Synergistic interaction earthworm-microbiota : a role in the tolerance and detoxification of pesticides?

Fatina Jouni

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Présentée pour obtenir le grade de docteur de

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Jury :

Claudia Wiegand	Professeur, Université de Rennes I	Rapporteur
Franck Vandenbulcke	Professeur, Université de Lille I	Rapporteur
Camille Dumat	Professeur, Université de Toulouse	Examinateur
Eric Peyretaillade	Maitre de Conférence, U Clermont-Ferrand	Examinateur
Yvan Capowiez	Chargé de Recherche, INRA Avignon	Examinateur
Magali RAULT	Maitre de Conférence, Avignon Université	Directrice

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to my parents,

for their constant support and unconditional love.

Thank you for believing in me

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Scientific Productions

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Influence of temperatures 12°C and 22°C on biochemical and behavioral biomarkers, in two endogeic earthworms exposed to ethyl-parathion in two differents soils.

Monitoring and analyzing the pesticide behavior and its transformation products in soil, casts and earthworms' gut and tegument.

Abreviation

AChE	Acetylcholinesterase
CbE	Carboxylesterase
ChE	Cholinesterase
CYP450	Cytochrome P450
DNA	Deoxyribonucleic acid
dNTP	**d**eoxy**n**ucleotide **t**riphosphates
GST	**G**lutathione-**S**-**T**ransferase
ITS	**I**nternal **T**ranscribed **S**pacer
LC_{50}	Lethal concentration for 50% of the individuals
MFO	**M**ulti **F**unction **O**xydase
OP	Organophosphate
OTU	**O**perational **T**axonomic **U**nits
PCR	**P**olymerase **C**hain **R**eaction
rDNA	Ribosomal Deoxyribonucleic acid

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Summary of the thesis (English)

Pesticides used to protect plants from pests, threat grievously non-target organisms such as earthworms. Due to their feeding and burrowing activities, earthworms are in direct contact with soil particles and microorganisms, as well as pollutants including pesticides. This work investigated (1) the effect of an organophosphate "ethyl-parathion" on the sensitivity of two endogeic earthworms' species, *Aporrectodea caliginosa* and *Allolobophora chlorotica*; and (2) the role of the gut-microbiota, in synergy with the earthworm's detoxification pathways, in pesticide tolerance or detoxification. In the first part, biochemical and behavioral responses showed that *A. caliginosa* is more sensitive to "ethyl-parathion" exposure than *A. chlorotica*. The endpoints measured ranged from physiological (weight), biochemical (AChE, CbEs, GST) to behavioral biomarkers (cast production and burrowing activity). Our findings showed that the sensitivity of *A. caliginosa* could be mainly due to the intrinsic sensitivity of its AChE to "ethyl-parathion". The role of the carboxylesterases, acting as bioscavenger of OP, and the role of the detoxifying enzymes GST did not appear to be efficient mechanisms involved in *A. chlorotica* tolerance. In the second part, we aimed to characterize the microbiome within the ingested soil, the cast and the gut tissue of *A. caliginosa* and *A. chlorotica* in control or polluted soils. Our results showed differences in the microbial composition between these compartments. In this line, we suggested that these two earthworms' species harbor a species-specific microbiome in their gut. In particular, our findings showed that the earthworm's gut acts as a "biological filter" for ingested microbial communities during the gut passage. At the level of the gut, we identified four dominated genus within the gut of *A. caliginosa* versus two dominated genus in the gut of *A. chlorotica*. Notably, we identified a *Rhodococcus* strain, which is highly abundant in the gut of *A. chlorotica*. Previous studies reported *Rhodococcus* strains for their ability to degrade some group of pesticides. We suggest that the presence of this strain could contribute to the tolerance of *A. chlorotica.* Finally, we showed that the effect of ethyl-parathion on soil enzyme activities mainly depend on soil texture rather than the presence and/or the species of earthworms.

According to our findings, it is of considerable importance to include more than one species to assess toxicity from organophosphorus insecticides, due to the interspecific differences that can occur within the same ecological category. Moreover, the identification and the functional analysis of the microorganisms found in the earthworm's gut and able to intervene in pesticide detoxification could enhance our knowledge about the fate of the pesticide inside the organism, and could be an important tool for bioremediation program.

Keywords: *Aporrectodea caliginosa, Allolobophora chlorotica,* organophosphates, biochemical biomarkers, behavior, enzymatic activities, microbiota, biological filter.

Summary of the thesis (French)

Les pesticides utilisés pour protéger les plantes des insectes nuisibles constituent une menace pour les organismes non cibles tels que les vers de terre. En raison de leur activité de bioturbation de sol, les vers de terre sont en contact direct avec les particules et les micro-organismes du sol, ainsi qu'avec les polluants, notamment les pesticides. L'objectif de ce travail est d'étudier (1) l'effet d'un organophosphoré (OP) «éthyl-parathion» sur la sensibilité de deux espèces de vers de terre endogés, *Aporrectodea caliginosa* et *Allolobophora chlorotica*; et (2) le rôle du microbiote intestinal, en synergie avec les voies de détoxification du ver de terre, dans la tolérance ou la détoxification des pesticides. Dans la première partie, les réponses biochimiques et comportementales ont montré que *A. caliginosa* est plus sensible à l'exposition à «l'éthyl-parathion» que *A. Chlorotica*. Les résultats portent sur l'analyse de biomarqueurs physiologiques (poids), biochimiques (AChE, CbEs, GST) et comportementaux (production de turricules et activité de creusement). Nous avons montré que la sensibilité de *A. caliginosa* semble liée à la sensibilité intrinsèque de l'AChE à «l'éthyl-parathion». De plus, le rôle des carboxylestérases, capables de piéger les insecticides OP, ainsi que le rôle de détoxification des GST notamment, ne semblaient pas être des mécanismes efficaces impliqués dans la tolérance de *A. chlorotica*. Dans la deuxième partie, nous avons caractérisé, en présence ou non d'insecticide, le microbiote dans le sol ingéré, les turricules et les intestins des 2 vers de terre. Nos résultats ont montré des différences dans la composition microbienne de ces compartiments. A cet égard, nous avons suggéré que chacune de ces espèces hébergent un microbiote spécifique de l'espèce dans leur intestin. Nos résultats ont notamment montré que l'intestin du ver de terre agit comme un «filtre biologique» pour les communautés microbiennes ingérées lors du passage dans l'intestin. A ce niveau, nous avons identifié, au niveau bactérien, quatre genres dominants dans l'intestin de *A. caliginosa* et deux genres dominants dans l'intestin de *A. chlorotica*. Nous avons notamment identifié une souche de *Rhodococcus*, très abondante dans l'intestin de *A. chlorotica*. Des études ont montré que des souches de *Rhodococcus* peuvent dégrader certains groupes de pesticides. Nous suggérons que la présence de cette souche pourrait contribuer à la tolérance de *A. chlorotica*. Enfin, nous avons montré que l'effet de l'éthyl-parathion sur les activités enzymatiques du sol dépend principalement de la texture du sol et non pas de la présence et/ou de l'espèce de ver de terre.

Selon nos conclusions, il est extrêmement important d'inclure plus d'une espèce pour évaluer la toxicité des insecticides organophosphorés, en raison des différences interspécifiques pouvant se produire au sein d'une même catégorie écologique. De plus, l'identification et l'analyse fonctionnelle des micro-organismes présents au niveau de l'intestin et susceptibles d'intervenir dans la détoxication des pesticides permettraient d'améliorer nos connaissances sur le devenir du pesticide dans l'organisme et pourraient constituer un outil important dans les programmes de bioremédiation.

Mots-clés: *Aporrectodea caliginosa*, *Allolobophora chlorotica*, organophosphorés, biomarqueurs biochimiques, comportement, activités enzymatiques, microbiote, filtre biologique

GENERAL INTRODUCTION

1. The soil inhabitants: biodiversity and functional contributions

1.1. Soil fauna: diversity and functions

Imagine walking through the nature and hearing the leaves crunching underfoot. Underneath those leaves, a complex ecological kingdom colonizes the soil and represents an impressively important reservoir of biodiversity. The soil is an anchor and support for the flora, fauna and microorganisms. In this area, many ecological functions are performed including: nutrient cycles, biogeochemical processes, phytoextraction, drainage, storage of water and many other elements and compounds like carbon, nitrogen, phosphorus, pesticides, heavy metals etc... Forcefully, the soil biota modifies considerably its environment by soil mixing, bioturbation, aeration and aggregate formation. Therefore, the properties of the soil maintain the biodiversity and the interaction between all its components, by providing a biological, chemical and physical habitat (Fig. 1) (Bardgett 2002; Epeide et al. 2008; Menta 2012; Ferris & Tuomisto 2015).

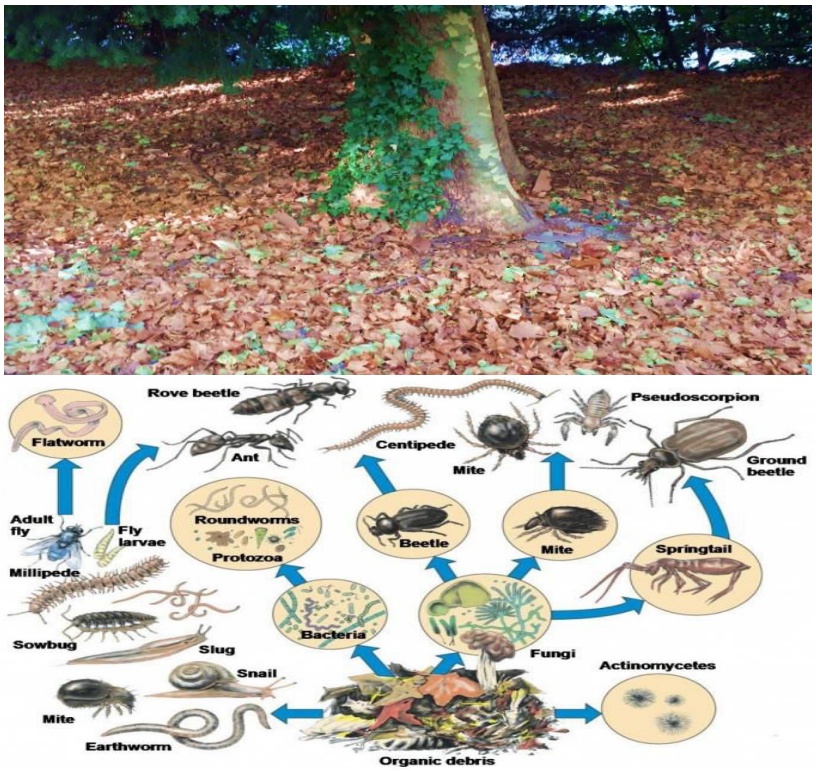

Figure 1: Soil biodiversity
(from https://www.microbesinmysoil.com/the-soil-food-web/)

Soil fauna organisms are numerous and diverse. They include microflora and microfauna (bacteria, fungi, nematode…), mesofauna (collembola, enchytraedae, diptera…) and macro-and megafauna (isopoda, mollusca, oligochaete…) (Figure 2) (Decaëns 2010; Swift et al. 1979).

