and 20 µl of the sample and was stopped after 10 min (22°C and continuous agitation) to add 50 µl of a solution containing 2.5% of SDS in 0.1% of Fast Red ITR and 2.5% of Triton X-100. Naftol–fast absorbance was read at 530 nm and the specific activity was calculated using an external curve made with α-NA in a range from 1.5 to 100 nmol/ml, absorption coefficient 13.6×10^3 $M^{-1}cm^{-1}$. All the hydrolytic reactions were performed in triplicate and with blank spaces (reaction medium without a sample). The enzymatic activities of acetylcholinesterase were expressed as nmoles of hydrolyzed acetyltiocholine per min by mg of protein. Carboxylesterase activity was expressed as nmoles of α-naphthyl acetate formed per min per mg of protein, and for the calculation, the absorption coefficient 16.4×10^3 $M^{-1}cm^{-1}$ was used. The quantity of proteins was calculated based on the calibration curve with bovine serum albumin using the method of Bradford (1976) and was read at 595 nm of absorbance.

2.3 Statistical analysis

The minimum sample size was calculated using the power analysis module in Statistica (version 6, StatSoft, Inc., Tulsa, OK, USA) with a significance level of 0.05 at a power of 0.5. Statistical analysis was done using the software IBM SPSS® statistics 21. Average was defined as the sum values divided by the size of the sample. The non-parametric Mann–Whitney test was used to test for significant differences in earthworm distribution between the control and conventional agricultural soil. Multivariate ANOVA (MANOVA) was performed testing the significance of the seasonal factor on avoidance response.

3 Results

3.1 Avoidance behaviour response test

No mortalities or escapes were observed during the periods of exposure using soils from four different seasons. In general, earthworms avoided the conventional agricultural soils in all season, which was reflected by the positive average net response (Fig. 1). A significant level of avoidance was evidenced in spring, reaching a maximum level of 90 ± 0.02%, followed by a 53 ± 0.021% of avoidance in summer. In the winter and autumn scenario, the levels of avoidance were lower compared to the spring-summer situation. In winter, the net responses of the avoidance tests was 19 ± 0,012% and 26 ± 0,031% in the autumn. The Avoidance behaviour response was not affected by the soil physicochemical parameters ($p > 0.05$). However the highest level of avoidance of avoidance coincides with the intensive pesticide application (Table 1).

3.1 Acetylcholinesterase and carboxylesterase activity

The results obtained for exposure in the different seasons show inhibition of both AChE and CbE, with the highest inhibition observed in spring and summer (Figure 2).There was a significant difference in acetylcholinesterase activity in *L. terrestris* exposed to conventional soils in the different seasons. In relation to the control (142.7 ± 14.5 nmolmin^{-1}mg^{-1}), the enzymatic activity decreased by 35% in autumn, corresponding to 91.9 ± 15.97 nmolmin^{-1}mg^{-1}. In winter, the lowest inhibition was observed. The values declined to 108.5 ± 28.0 nmolmin^{-1}mg^{-1}, which correspond to a 24% inhibition of the enzymatic activity. The strongest inhibition was registered in spring (29.6 ± 3.8 nmolmin^{-1}mg^{-1}), which represent a decrease of 77%. The enzymatic activity recovered by 5% in summer (36.3 ± 5.3 nmolmin^{-1}mg^{-1}), compared to the spring values.

CbE activity responded less pronounced to the seasonal exposure to conventional soils to that of AChE activity. Comparing CbE activity to that of the control, it was evident that the highest level of inhibition was in spring (152.4 ± 23.6 $nmolmin^{-1}mg^{-1}$), with a 43% reduction. In summer, the activity declined by a 52% (172.1 ± 31.3 $nmolmin^{-1}mg^{-1}$). The activity of CbE was almost not inhibited in autumn (334.8 ± 45.5 $nmolmin^{-1}mg^{-1}$) and in winter (338.9 ± 45.5 $nmolmin^{-1}mg^{-1}$), and the values remained similar to the control (363.9 ± 22.0 $nmolmin^{-1}mg^{-1}$). The activity of the enzymes AChE and CbE inversely correlated with the level of avoidance ($r = -0.95$). Thus, the highest level of avoidance corresponded to the lowest enzymatic activity of both AChE and CbE, indicating that the selected biomarkers are adequate for evaluating the effects of pesticides in conventional agricultural soils.

4 Discussion

Seasonal application of pesticides in agriculture may lead to a different degree of exposure for non-target soil species. This study demonstrated that the avoidance response and the esterase activity of *L. terrestris* were directly induced by the seasonal application of pesticides. According to the annual applications in this conventional agrosystem, organophosphates are applied intensively in spring and summer (Table 2), which should imply a higher level of exposition for *L. terrestris*, followed by a strong avoidance response and markedly decline of the enzymatic activity of both AChE and CbE. In the same way, less intense pesticide applications resulted in more favourable responses (figure 1 & 2). This is in line with previous studies where the intensity of the exposure of non-target species to pesticides in soils is determined by the seasonal applications (Reinecke and Reinecke 2007; Denoyelle et al., 2007). Interestingly, the pesticides use in spring and summer are more lipophilic than those applied in winter and autumn (Table 1). This may implicate an accumulation of the pesticides in soils and a reduced wash out from the soil due to the absence of rainfall in the summer (Silburn et al., 2013; Gomez et al., 2011). This indirectly may suppose that responses observed in autumn can be a joint effect of the direct application of the pesticides and a residual contamination from the spring and summer applications.

Avoidance tests are widely applied in ecotoxicology, with dissimilar outputs. Therefore the use of additional biomarkers for example AChE and CbE is highly advisable (Martinez et al., 2013; Pereira et al., 2010).

There are a few studies relating AChE activity (and one study on CbE activity) to exposure to pesticides. Pereira et al. (2010) reported significant AChE inhibition in *E. andrei* when it is exposed to concentrations of methomyl (0.86 mg/kg, while a significant avoidance response occurred with 5, 62 mg/kg. Similarly, Jordan et al. (2012) found that *E. andrei* was not able to avoid soils contaminated with 6 or 12 mg/kg of azinphos-methyl. However, AChE activity was strongly inhibited at these concentrations. Martínez et al. (2013) described the effects of different concentrations of chlorpyrifos in standardized ABR tests (two-chamber system), with 0.6 and 15 mg/kg of chlorpyifos, and another test with a modification that implied pre-exposure periods of 24, 48 or 72 hours in soils contaminated with 15 mg/kg of chlorpyrifos. In both protocols the earthworms were incapable of avoiding the contaminated soils. Acetylcholinesterase activity did not change in earthworms in the standardized behavior test (0.58 ± 0.20 protein U / mg, media ± DE; n = 72), while CbE activity was significantly inhibited (62 to 87% inhibition) in worms exposed to 3 and 15 mg/kg chlorpyrifos.

These studies cannot be compared directly to the current study owing to differences in field experiment procedures; the species of earthworm and finally the test in this study were conducted according to the seasons of the year. Despite the high level of inhibition of AChE activity in earthworms in the modified ABR design, there was a clear pattern of avoidance of the conventional soil by the earthworms, the level of avoidance being highest in spring and beginning to decline in summer. Supposing that cholinergic transmission is the main neuronal network involved in the capacity of the earthworms to detect and avoid soils contaminated with chemical residues (Rosenbluth, 1972). A high level of AChE activity should involve the over-stimulation of post-synaptic cells (for example muscular fiber), which leads to hyperactivity. This neurotoxic effect have been observed in organisms like the amphipod *Gammarus fossarum* exposed to methomyl (Xuereb et al. 2009), zebra fish larvae (*Danio rerio*) exposed to chlorpyrifos (Kienle et al. 2009) and fish (*Melanotaenia duboulayi*) exposed to profenofos (Kumar and Chapman 1998). A similar effect occurred with earthworms in the modified ABR test, where hyperactivity induced by the pesticide may have led to the worms adopting an irregular locomotion pattern, which leads to a clear avoidance response.

Another objective of this study was to determine the effect on earthworms of carboxylesterases. It is well known that carboxylesterases promote the non-catalytic detoxification of organophosphates by forming stable enzyme-inhibiting complexes (Sogorb and Vilanova, 2002). This hydrolytic enzyme metabolizes xenobiotics that contain ester, thioester or amide bonds (Haites et al, 1972; Stenersen, 1984). It has been shown that the inhibition of CbE activity is a stoichiometric mechanism to reduce organophosphate concentrations. Several studies have reported CbE inhibition of earthworms after exposure to PO (Sánchez-Hernández et al, 2009; Vejares et al., 2010). In this study CbEs activity was used as a biomarker of organophosphate exposure. The results were similar to those of AChE activity. Once again, significant inhibition was registered in spring and summer after natural pesticide exposure, while in autumn and spring CbE activity recovered, like that of AChE.

This study concurs with other studies (Wheelock et al., 2008; Collange et al., 2010; Vejares et al., 2010) that carboxylesterase is more sensitive than acetylcholinesterase.

There is scarce data currently available on the toxic effects of pesticides on edaphic organisms in natural agrosystems. Only a few pesticides and species have been tested in a wide range of soils. Consequently, it is difficult to make comparisons (Frampton et al., 2006). As well, complex mixtures of contaminants can exist in agrosystems with conventional crops, where synergistic and/or antagonistic effects can occur in the existing biota.

The inhibition responses to these esterases are closely related to avoidance of conventional agricultural soils in spring. However, earthworms cannot avoid conventional soils in summer owing to reduced enzymatic activity. This behavior may be a non-specific or delayed effect of locomotor stress on the displacement to the control soil, where the influence of climatic conditions are characterized by high temperatures and low humidity that increase stress (Martinez et al., 2013) as well as the physiochemical properties of the soils, given that the control soil (organic orchard soil) has a higher percentage of organic matter and a thicker texture than the conventional agricultural soil (Table 2).

According to these responses are stronger if there is a high degree of difference between the soils, independent of the level of soil contamination, also considering the application of organophosphate pesticides is higher in spring and summer and the avoidance response was clearly evidenced in the conventional orchard soils in those seasons.