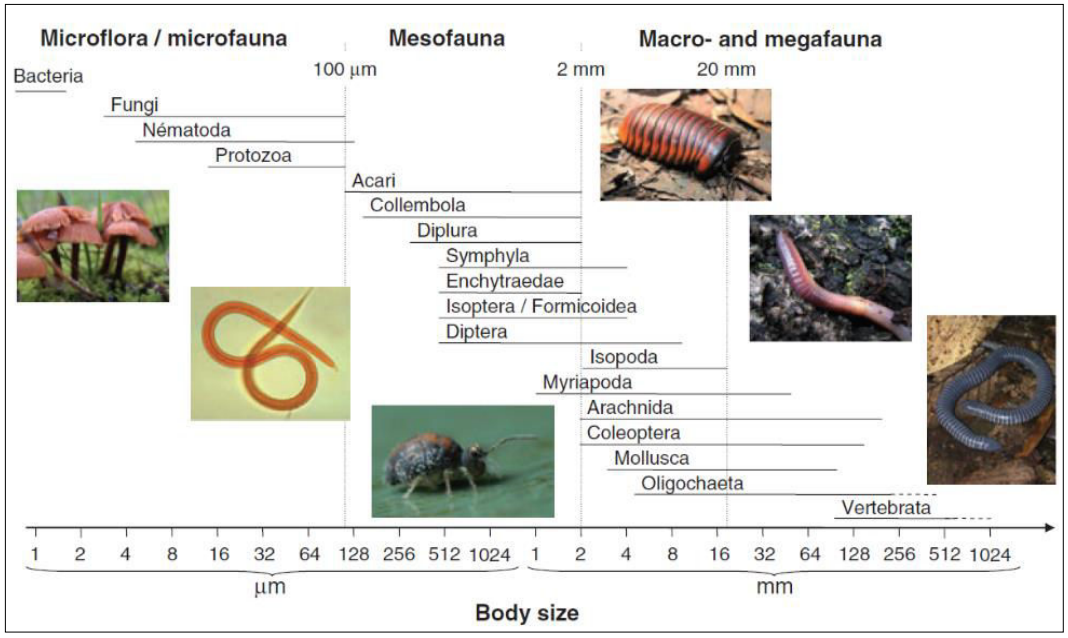

Figure 2: Taxonomic group's classification of soil organisms according to their body-size. By Swift et al. (1979), illustrated by Decaens (2010)

Natural history and biology of many groups of them are well known, while for others it is still misunderstood. In their habitat, they interact with microorganisms and have significant influences on many processes in agroecosystems. Particularly, they enhance the breakdown and dispersion of soil organic matter, nutrient cycling and dynamics, influence soil structure and porosity thus, they increase soil fertility and primary production (Huhta 2007; Crossley et al. 1989; Coleman & Wall 2015). Potentially, they are used as bio-indicator of soil quality and pollution (Cortet et al. 1999; de Lima et al. 2017). For instance, earthworms belong to the soil macrofauna and are known to be the major component of soil fauna communities and the largest of invertebrates' biomass in soils (Fragoso & Lavelle 1992; Shakir & Dindal 1997; Blouin et al. 2013 ; Salehi et al. 2013).

1.2. Earthworms: the masters of the soil

Extensively found in soil all around the world and contributing largely to its biomass (80%), earthworms are commonly the larger members of the oligochaeta class, belonging to the phylum of Annelida because of their segmented body (Fig. 3; Table 1) (Edwards & Bohlen 1996). These "old friends of farmers" (Darwin 1881; Leena et al. 2012) are hermaphrodites and count for more than 1800 species classified under their morphological and phylogenetic characteristics as well as their ecological and behavioral pattern (Lavelle 1988; Kooch et al. 2008; Römbke et al. 2015).

Figure 3: Apporectodea caliginosa

Table 1 : Earthworm taxonomy

Kingdom	Animalia
Phylum	Annelida
Class	Clitellata
Order	Oligochaeta
Family	Lumbricidae

In his final book, "*The formation of vegetable mould, through the action of worms*" Darwin published his findings and concluded**: "*The plough is one of the most ancient and most valuable of man's inventions; but long before he existed the land was in fact regularly ploughed, and still continues to be thus ploughed by earth-worms. It may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly organized creatures.*"** - Charles Darwin 1881 - We need to be grateful to Darwin, because of him earthworms have become valuable. He was fascinated and amazed by those invertebrates and spent considerable time studying them and their behavior (Darwin 1881).

1.2.1. Beneficial services of earthworms in soils

Earthworms are considered as major terrestrial ecosystem engineers for their ability to modify physical (aggregate structure, porosity), chemical (nutrient supply and cycling) and biological (soil fauna, microbial and enzymes activities) properties of the soil profile.

Effectively, due to the accumulation of their biogenic structures (casts, galleries…) they modify soil structure, porosity and aeration, and improve soil fertility and plants roots penetration. Hence, they improve water infiltration, drainage and storage in soils. Moreover, they are crucial drivers of soil organic matter dynamics by breaking down residues and debris, and regulation of the mineralization and humification processes by mixing soil layers (Rhea-fournier & González 2017; Chauhan 2014; Bhadauria & Saxena 2010; Grdisa et al. 2013;).

Earthworms interact with microorganisms and other living organisms in the process of soil organic material turnover, and contribute efficiently to the complex soil food web (Fig. 4). They mix debris into the mineral soil by their strong engineering effects, causing a dramatic alteration in soil properties and a redistribution of organic matter in whole profiles, which is beneficial for other organisms. However, little is known about the consequences of these engineering effects on soil food web structure (Frouz et al. 2013). Earthworms' feeding and burrowing activities help in the release of soil incorporated with organic matter, which afterwards, return to the soil via their casts. Casts are the excrements rejected behind the worm after digesting the soil, which has already passed through their digestive tract. It is a natural fertilizer rich in nutrients and may be deposit inside the soil or at the soil surface. Studies have documented that casts of earthworms contain higher mineral amounts of nitrogen, phosphate, potassium, sulfur, calcium and zinc, as well as, a higher microbial population and activity than the bulk soil (Haynes et al. 2003; Teng et al. 2012; Chauhan 2014).

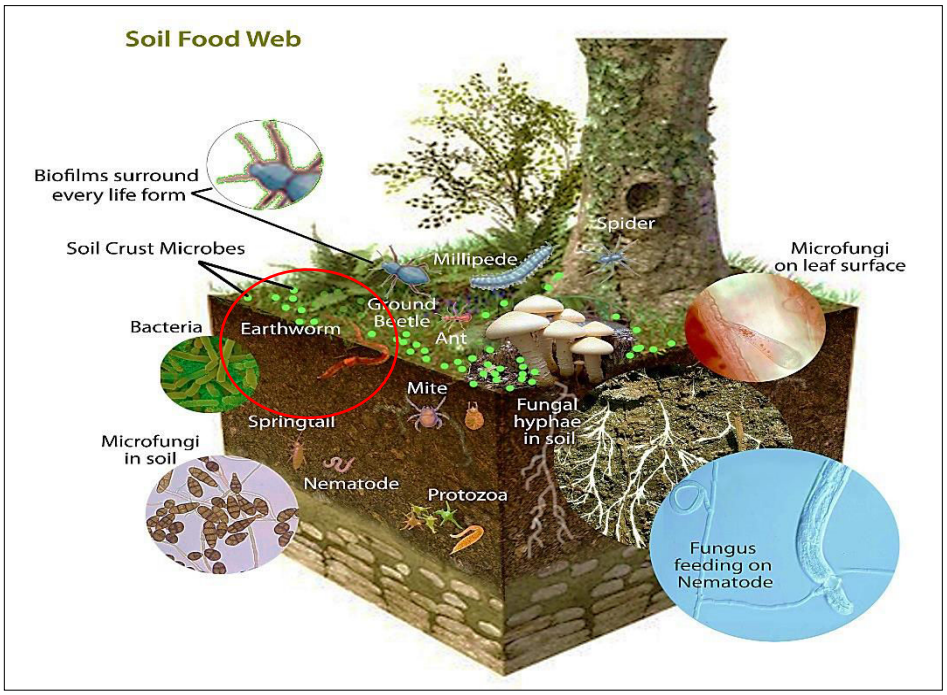

Figure 4: A complex food web including earthworms and other living organism of soil. http://endofite.com/

All of these miraculous services toward the soil make earthworms worthy to study. Evidently, earthworms are presumed to be potential biological indicator to evaluate soil fertility and pollution. They can be used in agricultural practices assessing ecotoxicologial risk and monitoring program of different contaminants. This can be done by studying their abundance, species composition and biochemical and behavioral stress-biomarkers (Paoletti 1999; Lionetto et al. 2012; Haeba et al. 2013). Thence, earthworms with their activities could potentially promote "the second green revolution" and spread benefits to the soil and farmers (Sinha et al. 2010).

1.2.2. Ecological groups of earthworms

To make identification easier, three ecological earthworms' categories have been described based on morphological, behavioral and ecological characteristics (Fig.5): epigeics, anecics and endogeics (Bouché 1977; Lee 1985; Lavelle & Spain 2001).

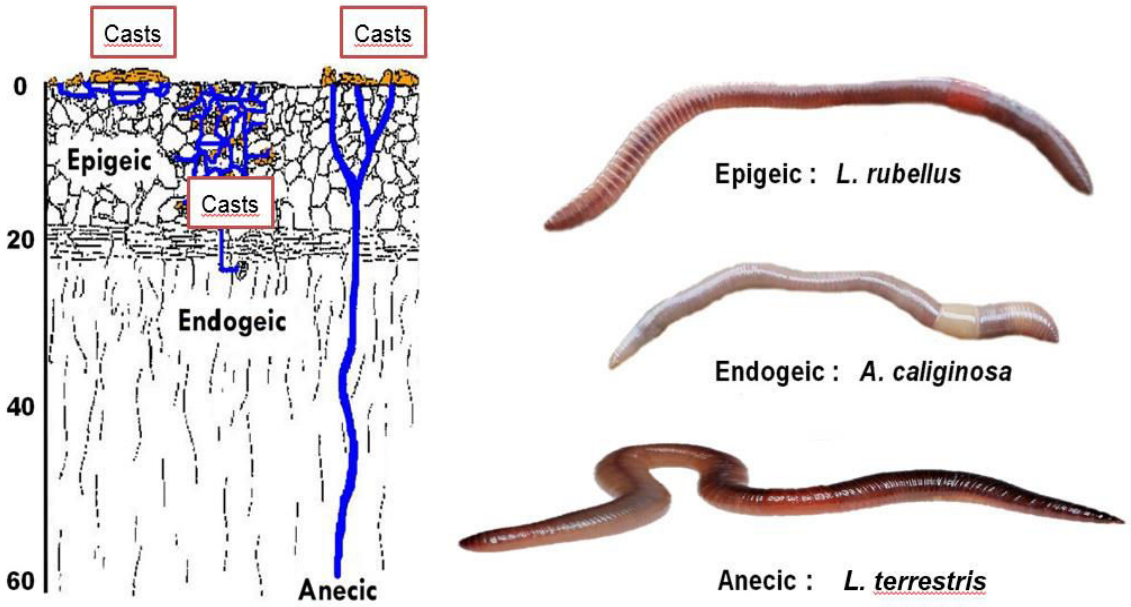

Figure 5: Earthworms niche groupings: Epigeic earthworm (Lumbricus rubellus). Endogeic earthworm (Aporrectodea caliginosa). Anecic earthworm (Lumbricus terrestris). Figure adapted from Fraser and Boag, photos of earthworms copyright Ross Gray. (Modified)

Epigeics (above the earth, surface dwellers) are small worms characterized by their highly pigmented skin usually red or brown. This coloration serves as a protection of UV rays and a camouflage from their natural predators. They live mainly on the surface of the soil in the litter horizon, feed on leaf litter and tend not to make burrows (poor burrowing ability). They play crucial role in the decomposition of leaves, other plant detritus and organic matter that falls in the land. Redworms also called manure worms, belong to this group and commonly used in the vermicomposting system. Examples: *Dendrobaena octaedra*, *Eisenia fetida* (ideal worm for vermicompost) and *Lumbricus rubellus*.

Anecics (out of the earth, subsoil dwellers) are among the largest varieties of earthworms that can grow up to several meters, and noted for their dark antero-dorsal pigmentation. They feed on decaying leaves and are known to emerge on the surface of the soil at night to search for food. Known as the powerful deep-dwelling class of earthworms, they migrate within the various soil strata by making vertical burrows, where they pull organic matter and decaying leaves in order to feed on them. Examples: *Lumbricus terrestris*, *Apporectodea longa* and *Dendrobaena platyura*

Endogeics (Within the earth, topsoil dwellers) are medium-sized and appear pale in color (pink, blue or grey). They lake pigmentation as they spend their entire lives in the dark beneath the ground and out of sun. Scientists know the least about their life cycle and behavior and are least recognizable to most people. In contrast to anecic earthworms, endogeic earthworms make horizontal burrows by swallowing large quantity of soil and organic matter. Crucially, they allow better plant root penetration and aeration of the soil. Examples include *Allolobophora chlorotica*, *Apporectodea caliginosa* and *Pontoscolex corethrurus*.