Conclusions

This study used an ecologically important species of worm, with the aim of investigating the real effects of pesticides on agriculturally beneficial species over the course of a year in a conventional orchard through the use of two biomarkers sensitive to exposure to the most widely used organophosphates in Chile. The majority of studies have only used epigeous species, probably owing to the facility for rearing and maintenance under laboratory conditions. However, given that the feeding strategy could significantly affect the rate of exposure to contaminants in research on environmental monitoring, it is important to include ecologically important species.

The standardized ABR test is not simply a sensorial response to exposure to toxic substances; it is a tool for the early detection of the possible effects of exposure to contaminants.

The significant changes in enzymatic activities demonstrate exposure to pesticides. AChE inhibition is a sensitive, rapid and widely used biomarker of exposure to pesticides. However, this study found that carboxylesterase activity proved to be more sensitive to inhibition in period with more pesticide use, including organophosphates. Consequently, acetylcholinesterase and carboxylesterase inhibition should be included in a battery of biomarkers to monitor soil toxicity. In conclusion, this study raises many questions about the effects of exposure to pesticides among non-target organisms under natural conditions. However, more studies are needed to address the effects of the treatment with pesticides in soil ecosystems.

Acknowledgments

Financial support was provided by FONDAP-CONICYT Project 15130015, with the Centro de Recursos Hídricos para la Agricultura y la Minería (CRHIAM).

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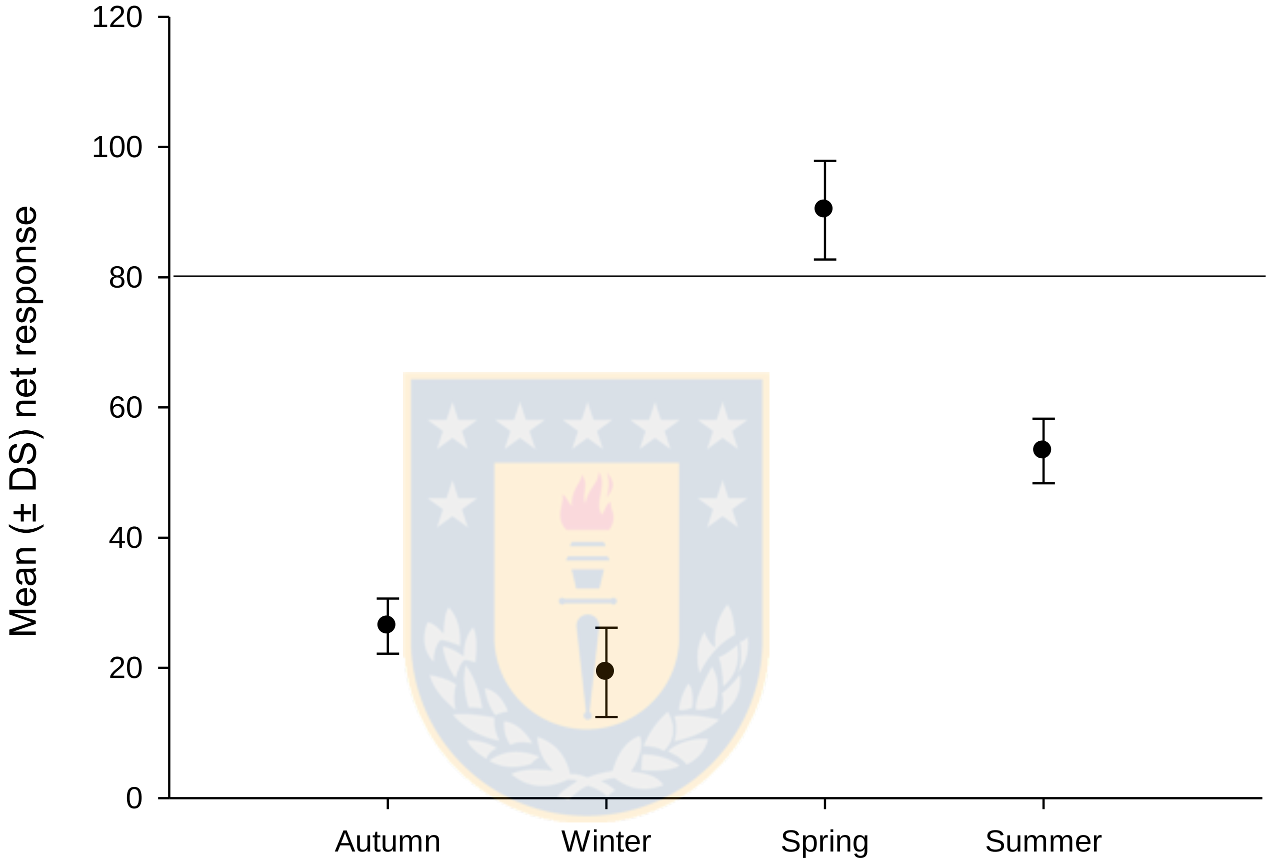
Figure 1. Net response of *Lumbricus terrestris* after the avoidance test



The results are expressed in mean values ± standard deviation of the individuals in the soil). The dotted line represents the threshold of the function of habitat according to Hund-Rinke and Wiechering (2001).

Figure 2: Relative activity in acetylcholinesterase and carboxylesterase in *Lumbricus terrestris* exposed for 48-h avoidance tests without duality in different seasons. Significant differences ($p \leq 0.05$) between the control (organic soil) and convention soil treatments.

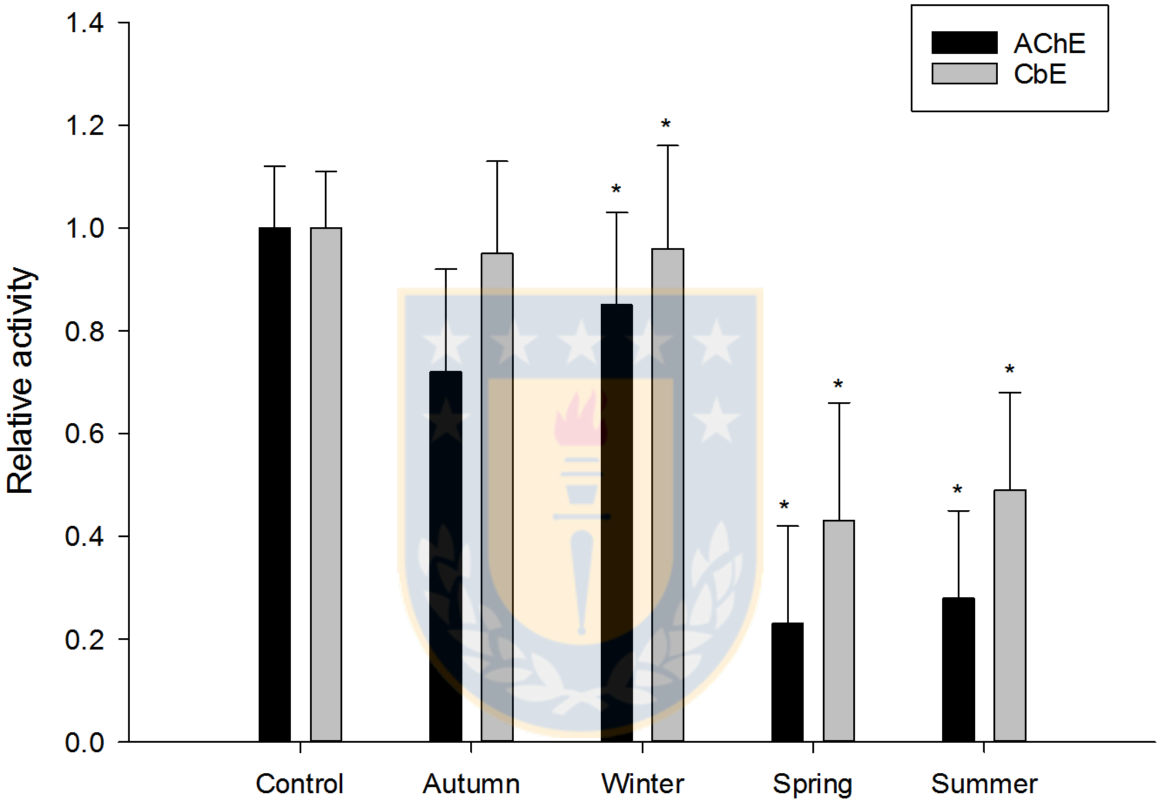


Table 1: Conventional apple orchard phytosanitary management

Seasonal application	**Chemical group**	**Active ingredient**	**log K_{ow}[1]**	**Clasification**
Autumn	Copper compound	Cuprous oxide		Fungicide
	Copper compound	Cuprous oxide		Fungicide
Winter	Oleophosphate	Fenitrothion		Acaricide
Winter- Spring	Dithiocarbamete	Alkylenbis	1.8	Fungicide
Spring	Oxymino acetate	Tifloxistrobina	4.5	Fungicide
Spring	Triazol	Fenbuconazole	3.22	Fungicide
Spring	Organophosphorus	Phosmet	2.95	Insecticide
Spring	Organophosphorus	Chlorpyrifo	3.42	Insecticide
Summer	Organophsphate	Methyl azinphos	2.75	Insecticide
	Organophosphorus	Chlorpyrifos	3.42	Insecticide
	Neonicotinoid	Thiacloprid	2-6	Insecticide
	Aminophosphonate	Glyphosate	- 3.5	Herbicide
	Organophosphorus	Methyl azinphos	2.75	Insecticide

1 Values were obtained from

Table 2: Seasonal physical-chemical parameters of conventional and organic orchard crops

	Conventional Orchard				Organic Orchard			
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
WC [%]	30.4±1.2	32.3±1.3	36.6±1.5	24.1±1.2	32.1±1.4	39.9±1.6	32.6±1.3	25.8±1.3
pH	6.1±0.0	5.98±0.0	5.9±0.0	6.0±0.0	6.42±0.02	6.44 ± 0.03	6.98 ± 0.02	6.55 ± 0.07
% OM	10.7 ± 0.10	10.4 ± 0.07	10.9 ± 0.06	10.7 ± 0.10	12.7 ± 0.10	12.9 ± 0.01	12.6 ± 0.10	12.0 ± 0.02
EC [dS m^{-1}]	1.33 ± 0.01	1.32 ± 0.01	1.33 ± 0.01	1.34 ± 0.02	1.26 ± 0.02	1.27 ± 0.01	1.30 ± 0.01	1.29 ± 0.02

CAPITULO V

ADOPCIÓN HACIA LA AGRICULTURA ORGANICA

Agroecology and Sustainable Food Systems
Submited: Mayo 2016

Organic producers in the Biobío Region, Chile: Perceptions about links to the public sector and support from scientific research

Ana D. Araneda[1], M. Rafaela Retamal[1], Melisa Villegas[1] and Barra, Ricardo[1]*

[1]Universidad de Concepción, Facultad de Ciencias Ambientales, Barrio Universitario s/n Concepción, Chile.