Over and above, earthworms are widely considered as precious candidates in microcosm experiments (Fründ et al. 2009). However, the epigean segmented Red wiggler species *Eisenia fetida,* is the mostly used as model soil invertebrates. Accordingly, it thrives widely in many habitats all around the world and it is adapted to variable climates (Wang et al. 2015; Plytycz et al. 2018). Used in ecotoxicological tests, it is also the favorite amongst earthworms in vermicomposting (Sparks 2014. Römbke et al. 2015; Hyunseong 2016). Despite the use of this species as a model, however, it is not found in mineral soil, and very uncommon in cultivated fields. Then, endogeic and anecic species such as *Lumbricus terrestris* and *Aporrectodea caliginosa*, which are more commonly found in cultivated fields, are ecologically important in terrestrial ecosystems of many temperate regions (Bouché, 1977; Bauer & Römbke 1997). This statement emphasis the need to develop analysis using ecologically relevant earthworms' species, as terrestrial models (Pelosi et al. 2013).

1.3. Microorganisms screening and functions in soils

Microbes can be found in almost any habitat, in every nook and cranny we could think of. They live in water, soil, digestive system of human and animals and sometimes in extreme environments. Inside our bodies, at microscopic level, a vast number of microbes colonized us. This microbiome consists of billions of microorganisms including bacteria, fungi, parasites, virus and other microbial and eukaryotic species (Schulz et al. 2013; Mora et al. 2016).

Further, a microscope is usually needed to see microorganisms, but their functions in soils are strikingly noticeable. Microorganisms play a critical role in maintaining good

soil health, structure and fertility. Collectively, they intervene in the breaking down of decaying plant biomass and fungal mycelia, humification, nutrients recycling (nitrogen, phosphorus, potassium, carbon…) (Fig. 6) (Llado et al. 2017).

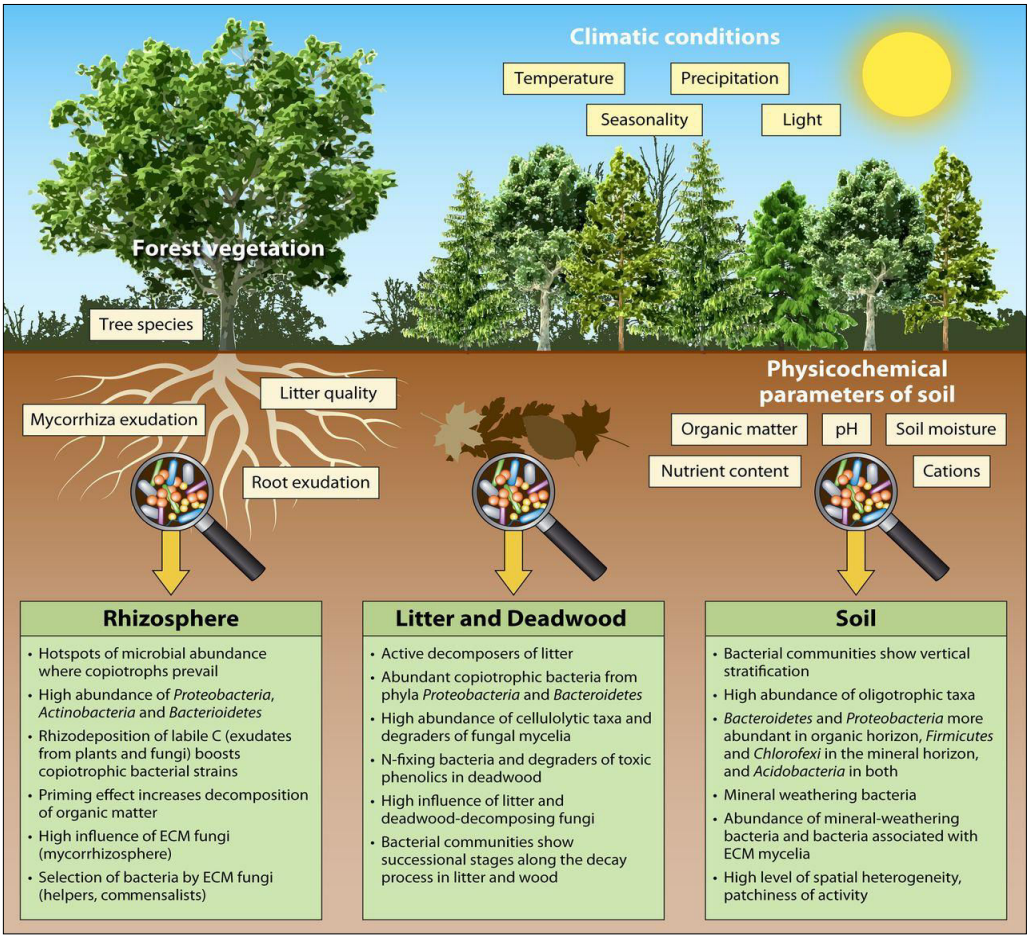

Figure 6: Bacterial community drivers of many ecological services in the rhizosphere, litter/deadwood, and soil compartments of the forest floor.

Connectedly, their crucial role in returning nutrients to their mineral forms helps the plants to take up again cycling nutrients, which favor their growth. In addition to supplying nutrients, microorganisms benefit plants also by producing a variety of substances that promote plant growth, including auxins, gibberellins and antibiotic (Gyaneshwar et al. 2002; Jacoby et al. 2017). Biochemical properties of soils associated to the cycles of elements such as carbon, nitrogen, phosphorus and sulfur, are used to point out soil quality. These properties include both general and specific biochemical parameters. In effect, general parameters involve microbial biomass, dehydrogenase activity, nitrogen mineralization potential and soil respiration; specific parameters are related to the activity of hydrolytic enzymes such

as phosphatase, urease and β−glucosidase. Most of these processes (Fig. 7) (Llado et al. 2017) are mediated by soil enzymes, which are produced by soil microorganisms, roots and to some extend by soil animals.

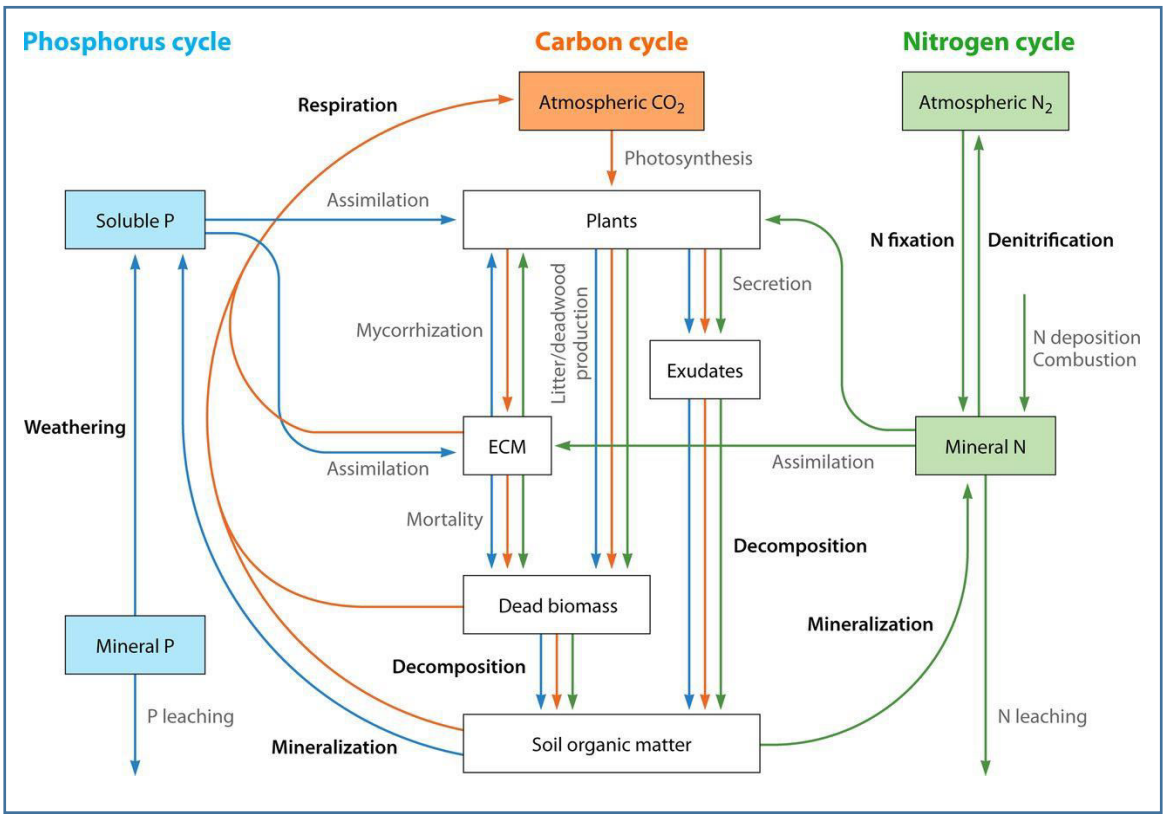

Figure 7: Ecological processes mediated by bacteria (highlighted in bold) and elements transfer (C in orange, N in green and P in blue) within the coupled biogeochemical cycles of carbon, nitrogen and phosphorus in forest ecosystems.

Therefore the composition of soil biota determines the potential of the community for enzymes synthesis. Soil enzyme activities directly influence energy and nutrients transformation and cycling. This feature is associated with the presence of extracellular and intracellular enzymes, which are essential in the process of decomposition and mineralization of organic material (Dilly' & Nannipieri 1998; Gil-Sotres et al. 2005; Friedlova 2010).

The soil matrix is considered as a favorable niche (Lavelle & Spain 2001). Added, in a typical healthy soil, in a single gram, live and reproduce a wealth of millions of microorganisms. Identification, characterization and classification of microorganisms were generally based on their morphological, physiological and cultural characteristics. This was and still performed using conventional culture-based

methods. These methods do not provide prompt results, because only a small fraction (99% of microorganisms in the external environment are uncultivable) of the various microbial community can be cultured on synthetic media (Janssen et al. 2002; Stewart 2012; Ling et al. 2015). Recent molecular methods, which are more sophisticated, offer better solutions in identifying and characterizing “uncultivable” microorganisms. These emerged methods continue to replace conventional culture techniques. Hence, molecular methods are increasingly incorporated in laboratories, despite being expensive, however, they are faster and offer reliable specificity in detection and identification (Rappé & Giovannoni 2003; Nichols et al. 2010; Fakruddin et al. 2013; Desai & Armstrong 2003).

It is of current concerns to understand interactions between microbes and soil components such as bacteria, fungi, roots and animals (Barea et al. 2002a). As specified above, these interactions could act either in nutrient cycling and plant growth, but also in the biological control of plant pathogens, improving soil quality (Johansson et al. 2004). Microorganism’s biomass and activity are greatly influenced by higher trophic levels of the soil food web (Fig. 5). Despite the fact that there is evidence that soil microbial communities are linked to ecosystem functioning, the understanding of the functional importance of different groups of the soil biota and the connections between them (the member soil food web) is limited (de Vries et al. 2013). One of the interests of understanding such interactions lies in the modification of microbial enzyme activities, which could be used as a potential tool for measuring soil quality.

1.4. Factors affecting soil organisms

1.4.1. Environmental factors and soil properties

Biotic factors and climatic conditions that influence the environment and soil, can also affect the biology and life processes of living organisms including, microorganisms and earthworms.

Earthworms devour and rely on their house comprising soil, organic debris, microorganisms and other materials. Soil temperature, moisture, pH and organic matter are factors of primary importance in the regulation of earthworm’s population, growth, reproduction and health. Earthworms do not have specialized breathing

system, they breathe through their skin. Adequate moisture is needed to keep their skin moist in order to facilitate their cutaneous respiration. Effectively, they take water from the ingested soil that must be moist, but not overly, unless they die suffocated. They survive in relatively low oxygen environment even when submerged in water if it contains dissolved oxygen. During global warming or drought or frost, earthworms ‘’the coldblooded organisms’’, may die because of the extreme conditions. High levels of rainfall cause the drowning of earthworms, while low levels of rainfall can dry out them. In both cases, heat or cold can affect the metabolism and reproduction of earthworms. Hence, the ideal environment for earthworms to thrive is a ventilated and drained soil, optimum temperatures (range from 10 to 20℃ for cool temperate species and 20 to 30℃ for tropical and subtropical species) and a neutral pH range 5.0 to 8 (Edwards & Bohen 1996; Lavelle 1988; Curry 2004; Wood 2018).