*Corresponding author: (ricbarra@udec.cl)

Abstract

In promoting organic agriculture in Chile a vitally important aspect is the attitudes and perceptions of agricultural producers toward adopting this type of production in economic and environmental terms. The objective of this work was to characterize agricultural producers in relation to the public and academic sectors and the existing biota. Interviews were conducted with organic apple producers in the city of Chillan, Biobío Region, Chile in 2015.

The producers presented a homogenous profile. The motivation for adopting organic agriculture was economic. They identified barriers like the limited relationship of producers with the public and academic sectors. Our conclusions indicate the need for a national policy to promote organic agriculture and that the main property of this policy should be the integration of the diversity of organic producers, agroecological zones and marketing systems.

Key words: Organic agriculture, apple production, semi-structured interviews, perceptions, Chile

INTRODUCTION

The change in vegetal cover for agricultural use has allowed feeding a growing global population (Keenan et al., 2015; Kim et al., 2015). This has configured a new landscape and generated a diversity of negative effects on ecosystems and human health. For example, deforestation is increasing greenhouse gas emissions (Tubiello et al., 2013). The increasing use of fertilizers to improve soil productivity and of pesticides to control weeds and pests has contaminated soils (Ondarza et al., 2011) and the infiltration of contaminants in surface (Donal et al., 2004) and groundwater (González et al., 2010), as well the biomagnification of these compounds in trophic chains (Evenset et al., 2016), with properties of persistence, bioaccumulation and toxicity are observed in these compounds. Since the 1960s, scientists, politicians and society in general have shown increasingly concern about these issues and have sought different ways to reduce or avoid the negative effects of agricultural on the environment. One of the most significant changes has been the adoption of new agricultural systems like organic agriculture that maintain monocrops dependent on external biological and/or botanical inputs (Altieri and Toledo 2011).

According to the UN Conference on Trade and Development (UNCTAD 2011), there are two million organic producers worldwide, of which 80% are in developing countries. Chile is among the countries exporting organic products and there are a growing number of agriculturalists adopting organic production. This scenario of the organic market, with increasing potential for expansion, has led to different lines of research. Two lines of scientific questions can be discerned from the market perspective:

- The subjective reasons for increased consumer demand for organic products, and
- The incentives and disincentives for producers to convert to organic agriculture.

In addition to these two broad research lines, a third has developed of experimental and comparative studies of the impacts (positive and negative) of conventional and organic agricultural production. As Díaz et al. (2015) indicated, this research line is less profuse and the results are not conclusive from the perspective of promoting certain agricultural policies over others.

In this context, there is a need for increased interdisciplinary research capable of combining at least two research lines, involving social and subjective variables about the decision by an agricultural producer to switch to organic production. Given the above, the objective of this interdisciplinary research is qualitatively characterize organic apple producers in the city of Chillán, Biobío Region, Chile, through the study of their perceptions, considering the relationship and links with the public and academic sector.

METHODOLOGY

Characterization of organic agriculture in Chile

Chile has certain advantages for organic production, namely:

- Varied climatic conditions that allow for diverse products produced largely during the winter of the Northern Hemisphere.
- Plant and animal health protected by the isolation provided by the desert, Andean range, Pacific Ocean and Antarctic.
- Equally exceptional level of plant and animal protection and oversight by the Agricultural and Livestock Service (SAG for its name in Spanish), which operates under the Ministry of Agriculture, which employs high standard to prevent the entry of pests.
- Law 20.089, which created the National System of Certification of Organic Products in January 17, 2006.

All these elements resulted in the sustained growth of organic production in Chile in recent years, with a total of 100,986 ha with certified organic production, 81,054 ha of which are for gathering certified wild crops and the remaining 19,932 ha are for organic agriculture. Of the latter, the most important crop is wine grapes (3,735 ha), followed by berry fruits with 3,600 ha and then tree fruits with 2,455 ha (ODEPA, 2015).

From the point of view of exports, SAG (2013) indicates that the major agricultural export product is fresh fruit, the most important fruit being apples with more than 16,000 tons per year, representing 74% of exported organic products, followed distantly by kiwi and avocado.

The Biobío Region has among the largest areas dedicated to certified organic production of the administrative regions in Chile (SAG 2013), and is viewed as a pioneering region in this field. The growth of organic agriculture in the Biobío Region is led by wild berry collection, with 80,870 ha and grassland pasture, with 14,341 ha. Another important sector in the Region is fruit production, including apples, olives, avocado and kiwi. The largest number of organic apple producers in Chile is located in the Biobío Region (ODEPA 2015). Apples are notably among the most difficult plants to grow organically given that under traditional cultivation apples require major applications of pesticides throughout the productive cycle.

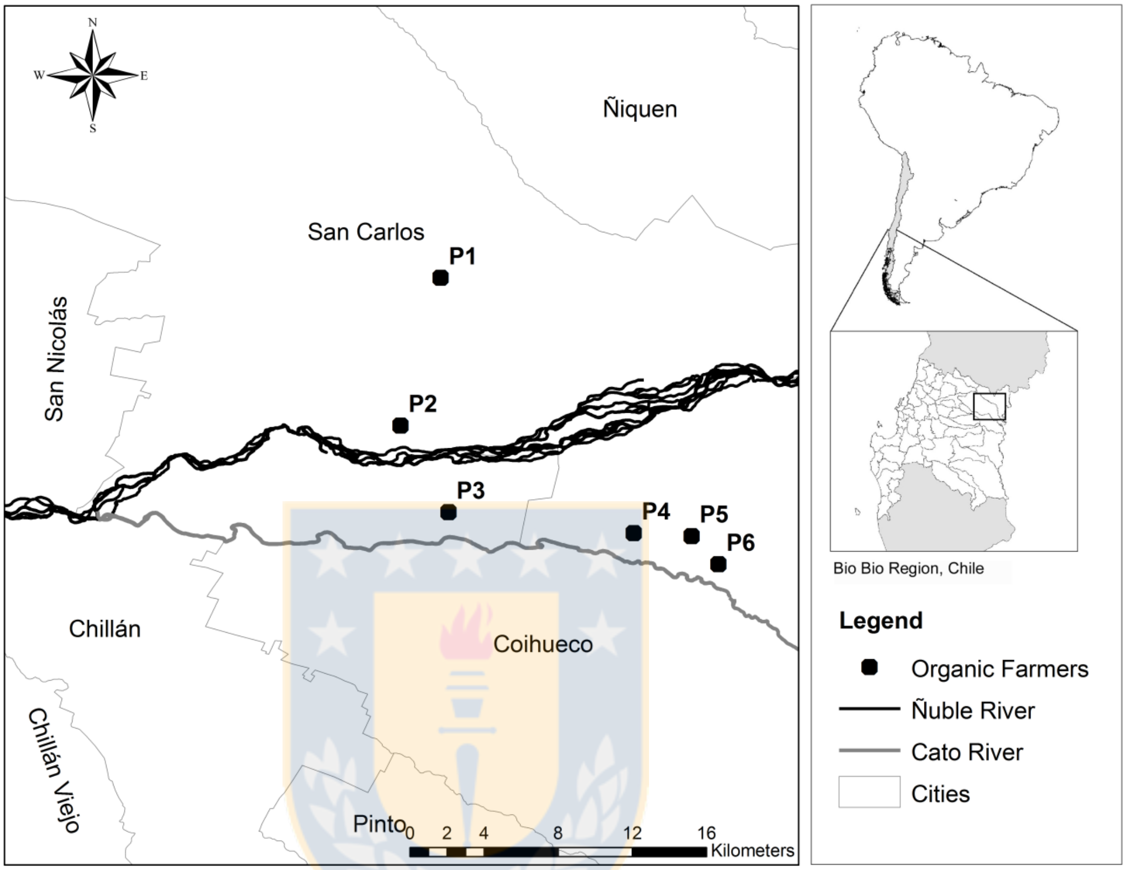
The main market for organic Chilean fruit is the US with 14,585 tons (71%) per year, followed by Europe with Europe with 6.946 tons (31%) (ODEPA, 2015), while the Biobío Region constitutes the main exporter region in Chile of organic apples to the US and Europe.

Structure of the interviews with organic producers

An instrument was designed to gather quantitative and principally qualitative information through a semi-structured interview based on perceptions, that is, the cognitive process of awareness that consists of the recognition, interpretation and significance in elaborating judgments about impressions obtained for the physical and social environment, with the intervention of other psychic processes like learning, memory, symbolization and interpretation (Vargas-Malgarejo 1994). The questions were defined considering the diagnostic made in the area of study in the city of Chillán, Biobío Region, Chile and based on national and international studies on the barriers to organic production (Hvitsand, 2016; Casagrande et al., 2015).

The semi-structured interview was applied to organic apple producers with farms with 2 to 19 kilometers of the city of Chillán, as shown in Figure 1. The producers were selected on the basis of databases of government bodies like SAG. Subjects were identified using the social networks of previously selected and interviewed producers using the snowball method (Morgan, 2008). The interviews were applied in person with six organic producers between October and November 2015 (Manojlovich et al., 2015).

Figure 1. Study area and location of organic apple crops



The interview was divided into six sections. The first section presented the research and its scope to generate confidence among the subjects. The second section dealt with identifying the interviewees. The other sections are described in Table 1.