Otherwise, soil microorganisms control soil organic matter decomposition and nutrient availability, by exerting a prevailing influence on the net carbon balance of ecosystems. A growing body of literature pointed out that climate, soil features, vegetation, substrate quantity and quality and land uses are relevant determinants of the abundance, structure and activity of soil microbial community. In effect, climate change has a direct effect on soil environments and can particularly alter temperature and moisture. These variations may have profound impacts on physiology and growth of some specific groups of microbes within communities. Therefore, it is anticipated that environmental parameters and management practices may influence the microbial community and activities in soil, but, the relationships between microbes and these alterations have been poorly understood, and convincing data are still scarce (Zhang et al. 2005; Nielsen et al. 2010; Tsiknia et al. 2014; Wu et al. 2018).

1.4.2. Agricultural practices and xenobiotic compounds

Intensive soil management, amendment and farming practices, tillage and conventional plough affect physical, chemical, and biological characteristics of soil. These practices have considerable impact on soil biota, productivity and sustainability. While organic management tend to favor microbial and earthworms biomass and activity, conventional practices lead to nutrients leaching, soil degradation and damage severely earthworm populations and microbial communities (Sheibani & Ahangar 2013; Smeaton et al. 2003; Mathew et al. 2012 ; Wang et al. 2012). By way of illustration, many studies have documented the negative effect of

tillage practices and soil disturbances on earthworms, by establishing a strong relationship between earthworm's abundance and no-tillage system. The decrease of earthworms abundance is due to loss of nutrient as soil organic matter, but also mechanical damage, burrows destruction, changes in soil physical properties and predation (van Capelle et al. 2012; Brown 2003).

Additionally, microbial population varies between soils, and depends on soil structure, pH, moisture, redox potential and other parameters. Organic management practices are recognized to affect positively the microbial biomass, however, microbial biomass, dynamic and respiration as well as enzyme activities have been noted to be negatively affected by tillage and conventional regimes. This is due to changes in carbon inputs and availability as well as soil organic matter concentrations (Wander et al. 1995; Shannon et al. 2002; Stark et al. 2007; Kallenbach & Grandy 2011).

Further, depending on their origin and fate in the water, air or soil, xenobiotic compounds represent a serious hazard to all living organisms. For instance, heavy metals in soil affect earthworms' population density and species depending on their ecological group. Thusly, the sensitivity of earthworms to heavy metals depended on species. Based on studies, *Lumbricus rubellus*, *Lurnbricus castaneus* and *Lumbricus terrestris*, were more tolerant to heavy metals than *Aporrectodea rosea*, *Aporrectodea caliginosa* and *Allolobophora chlorotica,* that showed a high sensitivity and were absent in sites near to smelter. This sensitivity was explained by a lower calcium secretion in their gut, due the capability of calcium to sequestrate and eliminate various metals through the chlorogogenous tissue (Spurgeon & Hopkin 1996; Nahmani et al. 2003). Concerning pesticides, many studies have investigated the effect of pesticides on earthworms (Pelosi et al. 2014). Those studies have been conducted mainly in laboratory, and shown that earthworms are impacted at all organization levels. For instance, pesticides disrupt the activity level of enzymes involved in oxidative stress (Schreck et al. 2008, Schreck et al. 2012), they cause acute toxicity to earthworms by inhibiting acetylcholinesterase and carboxylesterase activities, (Rault et al. 2008, Collange et al. 2010) leading to behavioral and physiological disturbances (Sanchez-Hernandez 2009). Field studies have shown that earthworms' density in orchards sprayed with organophosphates, was very low in comparison to adjacent uncultivated fields (Reinecke & Reinecke 2007). In addition, significant decreases in cholinesterase activity were found in earthworms

coming from the IPM (Integrated Pest Management) and conventional orchards (Denoyelle et al. 2007).

Withal, pesticides applications and other pollutants also affect soil microorganisms by decreasing their number and activity (Gianfreda & Rao 2008). Toxicants may interfere with vital function such as respiration, division and other processes. This may have undesirable impacts on non-target microorganisms. However, it was documented that it is difficult to predict the effect of pesticides on microorganisms because, some pesticides stimulate the growth of microorganisms but other decline it. Hence, in order to apprehend the effect of pesticides on soil microflora, it is necessary to inquire the concentration of the spread pollutants and the capability of the microorganism to adapt to existent pollutant or to degrade it (Lo 2010; Kalia & Gosal 2011).

Fauna and flora are not the unique inhabitants of the soil! Pollutants exist also!!!

2. Soil pollution: a global problem

Nowadays, the soil becomes a reservoir for industrial and domestic pollutants. The soil pollution is generally due to the deposition of a variety of contaminants in the atmosphere, in addition to human activities especially farming system. These pollutants are in high concentrations to be of risks to plants, animals, and humans (Fig. 8). They include metals, toxic chemicals compounds, organic and inorganic pollutants, pesticides… Once inside the soil, they can enter the food chain, threat food safety and alter significantly the soil composition, diversity and many critical functions of soils (Zalidis et al. 2002; Fabietti et al. 2010).

Figure 8: Pollution practices must be abandoned unless human will lose all contact with the world of instinct. https://fractalenlightenment.com/24754/issues/aggrandized-ego-alienated-soul-contesting-the-atrophy-of-instinct-in-an-age-of-anxiety

2.1. Pesticides: benefits and hazards

Pesticides represent a group of human-made chemicals covering a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides and others. They are applying as well in residences, gardens, agricultural fields and greenhouses, in order to harm devastating pests and vector borne diseases. Hence, they contribute to the protection of crop and improve yields and productivity. For these reasons, their role has become crucially important in increasing food production, to satisfy the high food demand of the growing population density (Aktar et al. 2009; Popp et al. 2013).

However, are they harming only pests at which they are target?

Pesticides can be applied by manual or hydraulic spraying in addition to aerial or truck based spraying techniques. They exert toxic effect to production workers, loaders, mixers, sprayers and farmers during manufacturing and formulation. When moving from the application site, pesticides can reach other ecosystems like soil and water, even downward through the soil to the groundwater (Fig. 9). Leaching, persistence and degradation of pesticides depend on their chemical and physical

characteristics and the transport processes. At this level, they threat target and non-target organisms including humans, mammals, fish, birds, plants, microorganisms and valuable species of soils (arthropods, earthworms…) (Kazemi et al. 2012; Carvalho 2017).

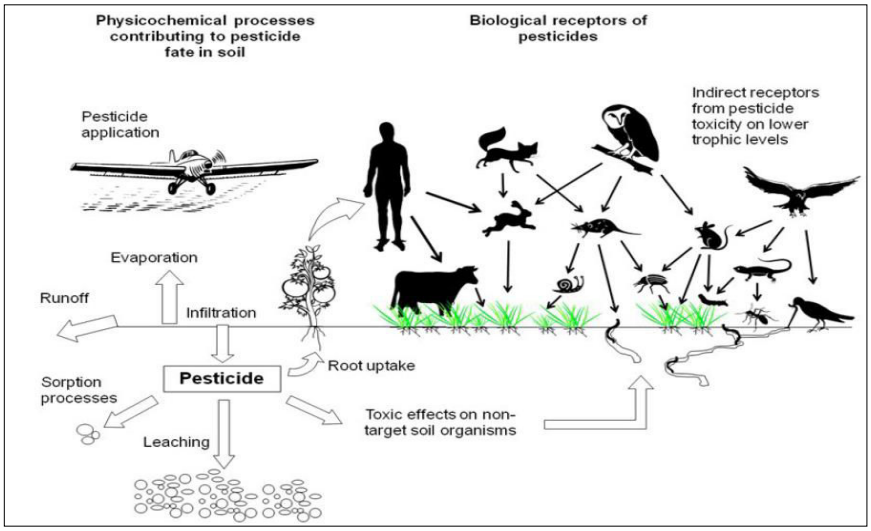

Figure 9: Main physiochemical and biological processes contributing to pesticide fate and toxicity (conceptual scheme elaborated from Köhne et al 2009, modified from Sanchez-Hernandez 2011).

According to the Pesticide Action Network (https://www.**pan-europe**.info) and despite the European strategies attempts to reduce pesticide inputs (Plan Ecophyto II), chemical control is still intensively used. In spite of much opinion about the sustainability of agriculture and the entering on the market of new product that can be used at low dose, the average of pesticide use did not decrease in recent years, as reported by the Eurostat on pesticide sales (Fig. 10). After a decrease observed in 2013, it can be implied that pesticide use has grievously expanded due to the increasing resistance of organisms and plants. Whilst, the initiation of organic methods and practices remained at a low level. In 2016, almost 400,000 tonnes of pesticides sold in Europe were used in the agricultural field.

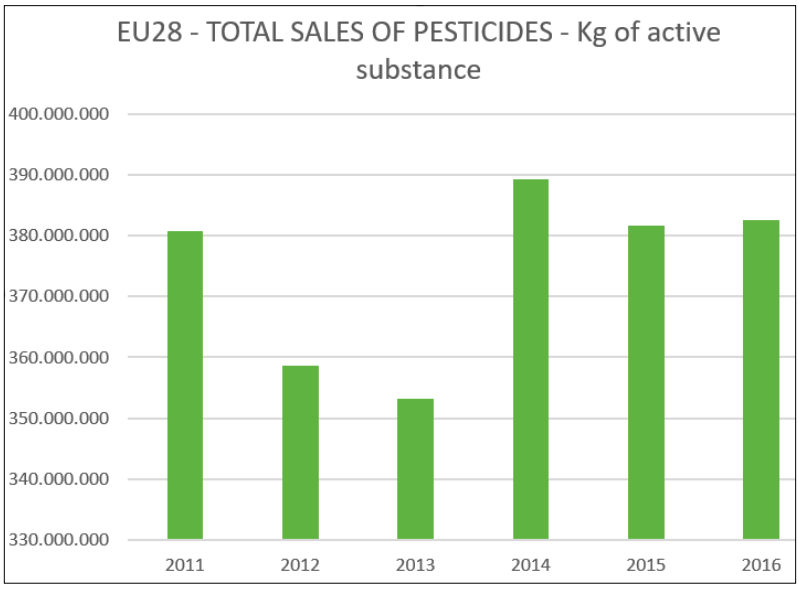

Figure 10: Total sales of pesticides
Data are collected from Eurostat last updated on 08/08/2018.
(http://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do)

Among pesticides, insecticides represent a group of highly toxic compounds and there effects on wildlife were extensively studies (Köhler & Triebskorn 2013). Their application rates are generally lower than other classes of pesticides such as fungicides or herbicides, however, they exert strong impact on non-target organisms. A particular progression of interest in the effects of neurotoxic insecticides (Table 2) such as organophosphates, pyrethroids, and neonicotinoids was observed (Köhler & Triebskorn 2013).

Table 2: Neurotoxic insecticides, mode of action and authorized doses[(1)] used in French apple orchards

Chemical classes	Commercial formulation	Active ingredient	Mode of action	Authorized dose (1)
Pyrethroïds	Decis Expert®	Deltamethrin	Sodium channel modulation	0.75 g hL^{-1}
Carbamates	Pirimor G	Pirimicarb	Acetylcholinesterase inhibition	37.5g.hL^{-1}
Organophosphates	Oleobladan® Pyrinex®	Ethyl-Parathion Chlorpyrifos	Acetylcholinesterase inhibition	250g.hL^{-1} 50 g hL^{-1}
Spinosyns	Success4®	Spinosad	Acetylcholine receptor stimulation	9.6 g hL^{-1}
Neonicotinoids	Supreme®	acétamipride	Acetylcholine receptor stimulation	5 g hL^{-1}

2.2. Organophosphates insecticides

2.2.1. Mechanism of action

Organophosphate insecticides (OP) display a variety of structures and are still the most widely used including chlorpyrifos-ethyl, parathion-ethyl, malathion, diazinon...

Once inside the organism, OPs are bioactivated through the cytochrome P450 mixed function oxidase enzymes. CYP450 constitute a family of enzymes mostly involved in the transformation and conversion of the P=S compound to its toxic oxon metabolite (P=O) (Fig. 11).