The summary evaluation method was used with a Likert scale that indicates the degrees of agreement or disagreement with a statement. The results of the Likert scale are presented in graphs showing the degrees of agreement/disagreement in relation to the diagnosis of organic agriculture and the strengths and weaknesses of the sector in Chile.

Table 1. Sections and questions applied in the interviews with organic apple producers in the city of Chillán, Biobío Region, Chile

Section		**Closed-ended questions**	**Open-ended questions**
I	Identification of the interviewee	Age	
		Gender	
		Educational level	
		Type of business	
II	Spatial characterization	Area under cultivation	Reasons for engaging in organic agriculture
		Area under organic cultivation	
III	Temporal characterization	No. of years of conventional agriculture	Area under organic production within the next 5 to 10 years
		No. of years of organic agriculture	
IV	Current and future diagnostic of organic agriculture		Perspective on organic production in Chile in terms of area
			Perceptions of Law 20.089 for the certification of organic products in Chile
			Perspective on entering the market in terms of costs
			Perspective on social legitimization
V	Relationship with the public sector	Relations with the public sector	Role of the public sector in promoting organic agriculture
			Specific instruments to promote organic agriculture
VI	Relationship with	Main technical problems	Aspects of organic agriculture that

	the academic sector		should be deepened through research
		Types of technical advisory services	Development of Chilean research in organic agriculture compared to research in other countries
V	Relationship with the existing biota and soil properties		Recognition of beneficial edaphic organisms in the soil
			Role of earthworms

RESULTS AND DISCUSSION

A total of six subjects were interviewed, representing 90% of organic apple producers in the city of Chillán, Biobío Region. The analysis of the first section of the semi-structured interview provided a profile of the organic producers in the area of study and subsequent sections allowed for studying their perceptions.

Profile of organic producers in the study

The most evident result of this research is the homogeneity of the data on the organic producers in the study area. The average producer is male, around 46 years of age, has a high level of education, at technical education, has been working in organic agriculture for twelve years of a farm with 95 ha and constitutes a private company dedicated to exporting its products.

All the interviewees were male, which was the same in studies on a larger geographic scale. For example, in a study covering a larger area of agricultural production (central-southern Chile), Acosta et al. (2001) found that the majority of organic producers are male. The interviewees ranged in age between 40-62 years, with an average of 46 years of age, which again is similar to the age in profiles obtained Acosta et al. (2001), which ranged between 40 and 50 years of age.

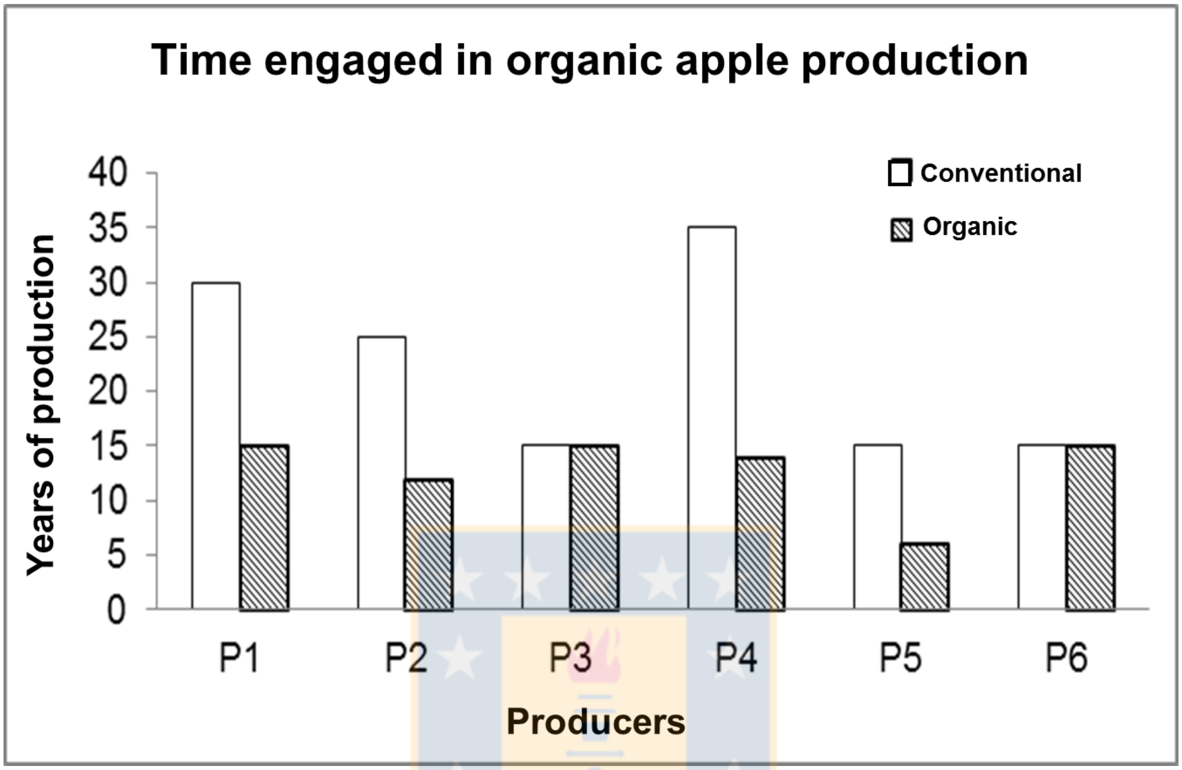
These two variables contribute to the defining the profile of organic producers in the area of study, similar to what was found by Ravi et al. (2015). Both the industry and academic studies have investigated the sociodemographic profile of organic producers around the world. To date these studies have yielded contradictory results that depend on the size of the area of study and the target market of organic products, which in this case is for export markets.

In relation to educational levels, all the interviewees have higher education, 66% have completed technical studies, 16% have incomplete university studies and 16% have completed university studies. These results reinforce two elements, firstly, and this distinguishes conventional from organic producers, is that the latter have higher levels of education than the former, which to a certain degree allows them to implement measures to ensure the productivity and quality of their crops (Bravo-Monroy et al. 2015). The second element is that since the 1980s it has been reported globally that organic producers have at least higher technical studies, which was recently reaffirmed by Alzaidi et al. (2013), who found that 80% of organic producers had higher studies.

In relation to spatial-temporal variables, interviewees have been engaged in organic agriculture for an average of 12.8 years, on farms with an average of 95.8 ha. The temporal variable is the first that allowed for making a distinction among the organic producers in Chillán. That is, 50% of the interviewees have been engaged in organic agriculture for well over the average of 15 years and the remaining 50% for much less time (Figure 2).

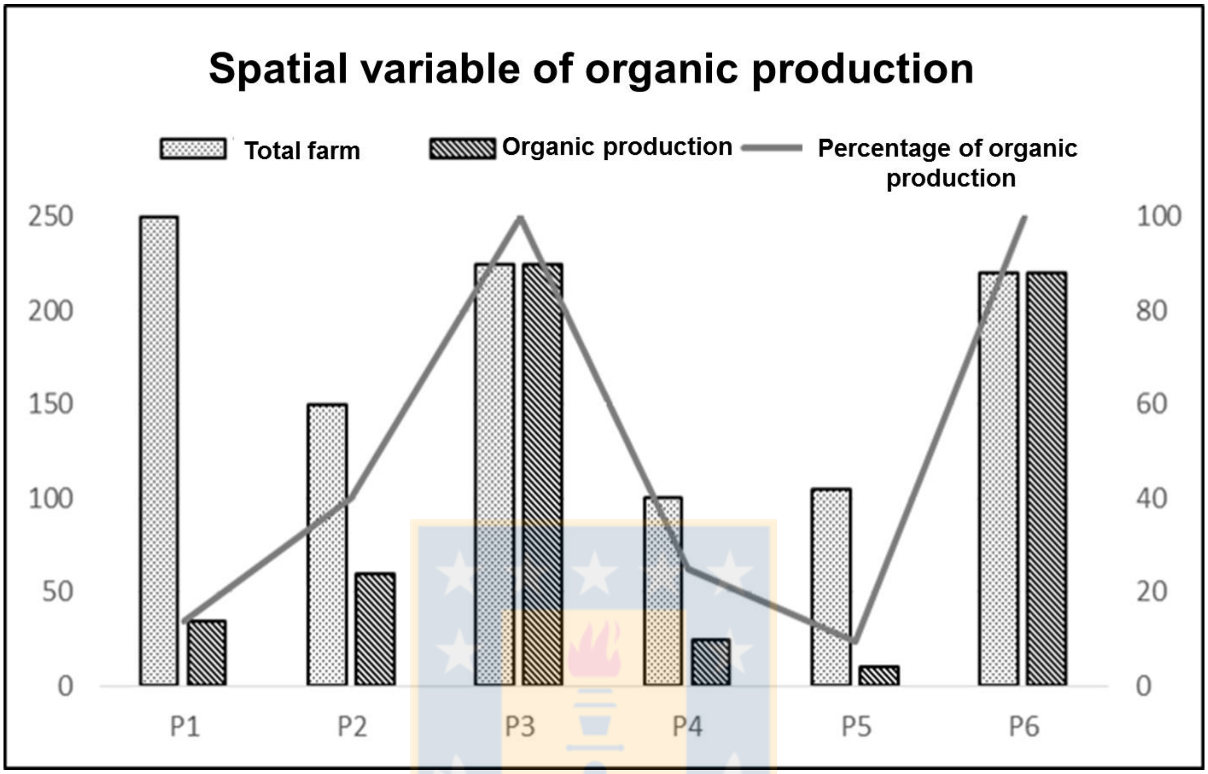
This result is corroborated by responses to the question “How long have you been engaged in organic agriculture?” and the question “Have you always been engaged in organic production or did you make the transition from conventional to organic agriculture?” Some 33% of interviewees have only engaged in organic agriculture while the remaining 66% made the transition from conventional production.

Figure 2. Time dedicated to organic apple production in Chillán, Biobío Region, Chile



The spatial variable is another differentiating element of the profile of organic producers in this study. On the one hand, the average area dedicated to agriculture, whether conventional or organic, is 175 ha, while the average area dedicated solely to the production of organic apples is 95.8 ha. In effect, the average producer dedicates around half of his farm to organic production. However, 33% of the interviewees dedicate all of their land to organic production and the rest dedicate much less than half to organic production (Figure 3).

Figure 3. Area dedicated to organic production in relation to total area dedicated to agriculture.



The results in terms of the area of organic production among the interviewees differ from those of other studies. For example, in other parts of the world the average size of organic farms is not greater than 10 ha, as is the case in the United States (Lockeretz and Anderson 1990) and Canada (Murphy 1992). However, organic farms in Germany tend to be larger than farms with traditional production. Consequently, the size of organic farms varies with geographic zones. In Chile, the waiting period of five years for organic certification and the lack of commercial networks hinder small-scale producers from converting to organic agriculture.

Perceptions of organic apple producers in Chillán, Chile

As described in the methodology section, the open-ended questions were grouped into four sections: (1) Current and future diagnostic of organic agriculture; (2) Relations with the public; (3) Relations with the academic sector; and (4) Relations with the existing biota and soil properties.

The results of this study indicate that the interviewees have homogenous attitudes and perceptions about adopting organic agriculture, as reflected in the similarities of their responses.

Current and future diagnostic of organic agriculture

We identified that producers convert to or initiate organic production because of the profitability of such crops. Other studies have reached the same conclusion, that the economic factor is preeminent in producers deciding to convert to or initiate organic agriculture (Offermann and Nieberg 2000): As well, according to the interviewed producers, the economic benefits are greater when their products are exported to overseas markets like the USA or Europe. This is similar to what occurs in other Latin American countries, where the majority of organic products are destined for export markets, like coffee and bananas from Central America, sugar from Paraguay and cereals and meat from Argentina (Garibay and Ugas 2009).

According to other authors, a lesser number of producers engage in organic production as a result of their environmental awareness, indicating that the contamination produced by the use of agrochemicals deteriorates human health in the short and long-term (Vogtmann et al., 1993; Wynen 1990).

An example of this result was identified by Elsebeth and Egelyng (2008), who concluded that Brazilian organic coffee producers consider that organic agriculture allows them to improve their quality of life, given that the use of agrochemicals has a negative effect on personal and family health, and that the decision to convert to organic production is based primary on this premise and secondly and less importantly on the profitability of organic production.

In response to a question about the future of organic production in their region, 83% of interviewees indicated that production would probably increase through expansion of organic products, such as organically produced meat, which will require organic pastures.

The organic producers in this study, who are basically exporters, were asked if increasing and diversifying organic production, it is possible to introduce their products in the internal market. The interviewees had basically two types of responses: that the internal market was not prepared to pay for certified organic products; and that there are sociodemographic variables that limit internal marketing of organic products.

In the first category, the situation is clear for all producers, that an organically produced apple costs three times as much as a conventional one, while the Chilean public is not willing to pay the additional cost for a variety of reasons, among them a lack of information about the relationship chemical-free food products and human health.

The second category incorporates the variables that producers consider as barriers to national markets for organic products. The low levels of environmental awareness of the Chilean population, which results in a smaller target population, added to which are the great distances between urban centers and low levels of connectivity, combine to impede the introduction of organic products in the internal market.

The interviewees responses indicate that organic producers in Chillán feel that their current production could be increased and diversified, but for the external market. Such growth is occurring in other countries, indicated by the increasing number of hectares converted from conventional agriculture. In Germany, all the organically produced crops are increasing by a maximum of 1-hectares per year with the support of subsidies, although this production is largely aimed at the internal market (Brenes- Muñoz et al. 2016), Similarly, the demand for and supply of organic foods has been increasing since the 1980s due to the preferences of consumers concerned about the nutritional value and quality of foods (Dalcin et al. 2014).

Perceptions about the public system

With respect to the interviewees' perceptions of the public sector, all of them indicated that Law 20.089, which created the National Organic Products Certification Service, is very restrictive. Interviewees stated that the law should be more flexible, reflecting the varied geographic and climatic conditions and soil types in the country, which affects organic production in the internal Chilean market. In effect, agriculture in northern Chile is different from that in the south.

The instrument for evaluating perceptions distinguished two aspects of organic production. Firstly, that Law 20.089 favors certification of organic products for export, and secondly that the same law does not promote internal markets for organic products, whether in popular or fair trade markets.

Unfortunately, these conditioning categories have also been identified in other countries. For example, Ahnströn et al. (2008) that there is an excess of regulation of organic agriculture in the United Kingdom, which threatens the recruitment of new producers. In Latin American it has been identified that to ensure internal markets and increase organic production it necessary to promote popular and fair trade markets. With this premise, government programs in Costa Rica ensure that 50% of organic food products are sold in supermarkets (Garibay and Ugas 2009). In this sense, there are other instruments in addition to organic certification that promote the recruitment of organic producers, increase organic production and increase sales in internal markets.

Interviewees were unanimous in their response to questions about their relationship with the public sector, who felt that there is no relationship, adding that the subsidies and programs provided by the government are for small-scale producers that translate into subsidies for survival and not for growth. They also noted that the personnel of public bodies that provide subsidies lack adequate training or a solid base of knowledge to aid small-scale produces to grow.

Comparing these results with global trends we find cases in Europe and Latin America that support the perceptions of the interviewees in this research. For example, many farms in southern Brazil are currently converting to organic production with support from the Ministry of Agriculture, which developed a participatory certification program (Honorato et al. 2014). In Saudi Arabia there are outreach programs and agents to increase and improve the capacity of organic producers (Oluwasusi 2014), and a national program to improve efficiency and productivity and to explore new local and international markets.

Exploring the theme of training and specific support programs, the interviewees indicated that there are no programs in Chile that integrate public health and healthy eating and quality of life, in particular with incorporating organic products in the diet.

More than 60% of producers are convinced that the government should include information on the relation between organically produced foods and health in educational programs. Some of the interviewees stressed that these educational programs should begin at an early age and that they should be accompanied by efforts to promote healthy living, organic agriculture, and urban farming, as well as information about the effects of agrochemicals on agricultural systems. Isin et al. (2007) emphasizes the important role of education in building support for organic agriculture.

Perceptions about the relationship with the academic sector

In another interview session interviewees were asked about their relationship with the academic sector. As with the closed-ended questions to establish a profile of organic apple producers in Chillán, the responses were homogeneous. All the interviewees agreed emphatically that there is no relationship with the academic sector, whether universities or research institutions, and that they draw on private professional support when needed.

This is in contrast to what can be observed in general in Latin America where there are technical-scientific programs to support the development of organic agriculture. For example, the Brazilian Biodynamic Institute offers education on organic agriculture and promotes access to land for biodynamic agricultural producers. The Agrecol Andes Foundation (AGRECOL 2016) in Bolivia provides services for knowledge management through training, systematization of experiences, promoting participatory methodologies, disseminating information, information and coaching to contribute to improving conditions of life in rural Andean communities. Colombia promotes the creation and development of capacities and training in organic agriculture. There are doctoral programs in Peru in agroecology and a degree program in managing organic enterprises was developed in Argentina.

The same occurs in countries in other continents. For example, in China there are programs for research and strategic development of organic agriculture funded by the Ministries of Science and Technology and of Agriculture (Egelyn et al. 2006). Organic production is supported in Saudi Arabia with feasibility studies (Alzaidi et al. 2013).

Exploring the causes of the lack of relationship between organic producers and academics, interviewees indicated that researchers are not interested in organic production and consequently there are few professional experts in this area and an absence of a network of contacts to develop long-term research projects on the climatic and edaphic diversity of Chile, this being Chile, the latter being a critical factor to ensure increase applied research on organic agriculture. Some 50% of the interviewees understand that this lack of relationship with academia could be due to the limited funding from the Chilean government for such scientific work. All the interviewees concluded that the role of the academic sector should be fundamental for organic agriculture considering that science allows for developing new and better technologies.

Without the need to ask interviewees about their information needs, they volunteered that they need more and alternative information about controlling weeds and fungi, more research on the soil as a dynamic resource, and finally a greater variety of available organic fertilizers.

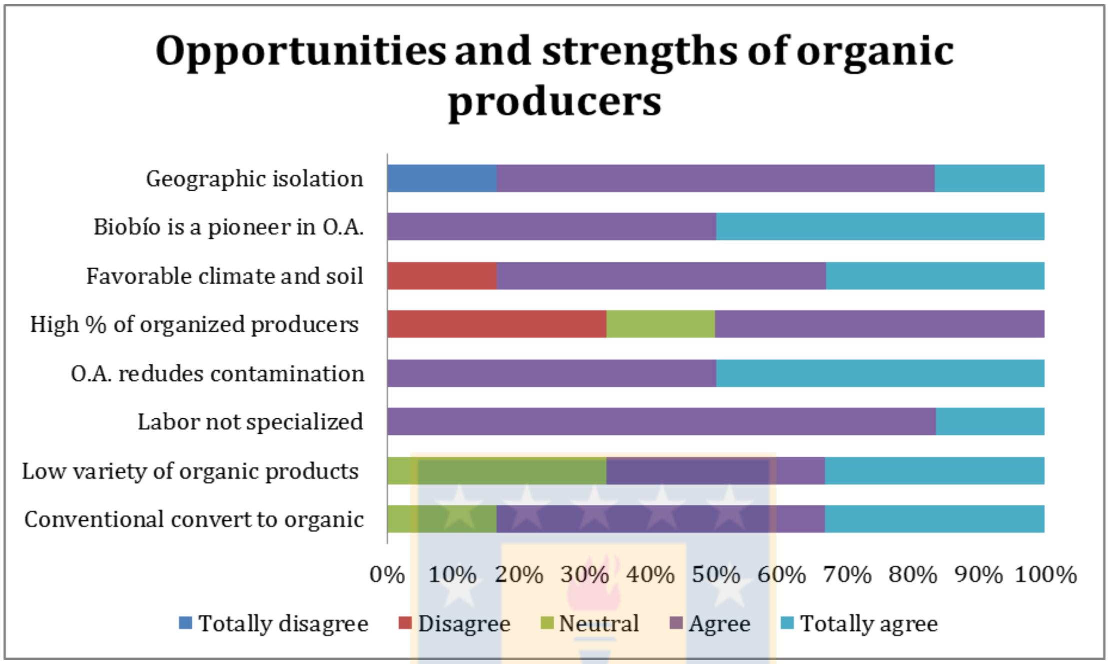
Perceptions about the existing biota and soil properties

The responses of interviewees to the questions in the last section of the survey dealing with perceptions about the existing biota and soil properties were encouraging in terms of the level of knowledge among producers. Some 80% of interviewees considered that successful production is accompanied by harmony and diversity of edaphic organisms and microorganisms, this being the most important characteristic for the total growth of the plants, permitting the soil to be dynamic and able to provide nutrients to the plant. Interviewees mentioned that earthworms are very beneficial in agricultural soil because of their agroecological characteristics, such as decompacting soil and oxygenating plant roots, as well as providing nutrients through excretions from their gastrointestinal tract. However, the most important challenge is to address pests and weeds to avoid crop loss without using pesticides. This perception of the organic producers is the most serious problem, because of which they stated that preventative work is always necessary. These results are consistent with studies by Al-Subaiee (2006) and Azaidi et al. (2013).