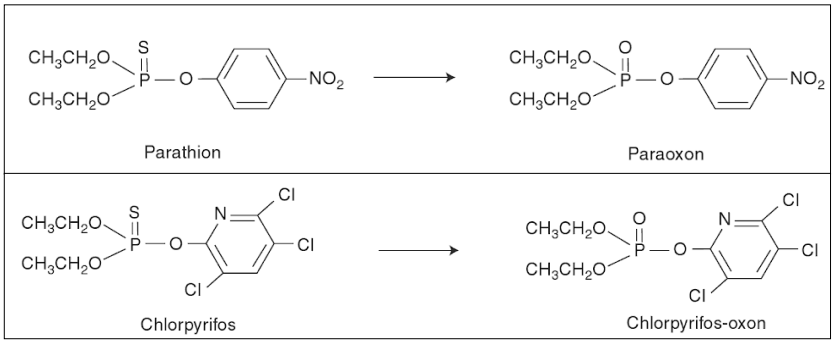

Figure 11: The conversion of the organophosphorus insecticides to their respective oxons

The inhibitory effect of oxon metabolites lies in a phosphorylation reaction, which creates a covalent bond with the catalytic serine group of esterase. Among this group, cholinergic enzymes called “Cholinesterases” are the target of OP compounds. Hence, due to the high affinity of the active site of serine esterase, the phosphorylation induces the destruction of the OP and then, the persistent inhibition of the serine esterase lasting hours to days. Whereas, this inhibition is usually considered irreversible if a process of “aging” (non enzymatically mediated dealkylation) takes place, some reactivation can occur if the aging process is not complete (Fig. 12).

Both target esterases “acetylcholinesterase” and non-target (butyrylcholinesterase and carboxylesterases) are affected by oxons (Satoh & Gupta 2010).

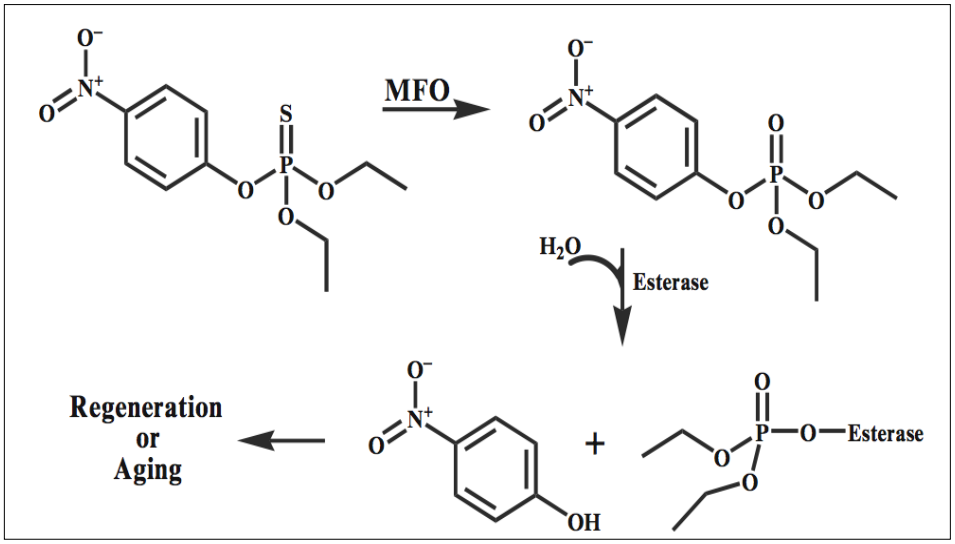

Figure 12: Esterase inhibition mechanism for the organophosphate parathion. The organophosphate (parathion) is first activated via mixed-function oxidases (MFO) to the "active" oxon-form, which is the inhibitory structure of the compound. Paraoxon then binds to the esterase and is hydrolyzed in the process by the addition of water, releasing p-nitrophenol. The phosphorylated esterase can then either release the phosphate group and regain catalytic activity, or become "aged" where the phosphate remains permanently bound and the enzyme loses catalytic activity (from Wheelock et al 2005).

2.2.2. AChE Target of OPs : a potential biomarker

The inhibition of Cholinesterases is the most used biomarker in ecotoxicological risk assessment and pesticide exposure. Cholinesterases (ChEs) constitute a group of hydrolases that are highly sensitive to organophosphate and carbamate pesticides. Found in plasma, mammalian erythrocytes, neuromuscular junction and other organs, CHEs include two types based on their substrate specificity acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE). AChE is considered critical to a normal control of nerve impulse transmission, because it catalyzes the hydrolysis of the neurotransmitter acetylcholine in the nervous system. Its inhibition leads to an accumulation of the neurotransmitter acetylcholine in the nerve endings thus, a continued stimulation of acetylcholine receptors. BuChE is found mainly in the plasma with no physiological functions assigned. In vertebrates, it appears in liver, lung, heart, at cholinergic synapses, in developing embryonic tissues and a functional role of BuChE can be found in regulation of cell proliferation (Mack & Robitzki 2000).

In addition, the activity of AChE is easy to measure and its sensitivity is dose-dependent (Lionetto et al. 2013). The classical use of AChE activity in ecotoxicology,

displays a wide range of methodological approaches that involve both laboratory toxicity bioassays and field monitoring surveys (Denoyelle et al. 2007; Yaqin et al. 2008; Yaquin & Hansen 2010; Otero and Kristoff, 2016). Withal, it is critical to apprehend the eventual consequences of AChE inhibition on the organism, especially at behavioral and physiological levels (Scott & Sloman 2004). For instance, several studies have shown that a direct relationship could be established between AChE inhibition and several functions including behavioral, biochemical and physiological disturbance (Carlock et al. 1999; Rickwood & Galloway 2004; Nunes 2011; Ghorab & Khalil 2015).

2.2.3. Vigourous protecting mechanisms

Generally, there are diverse detoxifying enzymatic systems that provide protection against synthetic and natural pollutants (Li *et al.,* 2007). The biotransformation of xenobiotic occurs in two stages. An initial phase of functionalization (Phase I), consists in the addition of a functional group to the exogenous molecule to make it hydrophilic, therefore more easily excretable. In the second phase of conjugation (Phase II), a polar group is added to the functionalized or original molecule. The first phase, primarily involves CYP450 and esterases such as Carboxylesterases (CbEs), whereas the conjugation phase involves the glutathione-S-transferases (GST), which is the most important enzyme group (Li *et al*., 2007). Both phases increase the polarity of the xenobiotic, further processing can take place for example the excretion (phase III).

2.2.3.1. Carboxylesterases

Carboxylesterases belong to the serine-esterase family of enzymes that are widely distributed in all living organisms (animals, plants, and microorganisms). As their name suggest, these enzymes are involved in the hydrolysis of carboxyl esters into the corresponding alcohol and carboxylic acid via the addition of water (Junge 1975). Alike cholinesterases, they belong to the so-called B-esterases family. CbEs have a wide distribution and are expressed in tissues known to maintain a barrier function, and which are exposed to xenobiotics. They are found in greatest amounts in liver, epithelia of the lung and intestine, skin, kidney and brain (Wheelock et al. 2008;

Hatfield et al. 2017). Effectively, CbEs consist of multiple isozymes with a variable levels and activities. Their expression and activity are tissue and organism-dependent, (Hosokawa 2008; Imai 2006; Wheelock et al. 2008).

In addition, CbEs physiological function remains unclear since no real endogenous substrates have been identified (Hatfield et al 2016), whereas, these enzymes play a substantial role in the metabolism and detoxification of many xenobiotic containing ester group including, agrochemicals and pharmaceuticals (Fig. 13). In particular, carboxylesterases hydrolyze pyrethroids (Abernathy & Casida 1973; Stok et al. 2004; Wheelock et al. 2005) and bind stoichiometrically to carbamates (Gupta & Dettbarn 1993; Sogorb & Vilanova 2002) and organophosphates (Casida & Quistad 2004).

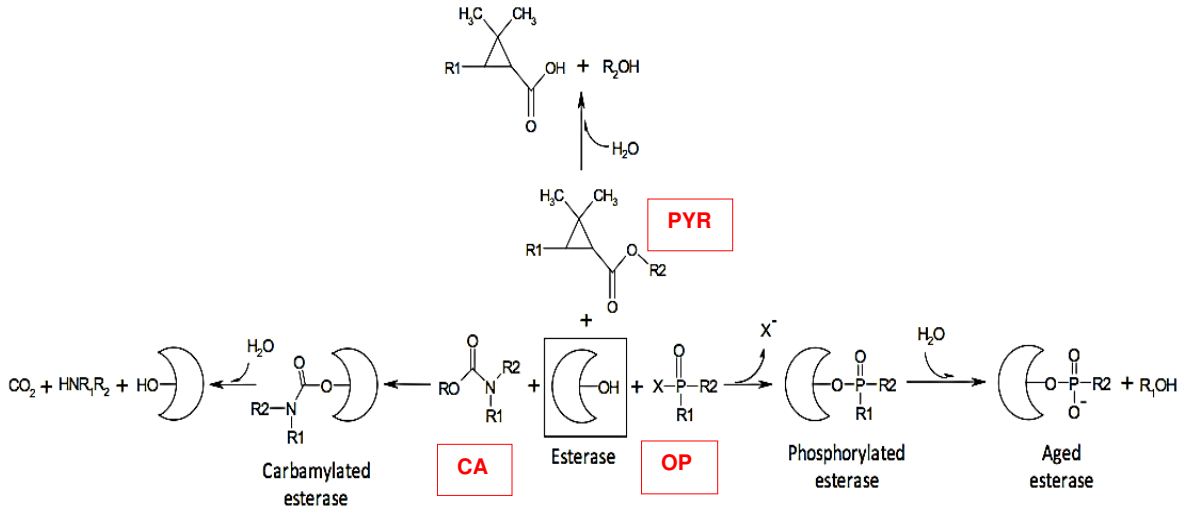

Figure 13: Interactions of esterases (cholinesterases and carboxylesterases) with carbamates (CA), the oxon metabolites of organophosphates (OP) and synthetic pyrethroids (PYR). Inhibition of esterases by CAs yields a carbamylated complex which is unstable and the esterase activity is rapidly recovered in the presence of water. Organophosphates inhibit irreversibly the hydrolysis activity of ChEs and CbEs by the formation of a stable phosphorylated complex. Under this condition, restoration of the esterase activity requires the synthesis of new enzyme. Synthetic pyrethroids interact only with CbEs, and these esterases hydrolyze them to yield the corresponding alcohol and carboxylic acid. Scheme elaborated from Sogorb & Vilanova (2002) and Thompson & Richardson (2004).

The inhibition of CbEs by OPs induces the formation of a stable enzyme-inhibitor complex, which is considered a stoichiometric mechanism for decreasing the OP concentration at the target site “AChE” (Maxwell 1992; Chanda et al 1997). Then, CbEs act as bioscavenger protecting organisms from organophosphates in several organisms (Maxwell 1992; Maxwell & Brecht 2001).

2.2.3.2. Cytochrome P450 (CYP450)

Phase I of metabolisation is catalyzed by the CYP450 group of enzymes. As specified above, these enzymes are involved in the bioactivation of organophophates insecticides. However, The CYP gene superfamily catalyzes mono-oxygenation reactions of a wide range of xenobiotic and endogenous substrates, which highly contributes to the detoxication mechanisms in all living organisms (Ortiz de Montellano 2015). CYP450 are found across diverse range of organisms including bacteria, plants, fungi, insects and mammals. They are involved in many cases of resistance of insect to insecticides (Després et al. 2007).

2.2.3.3. Glutathion-S-Transferase (GST)

Glutathione-S-transferases (GSTs; E.C.2.5.1.18) are members of multifunctional isoenzymes. They display various activities and participate in several types of reaction. Ubiquitously distributed in nature, they are found in organisms as diverse as animals, plants, microbes, and insects. These transferases are major phase II drug metabolizing enzymes. Added, their main biological roles consist of the detoxification of harmful electrophilic endogenous and exogenous compounds as xenobiotic, thus, they protect cells from oxidative stress (Armstrong 1991). These enzymes catalyze the addition of reduced glutathione (GSH≡γ-L-glutamyl-L-cysteinyl-glycine) to xenobiotic substrates. This reaction facilitates the elimination of the molecule that becomes generally less reactive and more soluble.

Further, GSTs could be either cytosolic or membrane-bound (Jakobsson et al. 1999) and their activities have been associated to insecticides resistance (Carvalho et al. 2013; Pavlidi et al. 2018).