Following the open-ended semi-structured questions a summary evaluation method was applied using a Likert scale indicating the degree of agreement or disagreement on a five-point scale in response to statements about the strengths and opportunities for organic production. We highlight the following:

Over 80% of interviewees consider that the geographic isolation of Chile allows for developing organic agriculture with low levels of problems with pests owing major geographic barriers, while the other interviewees disagreed, indicating that pests are changing genetically over time and becoming more resistant, Chile has a good regulatory framework for organic agriculture, and a good enforcement service in the form of the Agricultural and Livestock Service (SAG). All of the producers were in total agreement that the Biobío Region is a pioneer in organic agriculture in Chile, with the largest area under organic production and the highest level of production of organic apples. Some 50% of them thought that organic producers are well organized, 40% thought the contrary, and 10% had no opinion. Jacobson et al. (2003) had similar findings with organic producers in northern Florida, who consider themselves better organized than conventional producers (Fig 4).

Figure 4. Level of perception on the current diagnostic of organic agriculture in Chile and in the region under study



In response to a statement in relation to converting from traditional to organic agriculture, more than 80% of interviewees consider that such conversion occurs at the level of small- and medium-term, because the demand for organic apples is increasing steadily in international markets, with continuous growth in markets in Europe (Lampkin 2000), Saudi Arabia and other countries (Alzaidi et al. 2013). Some 90% of interviewees maintain that there is little development of the national market and 10% of them had no opinion.

Some 60% of interviewees consider that there is little variety among organic produces in Chile and 30% had no opinion, which is similar in other countries where there is limited variety in organic products, as in the case of Turkey (Tanrivermis 2008) In large measure the climatic, topographic and socioeconomic characteristics of Chile limit the possibilities of variety, in contrast to Spain, where there is a great variety of organic products owing to the different agroeconomic and geographic conditions in the country (Armesto-lópez 2008).

All of the interviewees were in agreement that the available labor force lacks any specialization in organic agriculture. Organic agricultural generally requires more labor and more qualified labor than conventional agriculture. The requirement for contracted labor is on average

9.1% higher with organic than conventional production (Tannvermi 2006). As well, agricultural machinery does not play an important role in organic agriculture. In their research in California, Altieri et al. (1983) concluded that the cost of labor for the production of organic apples was 20-30% higher than for conventional production, which is similar to what was found by Klepper et al. (1977) and Keipert et al. (1990) in the case of organic production in Germany.

Finally, 90% of the interviewed organic producers consider that organic agriculture reduces soil, water and air contamination, and 10% had no opinion. This positive attitude about organic agriculture concurs with findings of studies by Kotile and Martin (2002) and Alzaidi et al. (2013).

CONCLUSIONS AND CONSIDERATIONS

This research studied the perceptions of organic producers using qualitative methodologies to identify incentives and disincentives for organic production around the city of Chillán. Similar research in other countries indicates that it is difficult to define or characterize organic producers in a given region or sector. However, in this research both the profile of producers and their perceptions and attitudes were highly homogeneous. This is one of the main results of this research, given that perceptions and attitudes are in continual formation at the individual level and the differences are more evident when analyzed at the level of groups of individuals.

The analysis and interpretation of the results indicates the interviewed producers can be classified as large-scale organic producers given that they own large farms, have high levels of education compared to conventional producers and orient their products to international markets. This reality reduces the contamination of soils and the biota and increases rural development through the permanent employment of skilled labor, thus reducing rural-urban migration and the associated socioeconomic problems.

Organic agriculture has greater potential to contribute effectively to food security, human health, and meeting environmental standards at a low cost compared to conventional agricultural practices. While this framework for promoting organic agriculture is known in different areas of the society (academia, agricultural policy-makers, outreach workers, a significant part of the population, etc.), there is a need to explain the motivations for developing this type of production.

The second conclusion of this research is that the main motivation in adopting organic agriculture is economic, followed by concern for the environment and lastly human health considerations. The analysis of the results suggests that these large-scale organic producers were large-scale conventional producers in the past that made the transition to organic production because of potential economic returns. This is probably also the reason for the absence of small-scale organic apple producers in the area of study, and as well, the reason for the homogeneity of profiles and perceptions among the interviewees.

With respect to barriers to making the transition from traditional to organic agriculture, the interviewees identified pest control and fertilization. As well, the interviewees emphasized that one way to overcome this barrier is through the active involvement of the public and academic sectors in promoting organic production, for example through support in obtaining certification, the development of subsidies, training of expert of advisors and research into organic farming. At the same time, producers indicated that this link could constitutes an opportunity to increase the scope of organic agriculture, it is a limiting factor given that no close relationship exists between organic producers and the public and academic sectors.

A possible inference or working hypothesis of this research is that government support for organic agriculture in Chile encourages in greater measure large organic producers to be certified so that their products can be sold in international markets. This hypothesis concurs with the national agricultural policy as promoted with the slogan “Chile Potencia Agroalimentaria” (Chile empowers agrofoods), in which the marketing route is exports.

This research indicates that the most important characteristics of a policy to promote organic agriculture is the capacity to integrate the diversity of present and future organic producers, in effect, sufficiently flexible that producers can form alliances with universities and research institutes and public bodies to develop tools, subsidies and means of diffusion of sustainable agricultural practices. Policies should be sufficiently associative to allow conventional small producers to make the transition to organic production through the formation of cooperatives to facilitate access to credit.

The policy should be integral in the sense that it integrates a diversity of agricultural producers and all agroecological and climatic regions of the country. While this research defines the area of study in function of one of the most heavily exported organic products from Chile, the literature review to understand and interpret the perceptions and attitudes of interviewees indicates that the measures, practices, tools and training should be designed locally to ensure adoption by producers, as well as their efficient implementation. This strategy raises an even greater challenge for the country, that of more decentralized and participatory environmental governance.

National policy in Chile to promote organic agriculture should incorporate diverse marketing strategies and products. On the one hand, actions should be taken to favor growth of internal markets, and on the other, the state should play a leading role in developing outlets for marketing and organic agriculture service centers where inputs like improved organic seeds, organic fertilizers and herbicides are available at subsidized prices that affordable for producers in transition. Public funding should be defined at the level agroecological zones to favor decentralization.

Given all of the above, it is essential that there be a policy and long-term strategy to support the transition to or initiation of organic agriculture. Among the policy instruments, the state and research institutions should play a determining role in innovation, consultancy, training and the provision of financing in the first stages of transition, above all with small-scale producers.

ACKNOWLEDGEMENTS

Financial support was provided by FONDAP-CONICYT Project 15130015, with the Centro de Recursos Hídricos para la Agricultura y la Minería (CRHIAM).

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CAPITULO VI

DISCUSIÓN Y CONCLUSIÓN

DISCUSIÓN GENERAL

La sustentabilidad de la producción agropecuaria, también depende de indicadores que nos permitan verificar los impactos en el compartimento suelo de la carga de pesticidas ampliamente utilizados en la actividad. En Chile no existen aún normas secundarias de calidad de suelo, y esta tesis doctoral es un aporte en lo que concierne al uso de bioindicadores para evaluar el efecto de los plaguicidas agrícolas sobre la fauna edáfica. La aproximación de los biomarcadores puede ser un enfoque innovador para tener herramientas diagnósticas que permitan abordar el tema de los efectos a través de respuestas biológicas significativas medibles en los bioindicadores seleccionados como la lombriz de tierra, componente clave, que contribuye y mejora la estructura y la función de los suelos en beneficio para el crecimiento vegetal.

En este estudio, mediante el uso de la lombriz de tierra de la especie *Lumbricus terrestris*, como indicador de agrosistemas, junto a dos enzimas esterasas; acetilcolinestera (AChE) y carboxilesterasa (CbE) como biomarcadores de exposición se pudo evidenciar respuestas biológicas en un sistema agrícola con manejo convencional y en ensayos de comportamientos en laboratorio.

Se conoce que las pruebas de toxicidad con lombrices de tierra son importantes en los laboratorios para apoyar la autorización de los agroquímicos al comercio (Dinter et al., 2013). Sin embargo, ¿Cuáles son los efectos a largo plazo de los agroquímicos en los organismos no objetivos como las lombrices de tierra? En este sentido, este estudio evidencia las respuestas biológicas a través de las esterasas (CbEs y AChEs) asociadas a la exposición de los pesticidas como organofosforados principalmente, carbamatos, grupos químicos con mayor aplicación en la agricultura chilena. Esta exposición, está relacionada a la reducción e inhibición de estas enzimas que finalmente se traducen en el efecto selectivo de la función neuronal en el nivel sináptico, y el mal funcionamiento de las terminaciones nerviosas en los tejidos musculares de la lombriz de tierra (Pereira et al., 2010), provocando alteraciones en el sistema locomotor de la lombriz de tierra, efectos endocrinos a nivel individual y poblacional (Gonzales et al., 2010) y también puede verse bloqueado el rol de las carboxilesterasas en la transformación de los pesticidas a moléculas más solubles en el suelo como alcoholes, aminas y ácidos carboxílicos (Thompson et al., 2004).

Los biomarcadores son necesarios cuando se requiere realizar evaluaciones de riesgo o conocer el estado general de un sistema ambiental, es así, que muchas investigaciones apoyan que la inhibición de la acetilcolinesterasa (AChE) y carboxilesterasa (CbE) en las lombrices de tierra son buenos indicadores para evaluar respuestas asociadas a la exposición de los pesticidas en el suelo (Joordan et al., 2012; Martinez et al., 2013). La literatura existente estima que la inhibición de la acetilcolinesterasa producto de la exposición de pesticidas se produce cuando esta esterasa se ha reducido en un 30% respecto a un control o referencia (Booth et al., 2001; Martinez et al., 2013; Velki and Hackenberg 2103), además se ha estudiado que las carboxilesterasas pueden contribuir a reducir el efecto de los clorpirifós antes de que ocurra la inhibición de la actividad acetilcolinesterasa, y que promueven la destoxificación no catalítica de compuestos organofosfordos por la formación de un complejo enzima-inhibidor (Sogorb and Vilanova, 2002). En este estudio se ha demostrado que la mayor sensibilidad de la actividad de la exposición a pesticidas está dado por las carboxilesterasas y coincide con trabajos de Sanchez- Hernandez and Wheelock (2009) y Collange et al. (2010), que la detoxificación de clorpirifós por las carboxliesterasas fue más prominente que la acetilcolinesterasa en las lombrices de tierra que fueron expuestas a la pruebas de comportamiento.

En primer lugar, la selección de las especies de lombrices de tierra es muy importante. La mayoría de los estudios de laboratorio han utilizado especies del género *Eisenia*, para investigar la interacción entre la actividad de las colinesterasas en pesticidas organofosforados o carbamatos (Caselli et al., 2006; Gambi et al., 2007; Rault et al., 2007) y limitados son los estudios similares con especies de lombrices de tierra que realmente habitan y son relevantes ecológicamente en suelos agrícolas (Paoletti et al., 2008), como aquellas que tienen hábitos funcionales anécicas y endógeas que tienen la capacidad de desplazarse a mayor profundidad de la superficie del suelo, formando galerías verticales, y pudiendo entregar mayores respuestas respecto a la movilidad de un producto químico en el suelo, no así las lombrices epígeas como *Eisenia sp*., que desarrollan su ciclo de vida a pocos centímetros de profundidad de la superficie, siendo menos precisas para detectar alguna respuesta biológica en un agrosistema. (Paoletti et al., 2008) De hecho, la sensibilidad de actividad de las colinesterasas a los pesticidas y su tasa de recuperación varían notablemente entre las distintas especies de lombrices de tierra (Rault et al., 2008).

En segundo lugar, la selección del tejido diana para mediciones de esterasas es también un elemento importante en un programa de vigilancia o monitoreo. Aunque la mayoría de los estudios utiliza todo el organismo para ensayos de esterasas, otros estudios han demostrado que las acetilcolineterasas y las carboxilesterasas muestran una distribución dependiente del tejido, además las respuestas de las actividades de las esterasas a los pesticidas anti colinesterasas dependen de los tejidos donde se exprese mayormente. (Collange et al., 2010; Gonzales et al., 2010). Siempre que sea posible, se recomienda la disección de varios tejidos para la determinación de la carboxilesterasa y la acetilcolinesterasa.

En tercer lugar, el seguimiento de los efectos biológicos de los pesticidas en el campo, suelen utilizar la inhibición de la actividad de la acetilcolinesterasa como biomarcador decisivo para las respuestas biológicas. (Denoyelle et al., 2007) Sin embargo, la actividad de la carboxilesterasa ha demostrado ser un biomarcador complementario y altamente sensible a la exposición a los organofosforados y carbamatos (Collange et al., 2010; Gonzales et al., 2010)

Las carboxilesterasas son abundantes en el tracto gastrointestinal de *L. terrestris*. (Sanchez-Hernandez and Wheelock 2009) Muestran una alta sensibilidad a la inhibición por organofosforados. Estudios in vitro han demostrado que estas esterasas son secretadas por el epitelio intestinal, aunque los microorganismos intestinales que habitan en el intestino de la lombriz también puede contribuir al total de la actividad hidrolítica por CbEs. (Sanchez-Hernandez et al., 2009) Esta actividad luminal de las carboxilesterasas puede desempeñar un papel importante en la reducción de la toxicidad aguda anticolinesterasica. Del mismo modo, esta actividad esterasa luminal proporciona un importante campo de la investigación en la biorremediación de los suelos con plaguicidas contaminados. Sin embargo, estas suposiciones necesitan ser evaluadas para conocer su alcance real en un proceso de biorremediación.

Dentro de los objetivos de este estudio y como complemento a las respuestas biológicas de la exposición de las lombrices de tierra, se realizó la identificación de los residuos de pesticidas en el huerto de manzano con manejo convencional en una de las épocas de muestreo correspondiendo a la estación de primavera, periodo en el cual, el huerto recibe mayor aplicación de pesticidas del grupo químico de los organofosforados,

Las muestras fueron tomadas 30 días después de la aplicación de clorpirifós, mediante el método de extracción de fase sólida (SPE) y cromatografía líquida de alta resolución con detector de arreglo de diodos (HPLC-DAD) en el laboratorio de ensayos EULA-Chile. Los resultados no mostraron la presencia de clorpirifós (organofosforado). Los valores fueron <0,005 µg/g. La ausencia de residuos de pesticidas en el suelo se debe a su baja persistencia en el ambiente, entre 6 a 21 días dependiendo el suelo (Knuth et al., 2000; Alvarez et al., 2010) y sus posibles movilidades a otros compartimentos ambientales. Sin embargo la breve vida media podría ser ventajoso sobre los efectos de los organismos no objetivos, pero los clorpirifós carecen de especificidad y se ha demostrado que son muy tóxicos para muchos organismos no objetivos vertebrados e invertebrados (Fulton and Key 2001; Booth and O'Halloran 2001; Phillips et al., 2002).

Los resultados encontrados en este estudio realizado en cuatro épocas de muestreo durante los años 2014-2015 tanto en laboratorio como en agrosistemas con manejo convencional y orgánico de huertos de manzano de la Provincia de Ñuble, Región del Biobío indicaron que las carboxilesterasas en el tejido del tracto intestinal de *L. terrestris* había una mayor actividad de la CbE en el tejido del buche-molleja en el tratamiento control con 14.22 ± 1.00 µmol min^{-1} mg^{-1} en invierno y la menor actividad en el mismo tejido buche-molleja se registró en el suelo con manejo convencional en la época de verano con 6.15 ± 2.77 µmol µmol min^{-1} mg^{-1} ($p \leq 0.05$). La actividad enzimática evidenció una diferencia estacional, siendo mayor en invierno con 14.22 µmol min^{-1} mg^{-1} y en otoño con 13.93 µmol min^{-1} mg^{-1} y menor actividad en verano con 6.15 µmol min^{-1} mg^{-1} y primavera con 6.31 µmol min^{-1} mg^{-1} ($p \leq 0.05$). Para los bioensayos de comportamiento realizados en laboratorio, a través del ensayo de comportamiento de evitación por 48 horas, las lombrices de tierra evitaron los suelos en los periodos de primavera y verano, posteriormente las actividades de las esterasas evidenciaron que todas las lombrices fueron expuestas a los pesticidas utilizados en este cultivo convencional, la actividad acetilcolinesterasa se redujo en los periodos de primavera en 2,9 µmol min^{-1} mg^{-1} y verano con 3,6 µmol min^{-1} mg^{-1} y se recuperó en los periodos de otoño en un 72% e invierno en un 85%, similar es lo ocurrido con la actividad carboxilesterasa siendo más sensible su inhibición en el periodo de primavera con 15,2 µmol min^{-1} mg^{-1} y verano con 17,2 µmol min^{-1} mg^{-1}.

Los resultados encontrados tanto en laboratorio como en el campo evidencian efectos biológicos similares a través del uso de biomarcadores de exposición, (Tabla 1), en el laboratorio, las lombrices de tierra evitaron los suelos de las épocas de primavera y verano.

Tabla 1. Resumen de las actividades carboxilesterasas y acetilcolinesterasas en bioensayos de laboratorio y de campo durante las épocas de muestreo en el periodo 2014-2015. (Promedio ± desviación estándar)

	Ensayos en laboratorio		**Ensayos en Campo**	
	Actividad AChE (µmolmin^{-1}mg^{-1})	Actividad CbE (µmolmin^{-1}mg^{-1})	Suelo manejo Orgánico	Suelo manejo Convencional
			Actividad CbE (µmolmin^{-1}mg^{-1})	Actividad CbE (µmolmin^{-1}mg^{-1})
Control	14,2 ± 4,5	33,4 ± 2,0	13,1 ± 3,3	12,3 ± 1,2
Otoño	9,1± 5,9	34,2 ± 5,5	12,6 ± 3,7	8,6 ± 1,8
Invierno	10,4 ± 3,2	34,8 ± 5,5	13,9 ± 1,7	11,4 ± 4,0
Primavera	2,9 ± 3,7	15,2 ± 3,5	12,2 ± 3,3	6,3 ± 1,4
Verano	3,6 ± 5,3	17,2 ± 1,3	11,7 ± 3,9	6,1 ± 2,1

Los análisis de los biomarcadores en *L. terrestris* mostraron diferencias en las distintas épocas estacionales. De acuerdo a la aplicación anual de pesticidas en este agrosistema convencional, donde las aplicaciones de plaguicidas organofosforados se realizan intensamente en primavera y verano, lo cual, existiría una relación con la actividad de estas enzimas provocando una mayor inhibición cuando se encuentran expuestas en estos periodos del año, posteriormente habría una recuperación por parte de las enzimas en los periodos de otoño e invierno donde la actividad enzimática es similar al control y las aplicaciones de agroquímicos en estos cultivos es menos intensa. Además importante es mencionar que estos resultados podrían estar relacionados al mayor uso de los pesticidas organofosforados y carbamatos, aplicados en las estaciones de primavera y verano, en los huertos de manzano con manejo convencional y que pueden permanecer retenidos por la materia orgánica del suelo que osciló entre 10.2 a 10.7%, factor importante en la retención de los pesticidas en el suelo (Boivin et al., 2005; Alvarez et al., 2013). También hay autores que sostienen que los pesticidas en el suelo pueden ser biodegradados por organismos que se encuentran en la materia orgánica (Sorensen and Aamand 2001). Estos efectos contrastados están relacionados no solo con las propiedades de los pesticidas, sino también con la naturaleza de la materia orgánica. Otras de las posibilidades de la inhibición de estas esterasas en primavera y verano es el bajo lavado que tienen los suelos en estas estaciones por las bajas precipitaciones respecto al invierno, ocurriendo la permanencia de los residuos de pesticidas hasta su degradación (Silburn et al., 2013; Gomez et al., 2011; Sian et al., 2010).