2.3. Impact of OP on soil organisms

2.3.1. Impact on earthworms

Pesticides that are extensively used, threat target as well as non-target organisms amongst are earthworms. Besides, earthworms that are always in direct contact with their home that includes the soil and its component, they are at a high risk to absorb

such agrochemicals through the skin or to ingest them with food. Many literatures have documented the negative effects of pesticides on earthworms at different levels, because of their vulnerability to such applied chemicals and their crucial role as bioindicator. Pesticides act by hundred mechanisms thus, many studies have evidenced the impact of pesticides on earthworms each one at different organization level. They induced morphological, behavioral, physiological, reproductive, nervous and osmoregulatory disorders in earthworms. They decrease biomass and density by increasing mortality of individuals and inhibiting feeding behavior, reproduction and growth. Along with, they disturb the activity of several enzymes including those immersed in oxidative stress (Dureja et al. 1999; Pelosi et al. 2014).

The first ecotoxicological studies, had mainly focused on mortality (Edwards 2004; Capowiez et al 2005), then a growing interest in the development of sublethal biomarkers pointed out the importance to study the effect on both reproduction and growth (Bauer and Römbke 1997). For example, triazine herbicide and the organophosphosphate insecticide Galition were found toxic to *Eisenia fetida*, due to their significant impact in the growth inhibition of this species (Milanovic et al. 2014). Moreover, two organophosphates pesticides diazinon and chlorpyrifos were reported to cause toxic effect on growth, maturation and fecundity of *Aporrectodea caliginosa* (Booth et al. 2000), while the OP parathion and a carbamate propoxur, impact the reproduction of *Aporrectodea caliginosa, Aporrectodea longa* and *Eisenia fetida* (Kula & Kokta 1992). In order to investigate different organization levels, changes in behavior are of growing interest to link the toxic effects at the ecosystem level (Hellou 2011). In this context, impact on weight loss and burrowing activity of a neonicotinoid insecticide such as imidacloprid, has been observed on two earthworms species: *Aporrectodea nocturna* (anecic) and *Allolobophora icterica* (endogeic) (Capowiez et al. 2005, Capowiez et al. 2006). However, according to the concept of a hierarchical cascade of biological responses to pollutants, sub-individual biomarkers should be linked to behavioral responses. OP and carbamate pesticides are a good model to test this hypothesis as their primary mechanism of acute toxicity is the inhibition of AChE activity involved in the functioning of the nervous system. In chlorpyrifos-spiked polluted soils, the avoidance response of the earthworms *Lumbricus terrestris* was not significant and no correlation could be established either with the dose of OP pesticide used or with AChE inhibition (Martinez-Morcillo et al. 2013). Other studies

have shown that the outcome of avoidance behavior response test is pesticide concentration-dependent, using the carbamate insecticide methomyl (Pereira et al. 2010). However, most of the studies concerning the impact of pesticides were conducted under laboratory at the molecular level. *Eisenia fetida* treated with the organophosphate insecticide malathion, recorded a genotoxicity by the alteration of the DNA structure of spermatogonia, the disruption of sperms disposition and viability accompanied by a significant decrease in the body weight (Espinoza-navarro & Bustos-obregón 2005).

Under laboratory conditions, AChE inhibition in response to OP or Carbamates pesticides has been validated in earthworms (Booth et al. 2000, Venkateswara Rao et al. 2003, Caselli et al., 2006). Some inhibitions were observed in orchards after one spraying event (Reinecke & Reinecke, 2007) or repeated treatments (Denoyelle et al., 2007) but sometimes, no effect could be obtained under field conditions (O'Halloran et al. 1999, Booth and O' Halloran, 2001). Over-and-above, studies suggest that the toxicity of a same pesticide is depended on the ecological group of the worm, its behavior and feeding manners (Kammenga et al. 2000). The impact of the pesticide methyl-parathion on the growth of three different earthworms was species-specific. The substantial impact of this pesticide was observed in the anecic species *Lampito mauritii* and the endogeic *Metaphire posthuma*, in comparison with the epigeic *Allolobophora parva* (Suthar 2014). Finally yet importantly, organophosphates pesticides methyl-parathion, chlorpyrifos and phorate showed devastating effects on the nervous system of earthworms (Reinecke & Reinecke, 2007; Mhamane & Reddy 2014).

More recently, some laboratory studies have suggested the inclusion of CbE activity in the assessment of pesticide exposure and toxicity in these soil organisms (Collange et al. 2010; Sanchez-Hernandez & Wheelock, 2009). Many authors postulate that the sensitivity of CbE activity to both OP and Carbamates insecticides modulates the acute toxicity of these agrochemicals. Thus, CbE activity measurements in some terrestrial invertebrate such as earthworms exposed to chlorpyrifos-spiked soils showed a higher percentage of CbE inhibition than AChE activity, irrespective of the tissue used for esterase measurements (Collange et al. 2010; González Vejares et al. 2010). Changes in enzymes involved in oxidative stress have also been observed under pesticide exposure on *Aporrectodea*

caliginosa. An increase in GST and catalase activities revealed the metabolisation of the pesticides while neurotoxic effects were assessed towards the inhibition of AChE (Schreck et al. 2008).

While there is a lot of published literature on the effects of pesticides on earthworms at different levels, it remains difficult to draw general conclusions about these effects (Pelosi et al. 2014). One of the main questions lies in the difference of sensitivity to pesticides between earthworm ecological groups (epigeic, endogeic or anecic) or between species within these groups (Pelosi et al. 2014; Pelosi et al. 2013). Moreover, the age and development stage of earthworms may influence their sensitivity to pesticides (Lowe & Butt 2007).

2.3.2. Impact on microorganisms

Organophosphates application in soil can affect the population of diverse organisms from bacteria, actinomycetes, fungi and algae to protozoa and other soil components.

While, understanding the effect as well as the relationship between soil biota and chemicals such as organophosphate may be of practical pertinence, because of possible inhibition in microbial activities contributing to soil fertility. In general, the effect of organophosphates on soil microorganisms has been covered in some researches and studies. Direct or indirect consequences can be linked to both qualitative and quantitative variations of soil microbial populations (Usman et al. 2017). As a case in point, organophosphate herbicides affect the size and composition of microbes, and when applied in high doses, they cause the death of susceptible groups of microorganisms. Added, both glyphosate and paraquat have been reported to cause a decrease in bacterial, fungal and actinomycetes populations while paraquat increased fungal populations (Sebiomo et al. 2011). Other studies have shown that some organophosphates had no inhibitory effect on the soil microorganisms. In this event, it has been noticed that certain kinds of microorganisms may have the ability to degrade pesticides and overcome the direct or immediate effects. Rigorously, microbial population may either be stimulated or suppressed due to many factors including the mode and type of application, biomass of biota involved and soil environmental condition. Whilst, catabolism and detoxification metabolism take place when a soil microorganism benefit from the pesticide by using it as a carbon and energy source. Because OPs have been

declared as highly toxic pollutants, their degradation has attracted the attention of many scientists. In parallel, biodegradation by microorganisms is the predominant mechanism of pesticide breakdown and detoxification, thus microbes may have a considerable effect on the persistence of most pesticides. Notably, many workers have isolated indigenous microorganisms capable of metabolizing OP compounds, because these organisms provide an environmentally friendly method for detoxification. In addition to many studies on enzymes that intervene in the degradation of these compounds including carboxylestrases, other bacterial organophosphorus hydrolase (OPH) enzymes are involved in the hydrolyses and detoxification of a broad range of toxic OPs, through the cleavage of P-O and P-S bonds in these OP (Ortiz-Hernandez & Sanchez-Salinas 2010; Singh et al. 2014). On the other hand, the processes of adsorption or desorption of pesticides depend also on the physical and chemical soil properties. This highly depends on the concentration and solubility of the pesticides in soil solution, organic matter content, pH, moisture and soil temperature (Digrak & Özçelik 1998; Milosevic & Govedarica 2002). Hence, investigations and knowledge in microbial ecology, biochemical properties of soils and catabolic pathways of degradation in pesticides contaminated environments, can shed some insight that can be applied for bioremediation.

3. Earthworms-microorganisms interaction: this synergy must be maintained

Microorganisms dispersal in the environment and soil is more or less limited, numerous microorganisms of soils are inactive at a given period, waiting for suitable conditions to promote their activity (Lavelle 1997). Soil invertebrates' activities boost their dispersal within the soil to reach inaccessible resources. Earthworms represent an ideal example of promoting microorganisms' activities and dispersals. Their crucial activities of casting and burrowing enhance soil mixing then, the spreading of microorganisms in the soil profile. In the drilosphere, the part of soil influenced by earthworms, the interactions with microorganisms are perceived in the burrows, casts and gut (Brown & Doube 2004). On the spot, earthworm's gut provides an ideal environment and conditions to stimulate microorganisms. On one hand their survival,

activity and multiplication, on the other hand their digestion and elimination. Therefore, this gut can be considered as a biological filter of microorganisms. The mechanisms of these interactions and the processes driving the activation or elimination of microorganisms, is however largely unknown (Kim et al. 2010).

Since microorganisms colonized almost any habitat, many studies have focused on the effect of earthworms on the soil microbiome and the eventual changes of the number and diversity of microorganisms in the ingested soil, passing by the intestinal transit inside the worm, until the excretion of soil as casts. Soil ingestion, digestion, assimilation of organic material and secretion of enzymes in the gut and then casting, constitute the first points of earthworm-microorganism interactions. Earthworms functions in soil such as casting and burrowing, result from interaction with microbes and living organisms inside out the earthworms (Sampedro et al. 2006). Within the digestive tract of earthworms, microbes intervene in the digestion and the biochemical degradation of organic matter of soil and compost (Dominguez et al. 2003). Earthworm-microorganisms interactions seemed to be crucial for many soil functions, while a lot remains largely unknown.

3.1. Earthworms' gut microzone: a biological filter

An earthworm digestive system runs throughout the length of the body from the mouth or prostomium to the anus. It is divided into many regions each one with a function. This system comprise the mouth, the pharynx followed by the esophagus, the crop, the gizzard the intestine and then the anus (Fig. 14).

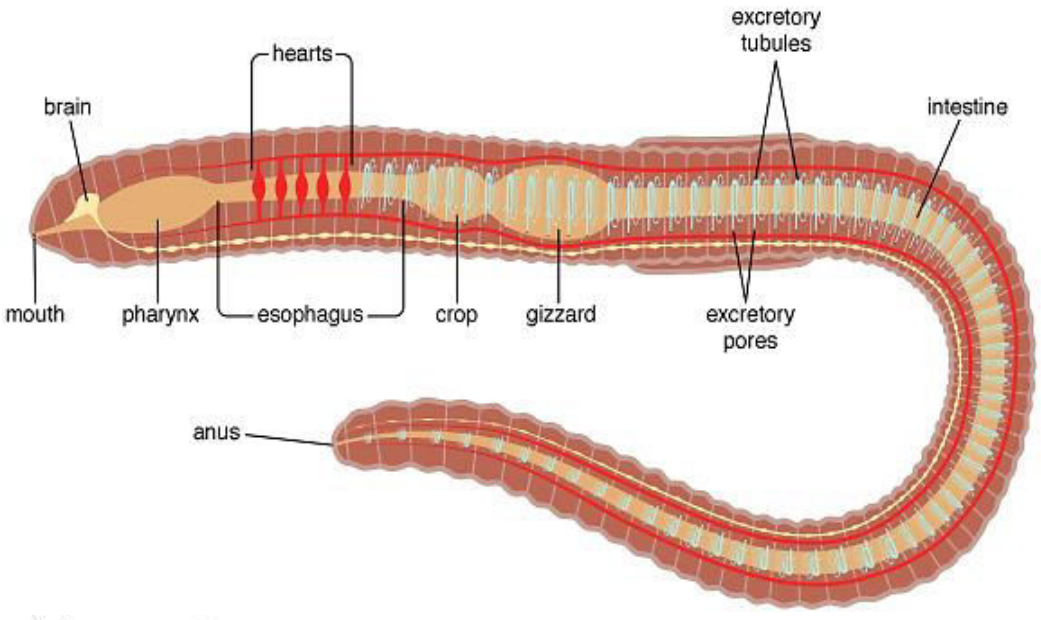

Figure 14: Earthworm digestive system © Encyclopædia Britannica, Inc. https://kids.britannica.com/students/assembly/view/193074

It is generally agreed that earthworms feed on soil containing minerals, decaying organic materials, humus, plant residues, living microbial biomass and animals. This rich food is transported throughout body parts from the mouth to the rest of the anus. The food is modified in every part to facilitate its absorption. When entering by the mouth of the worm, food is swallowed by the pharynx. After that, muscular contractions move the food to the esophagus. At this point, calciferous glands of many earthworm species produce granules of calcium carbonate (Darwin 1881; Canti & Piearce 2003) ($CaCO_3$). Their function is unknown but may be linked to the regulation of pH and CO_2 concentration, reproduction (egg formation), digestion or to rid earthworm's body of excess calcium (Ca^{2+}) and toxic cations (Briones et al. 2008). Then, the food passes into the crop where it is stored and then moves into the gizzard, a strong muscular food processor. It is known that the gizzard acts as a blender to grind the food completely into very small particles using stones and seeds that the earthworm eats and also by the secretion of enzymes helping in the breaking down of chemicals in the food. The finely divided food is then pushed down and the digestion process continues in the intestine. Here more enzymes are added to help in further breakdown of the food into simple nutrient molecules. Digested food is

absorbed and transported to the rest of the body by blood vessels lining the intestinal wall and nutrients carried to all parts of the earthworm's body by the blood, while wastes and nutrients not absorbed in the intestine are eliminated through the anus as casts (Parle 1963; Daniel & Merrill 2013). It was shown that the digestive tract of earthworm reflected the composition of the ingested soil or materials and evidenced the presence of protozoa, bacteria, fungi, algal cells, plant material, actinomycetes and also seeds (Clause et al. 2011; Zirbes et al. 2012).

3.2. Fate of ingested microorganisms in earthworms' gut

Aristotle called earthworms as the "intestines of the soil" because they act like intestines by processing and turning the soil more nutritive. Earthworms' gut is divided into 3 parts: fore gut, mid gut and hindgut. Inside their gut, independently of the ecological group, they modify the initial composition of ingested soil by stimulating or inhibiting the growth of microorganisms (Winding et al. 1997; Byzov et al. 2007). While microbes intervene in the biochemical degradation of organic matter, earthworms are important in providing suitable conditions to promote microbial activity. It is well known that microorganisms constitute an essential component of the earthworm's diet (Zirbes et al. 2012). In particular, protozoa and fungi are capital sources of nutrients while, algae represent a moderate sources and the importance of bacteria is minor (Edwards & Fletcher 1988).

Previous studies have attempted to characterize and apprehend the microbial functioning and activities inside the earthworm's gut. It is generally agreed that a mutualism occurs in the digestive tract within earthworms, since earthworms regarding their ecological group, stimulate or inhibit microbial growth. This mutualism is very important in the structure and function of food webs (Barois 1992). Inside earthworms' intestine, this mutualistic relationship between soil microorganisms and earthworms promote the digestion of organic compounds. At this level, soil microflora find suitable conditions for their activity, the earthworm's gut environment provides high water content (80 to 150% of the dry weight of soil), neutral pH and large quantities of intestinal mucus (5 to 43%). This mucus is an easily metabolizable organic matter resource, a mixture of low molecular weight hydrosoluble compounds readily degraded by microbes. Its secretion plays a central role in this mutualistic

digestion system. In parallel, digestive enzymes including lipases, chitinases, and cellulases are secreted by both the worm and ingested microorganisms in order to digest soil organic matter, through the famous mutualism earthworm-microorganisms digestion process. All of these conditions are advantageous for growth of some microorganisms and the activation of many prokaryotic cells displaying a little activity or a dormancy state. Hence, earthworm's gut microenvironment and its effect is called ''priming effect'' (Barois 1992; Lattaud 1997; Trigo et al. 1999; Brito-Vega & Espinosa 2009).

It was shown that microbes are digested in the earthworms gut, this microbial biomass leads to the formation of organic carbon reused by the earthworm. More precisely, Sampedro et al. (2006) demonstrated that the microbial community in the earthworm gut provides fatty acids that can be assimilated by earthworm.

This cooperation earthworm-microorganism has been described as a "mutualistic digestive system" to improve the degradation of organic matter for a better assimilation of nutrients by the earthworm.

Moreover, the alimentary canal of earthworms has been conceptualized as an anoxic microzone in the midst of aerated soils. Ingested microorganisms encounter this anoxic niche rich in organic compounds, large amounts of amino sugars, maltose and glucose that is likely derived from the degradation of the mucus. Thus, the in situ conditions of the gut are ideal for diverse ingested soil microorganisms capable of growth under anoxic conditions, including denitrifying and fermentative bacteria. This selective stimulation of ingested soil microorganisms, results in diverse anaerobic activities in the gut linked to the in-vivo emission of the greenhouse nitrous oxide (N_2O), dinitrogen (N_2), molecular hydrogen (H_2), and methane (CH_4) by earthworms (Drake & Horn 2007).

Despite the deficiency of molecular oxygen (O_2) and anoxic conditions of the gut, it has been demonstrated that the number of cultured ingested aerobes (including nitrifying bacteria) increases during gut passage. Hence, the viable counts of microbes capable of aerobic growth were also higher in the alimentary canal than in preingested soil. Such findings support the contrasting in situ conditions of the earthworm gut capable to greatly stimulate aerobes and anaerobes of aerated preingested soil at the same time (Ihssen et al. 2002; Zeibich et al. 2018).

4. Aims of the study

The soil, this cardinal part of the environment, maintains numerous and divers living organisms in a complex web. Along with, earthworms are the most macroscopic representatives' invertebrates. Interactions between earthworms and all soil components, including microorganisms are essential to perform several symbiotic and synergistic functions and services, either inside or outside the worm. However, agrochemicals especially insecticides, continue to be applied in the environment and affect these interactions. Inevitably, earthworms and microorganisms are endangered at many levels because of the toxicity of these pollutants that disturb this natural balance. Perhaps this figure of Brown et al. (2000) summarizes and represents our field of study, and highlights the crucial role of earthworms at many positions inside the soil and their intestines (Fig. 15).

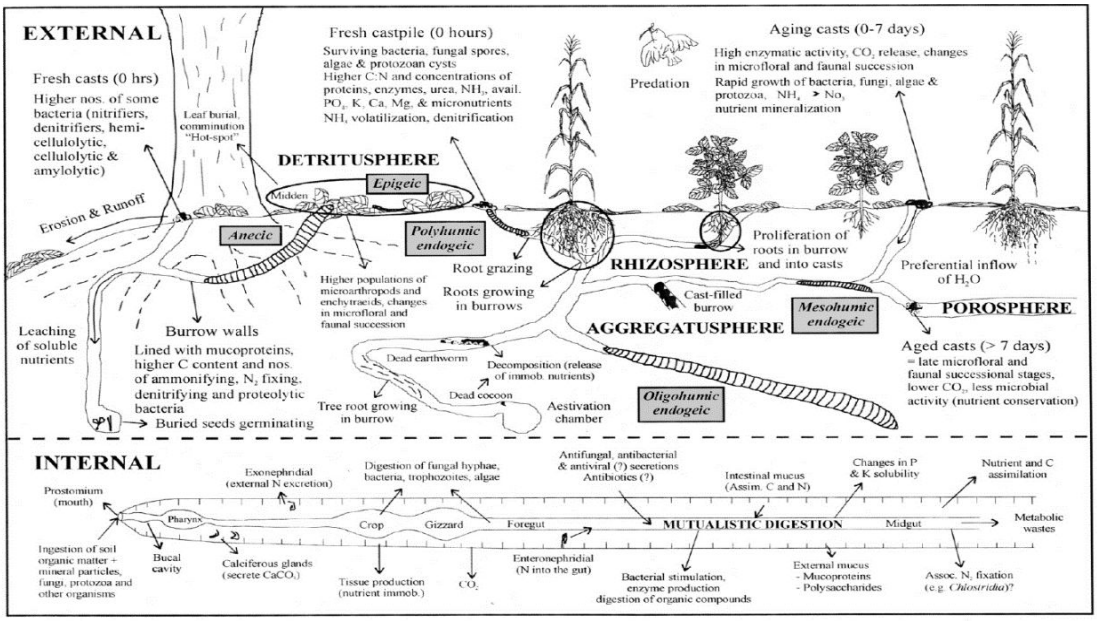

Figure 15: Consequent effect of the drilosphere structures (internal and external) along with the surrounding functions on the dynamic of soil organic matter and microbial activity (From Brown et al. 2000).

These interactions and links between soil, earthworms, microorganisms and pollutants constitute the basic topics of this work. Likewise, the study of biochemical, physiological and behavioral biomarkers, besides the microbiota of several compartments (soil, earthworm's intestinal microbiota and casts) when exposed or not to insecticides, underlies the aims of this work.

The main purpose of my thesis is to assess the role of the biochemical biomarkers of earthworms and its gut microbiota on either the sensitivity of earthworms towards insecticides or their ability to modify the bioaccessibility of insecticides. Two different endogeic species were used as earthworm model *Apporectodea caliginosa* and *Allolobobophora chlorotica*. These two species are largely found in agricultural fields. Moreover, according to sublethal responses, previous results evidenced that the earthworm *A. chlorotica* was a more tolerant species to OP-contaminated soils than *A. caliginosa* (Rault et al. 2008). Then, my work covers two main domains ecotoxicology and microbial ecology presented in 4 chapters:

Chapter 1: studies the effect of the organophosphate "ethyl-parathion" on two endogeic earthworm species *A.caliginosa* and *A.chlorotica* after 7 days of exposure. This was performed by analyzing potential biomarkers including physiological (body weight), behavioral (cast production and burrowing activity) and biochemical biomarkers (acetylcholinesterase and carboxylesterases).

To follow up previous results of the chapter 1,

Chapter 2: an *In-vivo* and *In-vitro* study and characterization of enzymes, trying to understand the difference in the sensitivity to this insecticide in both earthworms' species. This was performed by the investigation of detoxifying enzymes: GST, carboxylesterases and their implication in the tolerance of *A.chlorotica* or sensitivity of *A.caliginosa*.

Then, due to the dynamic interactions earthworms-microbiota, especially in the drilosphere area, it was critical to evaluate the microbial communities and dynamic (community, biomass, enzymes activities) in soil exposed or not to earthworms and insecticide.

Chapter 3: a taxonomic study of the microbial community of the ingested soil, gut and cast to confirm the presence or not of a specific microbiome at the gut level. In addition, we tried to investigate if the earthworm's gut microbiota harbors some microbes that can detoxify the insecticide.

Chapter 4: examine the impact of both species on the activity of soil enzymes in two different soils contaminated by ethyl-parathion.

Chapter 1: Interspecific differences in biochemical and behavioral biomarkers in endogeic earthworms exposed to ethyl-parathion

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Interspecific differences in biochemical and behavioral biomarkers in endogeic earthworms exposed to ethyl-parathion

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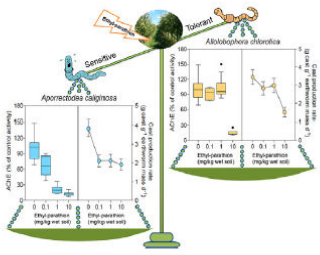
HIGHLIGHTS

- Marked interspecific differences in acetylcholinesterase (AChE) activity in earthworms.
- Ethyl parathion was more toxic to *Aporrectodea caliginosa* than *Allolobophora chlorotica*.
- Cast production was a sensitive biomarker of ethyl parathion exposure.
- There was a strong relationship between AChE inhibition and cast production.