Las respuestas de inhibición de estas esterasas están en estrecha relación con la evitación de los suelos agrícolas convencionales en los periodos de primavera, pero en verano las lombrices de tierra no son capaces de evitar los suelos convencionales considerando la reducción de las actividades de las enzimas, este comportamiento podría tratarse de un efecto no especifico o retardado de estrés locomotor sobre el desplazamiento al suelo control, donde la influencia de las condiciones climáticas en verano se caracterizan por tener altas temperaturas y baja humedad aumentando aún más este estrés.

Estas respuestas asociadas a la exposición de pesticidas conllevan muchos cuestionamientos cuando se desconoce la cantidad y persistencia de los residuos de los pesticidas en el suelo. Este es un debate que ha llevado a muchos investigadores a buscar aproximaciones reales para la validación en el campo de las esterasas en las lombrices de tierra, siendo un paso esencial para poder realizar una evaluación de riesgo, sin embargo los estudios de campo que han determinado efectos biológicos a la exposición a pesticidas no siempre ha proporcionado las respuestas correctas, debido a la complejidad del ensayo de campo, el indicador apropiado, como con otros muchos organismos la actividad de las esterasas responden generalmente a una dosis dependiente, pudiendo fácilmente realizarse en condiciones controladas en laboratorio.

Pocos estudios de campo han abordado el uso de las acetilcolinesterasas y carboxilesterasas como biomarcadores. Por ejemplo, la lombriz de tierra *A. caliginosa* se utilizó para la prueba de inhibición de la acetilcolinesterasa siguiendo las tasas de recomendación establecidas para la aplicación de diazinon y clorpirifós (Booth et al., 2010), no se observó inhibición de la AChE durante el periodo de seguimiento de 28 días, sin embargo Reinecke and Reinecke (2007) registraron una inhibición significativa de la acetilcolinesterasa con la lombriz de tierra *A. Chlorotica* que se encontraba enjaulada en huertos de ciruelos dos semanas después de la aplicación de clorpirifós. Por lo tanto, se necesita un enfoque complementario para el estudio de campo del impacto de pesticidas en poblaciones naturales de lombrices de tierra pudiendo ser el uso de mesocosmos (sistema hecho por el hombre al aire libre) que simulan las condiciones de campo tan cerca como sea posible.

Agricultura Sostenible

La agricultura local y nacional actualmente tiene varios desafíos que enfrentar, primero, la creciente demanda de productos nacionales al extranjero y estar dentro de los límites permisibles de residuos de agroquímicos que permiten la entrada de los productos a mercados internacionales. Chile denominado Potencia agroalimentaria ha tenido escasos pronunciamientos legales y ha recibido cuestionamientos por parte de la organización para la cooperación y el desarrollo económico (OCDE 2005) por el uso y gestión de los recursos naturales, estos cuestionamientos han permitido considerar nuevos aspectos en el sector productivo agrícola, centrándose en la sustentabilidad ambiental, es así que este nuevo concepto de sustentabilidad es útil porque recoge un conjunto de preocupaciones sobre la agricultura, concebida como un sistema tanto económico, social y ecológico.

La comprensión de estos tópicos más amplios acerca de la agricultura requiere entender la relación entre la agricultura y el ambiente global, ya que el desarrollo rural depende de la interacción de subsistemas biofísicos, técnicos y socioeconómicos.

Por lo tanto, la agricultura orgánica tiene un gran potencial de contribuir eficazmente a la seguridad alimentaria, la salud de los ciudadanos, el aumento de la salud familiar y las normas ambientales a un bajo costo, respecto a las prácticas agrícolas convencionales. Si bien, este marco de promoción de la agricultura orgánica es conocido en diferentes áreas de la sociedad Chilena (academia, la política agraria, los extensionistas, una parte importante de la población, etc.) surge la necesidad de explicar las variables que explican las motivaciones a desarrollar o no este tipo de producción agrícola.

El nivel de integración de la política nacional que promocione la agricultura orgánica en Chile, debe además incorporar diversas estrategias y productos de comercialización. Esto es por un lado, favorecer las acciones que favorezcan el comercio interno, pero por otro, un rol más protagónico del estado que permita desarrollar puntos de venta y centros de servicios agrícolas orgánicos donde los insumos orgánicos, como semillas orgánicas mejoradas, fertilizantes orgánicos y se encuentren a precios subsidiados y asequibles a los agricultores en transición. El financiamiento estatal de estos productos subsidiados puede ser definido a nivel de zona agroecológica y así favorecer la descentralización y también, uno de los desafíos importantes es el de analizar estos costos ambientales como parte del análisis económico que se realiza rutinariamente en actividades agrícolas. La contabilidad ambiental que incluye por ejemplo los costos de erosión, la contaminación por plaguicidas, etc., debiera ser un aspecto crucial del análisis comparativo de diferentes tipos de agrosistemas.

Es claro que no será posible lograr simultáneamente todos estos objetivos en todos los proyectos de desarrollo. Existen intercambios entre los diferentes objetivos, ya que no es fácil obtener a la vez alta producción, estabilidad y equidad. Además, los sistemas agrícolas no existen aislados. Los agrosistemas locales pueden ser afectados por cambios en los mercados nacionales e internacionales. A su vez, cambios climáticos globales pueden afectar a los agrosistemas locales a través de sequías e inundaciones. Sin embargo, los problemas productivos de cada agrosistema son altamente específicos del sitio y requieren de soluciones específicas. (Altieri and Nichols 2000). El desafío es mantener una flexibilidad suficiente que permita la adaptación a los cambios ambientales y socioeconómicos impuestos desde afuera.

Por todo lo anterior, es substancial que exista una política y una estrategia a largo plazo para el apoyo de la conversión o iniciación de la agricultura orgánica. En estos instrumentos políticos debe quedar de manifiesto que tanto el Estado como las instituciones de investigación, juegan un rol determinante en la innovación, el asesoramiento, la formación y apoyo financiero durante los primeros estadios de conversión, sobre todo de pequeños productores. Este vínculo o alianza entre el sector económico de la agricultura orgánica, el sector público a cargo de su promoción y control y la generación de conocimiento que ayude a la toma de decisiones permitirían que los beneficios de la agricultura orgánica traspasen el sector económico y se extiendan a la sociedad en su conjunto.

Muchos organismos han forjado un nicho al adoptar el lema de «producir conservando y conservar produciendo»; lo importante es que se aclare que no se trata de un intento más de cómo encajar la cuestión ambiental dentro de regímenes agrícolas ya establecidos, sino de buscar una sinergia real entre ecología, economía y ciencias silvoagropecuarias. Concretar esta visión significará reorientar la investigación y la enseñanza agrícola para enfrentar los desafíos de la gran masa de agricultores vulnerables y sus ecosistemas frágiles, pero asegurando también la sustentabilidad de las áreas intensivas de producción. Para esto será necesario introducir una racionalidad ecológica en la agricultura para minimizar el uso de insumos agroquímicos, complementar los programas de conservación de agua, suelos y biodiversidad, planificar el paisaje productivo en función de las potencialidades de los suelos y cada ecorregión, y promover el manejo sustentable de bosques y otros recursos renovables y no renovables.

CONCLUSIÓN GENERAL

De acuerdo a la hipótesis en esta tesis doctoral, que las aplicaciones de agroquímicos en agrosistemas de la Región del Biobío dominadas por cultivos hortofrutícola causan efectos adversos en organismos no diana como la lombriz de tierra, cuya actividad genera importantes beneficios para la estructura y dinámica del suelo. Conforme a lo realizado en las investigaciones del capítulo III y IV, trabajos realizados anualmente tanto en el campo como en laboratorio evidenciaron inhibición por parte de las enzimas carboxilesterasas y acetilcolinesterasas en los periodos de mayor aplicación de pesticidas organofosforados. Esta disminución de las actividades de estas enzimas, reflejan que las lombrices de tierra han sido expuestas a pesticidas como los organofosforados. Por lo tanto se acepta la hipótesis de trabajo planteada.

Muchas investigaciones han demostrado que las acetilcolinesterasas son buenos indicadores de pesticidas de los grupos organofosforados y carbamatos en organismos que habitan en distintos compartimentos ambientales, sin embargo el uso de las enzimas carboxilesterasas de *Lumbricus terrestris* evidencian ser buenos indicadores y complementarios de la exposición a pesticidas como los organosforados y carbamatos que se reflejó con la menor actividad de la enzima en la estación de primavera y verano coincidiendo con la mayor aplicación de pesticidas en el huerto con manejo convencional. Esta actividad enzimática fue más contrastante en el tejido buche-molleja que en el intestino anterior e intestino medio que no presentaron diferencias de actividad con el manejo convencional y orgánico de los suelos.

Bajo estas consideraciones, las esterasas de lombrices de tierra pueden ser una herramienta adecuada para estos efectos regulatorios y ambientales. En esta investigación, se sugiere el uso de esterasas de lombrices como biomarcadores para ser incluidos en una prueba de toxicidad de campo. Por otra parte, se postula que la secreción de las CbEs en el intestino lombriz de tierra podría ser una metodología respetuosa con el medio ambiente en la biorremediación enzimática de los suelos de plaguicidas contaminados.

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