GRAPHICAL ABSTRACT



1. Introduction

Cholinesterase-inhibiting pesticides such as organophosphates (OP) continue to be an important group of agrochemicals used in the chemical control of agricultural pests. Although these pesticides show a relatively low environmental persistence, they lack target specificity and generally display a high acute toxicity towards non-target organisms such as earthworms (Pelosi et al., 2013a). The mechanism of acute toxicity of these chemicals is one of the best described in the toxicology literature and involves the inhibition of acetylcholinesterase, (AChE, EC 3.1.1.7); a key enzyme in the nervous system (Fukuto, 1990; Thompson and Richardson, 2004; Casida, 2017). This enzyme hydrolyses the neurotransmitter acetylcholine at the synaptic cleft, thus ending the transmission of the nerve impulse. Species-specific differences in OP sensitivity and resistance is due mainly not only to the affinity of the OP by the active site of AChE, but also by the number of OP molecules that interact with AChE (Maxwell, 1992; Küster, 2005). In this context, the enzymes carboxylesterases (CbE, EC 3.1.1.1.) play an important role in the detoxification of OPs. This serine hydrolase is often more sensitive to inhibition by OPs than AChE (Wheelock et al. 2008), so this chemical interaction is considered an efficient mechanism of OP detoxification by which fewer OP molecules may reach the active site of AChE (Chanda et al. 1997). Indeed, CbE activity participates in the tolerance of some insect pests to OP pesticides (Farnsworth et al. 2010). Despite this potential role of CbE activity in the interspecific differences in OP susceptibility, its implication in the OP toxicity in earthworms is still very limited.

The classical use of AChE and CbE activities in ecotoxicology is to be biomarkers of OP exposure, using a wide range of methodological approaches that involve both laboratory toxicity bioassays and field monitoring surveys (Otero and Kristoff, 2016). However, it is necessary to know what consequences for the organism and its population will come from the inhibition of AChE activity (Scott and Sloman, 2004). Because this enzyme is implied in animal locomotion and sensorial functions, pesticide-exposed organisms would manifest some kind of neurobehavioral disruption. However, this functional link between biochemical and behavioral toxicological endpoints has not been well established for earthworms. Indeed, many investigations have linked behavioral changes with inhibition of AChE activity in OP-exposed birds (Walker, 2003), fish (Tilton et al. 2011; Khalil et al., 2017), and

invertebrates (Jensen et al., 1997; García-de la Parra et al. 2006; Malagnoux et al. 2015). In the case of earthworms, which are recommended organisms in terrestrial ecotoxicity testing (EC, 2002), some studies have developed simple and low cost procedures to assess behavioral perturbations in polluted environments, but they do not include the measurement of exposure biomarkers as initiating molecular events of toxicity. For example, the capability of earthworms to avoid unfavourable or contaminated soils has been implemented in a standardized test named avoidance behavior response test (ISO, 2008). Similarly, Capowiez et al. (2010) developed a simple and quick procedure to measure the cast production (egested soil after gut transfer) by soil-dwelling earthworms (i.e., endogeic and anecic species), which was a sensitive behavioral endpoint to soil contamination. Despite these methodological advances in earthworm behavioral toxicology, little is known on species-specific differences of earthworm behavior related to chemical stressors such as pesticides. This is probably because *Eisenia* sps. are the recommended organisms in soil toxicity testing so far. Nonetheless, there is a growing concern in the inclusion of soil dwelling species because of their significant impact on physicochemical and biological properties of soil, their population density in the agroecosystems, and their higher sensitivity to agrochemicals compared with *Eisenia* sps. (Pelosi et al., 2013b)

Our study is focused on endogeic earthworms because of a set of environmental and biological features. These earthworms change the soil structure, nutrient dynamics and microbial communities by their continuous burrowing, feeding and casting activities (Jégou et al., 2001; Lipiec et al., 2016). They represent the largest soil biota biomass, although their abundance and biodiversity are often negatively impacted by conventional agricultural practices that are characterized by a high pesticide input (Pelosi et al. 2013a). However, reservations about pesticide toxicity in earthworms should be taken into account because marked species-specific differences in pesticide exposure level (digestive and/or dermal uptake), earthworm ecology (endogeic, anecic and epigeic species), life traits (e.g., reproduction rate, growth), behavior (e.g., feeding mineral soil to obtain organic matter or feeding on decay organic matter), and basal levels of ecotoxicological biomarkers (Rault et al. 2007).

This study sets out to establish a functional link between initiating molecular events of OP toxicity (i.e, esterase inhibition) with changes at individual level (i.e.,

behavior). Considering the role of these soil-dwelling earthworm species in soil quality, we selected the burrowing activity and cast production as behavioral measurements with direct implications in promoting soil fertility. Therefore, the aims of this study were: 1) to compare the response of AChE and CbE activities between both earthworm species, and to evidence whether a higher percentage of AChE inhibition corresponded to a lower level of CbE activity or less sensibility of CbE activity to the OP, 2) to assess whether OP-induced changes in the earthworm behavior are linked to inhibition of AChE activity. This research will bring insight in ecologically consequences of adverse effect on behavioral implications from toxic effects at molecular initiating events.

2. Materials and methods

2.1. Chemicals

The substrates for esterase activity assay (acetylthiocholine iodide [AcSCh], 4-nitrophenyl acetate [4-NPA], 4-nitrophenyl valerate [4-NPV], 1-naphthyl acetate [1-NA]), the product of carboxylesterase-mediated hydrolysis (1-naphthol), the chromogens (5,5´-dithiobis-2-nitrobenzoic acid [DTNB] and Fast Garnet), the OP pesticide ethyl-paraoxon, and a protease inhibitor cocktail were purchased from Sigma–Aldrich (Saint Quentin-Fallavier, France). For in vivo tests, we used a commercial liquid formulation of the OP insecticide ethyl-parathion (Oleobladan®, active ingredient 93g L^{-1}, Bayer).

2.2. Soil and earthworms

A silt loamy soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH 8.3), and healthy adults of *A. caliginosa* and *A. chlorotica* were collected from an apple orchard in Montfavet near Avignon (France). This orchard has received no pesticide treatments for the last 10 years. Earthworms were collected manually, weighed and kept in the laboratory into plastic pots filled with fresh soil collected from the same orchard.

Soil was sieved at <2 mm, and the water content was adjusted to 20-21% (approximately 81% of the maximum water holding capacity) with distilled water. Afterwards, wet soil samples (1 kg) were spiked with ethyl-parathion solutions (40 ml)

to yield the final concentrations of 0.1, 1 and 10 mg active ingredient (a.i.) kg^{-1} wet soil. These ethyl-parathion concentrations were chosen according to the recommended application rates by the supplier, and the calculations of the Predicted Environmental Concentration (PEC) using the equation (FOCUS, 2006): $PEC_{S,O}=A \times (1\text{-}fint)/(100 \times depth \times bd)$, where $PEC_{S,O}$ is the initial soil concentration (mg kg^{-1}) of the insecticide immediately following a single application, *A* is the application rate (g ha^{-1}), *fint* is the fraction of pesticide intercepted by the crop canopy, *depth* is the mixing depth (cm), and *bd* is the dry bulk soil density (g cm^{-3}). The $PEC_{S,O}$ was calculated according to a bulk soil density of 1.5 g cm^{-3}, a depth soil layer of 5 cm of pesticide penetration, and no crop interception. Therefore, recommended application rates of ethyl-parathion corresponded to an initial PEC of 0.7 mg a.i. kg^{-1} soil, which is close to the nominal concentration of 1 mg kg^{-1} we used. A control soil was prepared under the same conditions but free of pesticide. All treated soils were split into 100-g subsamples, which were placed into plastic pots (10-cm d × 3-cm h), to yield 20 replicates per treatment.

The earthworms were washed in tap water, blotted dried on filter paper, weighed (without gut voiding) and individually placed into the test containers (control and ethyl-parathion-treated). For each treatment (control, 0.1, 1 and 10 mg ethyl-parathion) 20 earthworms were place individually into the containers to prevent cascade death (Sheppard et al., 1992), and they were kept in a dark cold chamber (12±1 ℃) for 7 days. After this incubation time, earthworms were weighed and either frozen at -20 ℃ until biochemical measurements (n=10 per treatment) or placed in a two-dimensional terrarium for recording behavioral responses (n=10 per treatment). Earthworm-free containers (n=10) containing 100 g of soil were used as controls to assess cast production. Body weight variations were expressed as a percentage of the initial average weight.

2.3. Earthworm behavior

Two behavior responses were assessed: the burrowing activity and the cast production. The former behavior was monitored using two-dimensional (2D) terraria (Evans 1947), which consisted of two glass sheets (30×42 cm) separated by a 3-mm apart and filled with 550 g of 2 mm sieved uncontaminated soil, allowing thereby the recording of earthworm burrowing (Capowiez, 2000). Forty 2D terraria were used

taken into account the four treatments: control, 0.1, 1 and 10 mg a.i. kg^{-1} of the insecticide. At the beginning of the assay, one earthworm was introduced into each 2D terrarium. The location of the earthworm and the burrows architecture was recorded daily on a transparent sheet for 6 days. Once the assay was completed, information gathered on each 2D terrarium was digitized (Capowiez, 2000). Burrow lengths in each terrarium were computed using ImageJ. After a calibration step, the area was transformed in burrow lengths for the 6 days of continuous monitoring.

Cast production was determined according to Capowiez et al. (2010). The casts were separated using a set of four 15-cm sieves (mesh sizes = 5.6, 4, 3.15, 2.5 mm), because earthworm activity strongly modifies the soil texture by increasing aggregate fractions. The soils from Petri dishes, including that adhered to the dish wall which was removed using a knife, was carefully sieved avoiding the fragmentation of casts. The set of sieves was manually shaken for 10 s. The soil fractions retained in each sieve was weighed. The effect of earthworm bioturbation was then examined by calculating the changes in the particle size distribution, i.e. weight of fresh soil in each sieve minus the corresponding weight of soil for the control soil (earthworm free). The cast production was expressed as wet cast weight per earthworm fresh body mass per day.

2.4. Homogenate preparation

Frozen individuals were sliced in 1 cm length pieces and homogenized individually on ice using an Ultra Turrax IKA T18 basic apparatus at 14000 rpm, 4 times of 10s duration with 1-min interval, in 20% (w/v) low-salt buffer containing 10 mM Tris-HCl pH=7.3, 10 mM NaCl, supplemented with protease inhibitors (aprotinin, leupeptin and pepstatin=5 µg mL^{-1}, antipain=1 µg mL^{-1}, trypsin inhibitor=1 mg mL^{-1}). The homogenates were centrifuged (3000 g, 10 min, 4°C) to obtain a post-mitochondrial fraction (supernatants) which was supplemented with glycerol (10% v/v, final concentration) as an enzyme stabilizing agent, and frozen at -20°C.

2.5. Esterase activity assays

Enzyme activities were measured by microplate-scale spectrophotometric procedures using the tissue homogenates, and the products of the hydrolysis reaction were measured using a microplate reader (Synergy HT, Bio-Tek). The

activity AChE was monitored at 412 nm and 25ºC following the method by Ellman et al. (1961), and adapted by Rault et al. (2008), using a millimolar extinction coefficient (6,800 M^{-1} cm^{-1}) calculated according to a dithiotreitol-DTNB external calibration curve. The reaction medium (200 µL, final volume) contained 4 µL of homogenate, 0.375 mM DTNB and 3 mM of AcSCh in 0.1 M sodium phosphate buffer (pH 7).

Because the sensitivity of carboxylesterase activity to OP is highly dependent on the substrate, this activity was assayed using three different substrates, which differ in their carbon-ester chains: 1-naphtyl-acetate (1-NA), 4-nitrophenyl acetate (4-NPA) and 4-nitrophenyl valerate (4-NPV) to characterize multiple CbE isoenzymes following the microplate-scale assay by Thompson (1999). The hydrolysis of 1-NA was determined in a reaction medium (200 µL final volume) contained 25 mM Tris-HCl (pH 7.6), 1 mM $CaCl_2$ and 2 µL of diluted (1/20) homogenate. The reaction was initiated by adding 2 mM 1-Na (final conc.), and stopped after 10 min of incubation at 25ºC by the addition of 50 µL 2.5% SDS containing 0.1% Fast Garnet in 2.5% Triton X-100. The solutions were left to stand for 10 min at room temperature and dark, and the absorbance of the 1-naphthol-Fast Garnet complex was read at 530 nm. Enzyme activity was calculated from a standard curve (A_{530}=f([1-naphtol]) containing α-naphthol in the same reaction mixture free of substrate. Hydrolysis of 4-NPV and 4-NPA was determined in a reaction mixture (250 µL, final volume including 2 µL of enzyme extract) contained 1 mM 4-NPV and 50 mM Tris-HCl (pH 7.5), or 5 mM 4-NPA in 20 mM Tris-HCl (pH 8.0) and 1mM EDTA (to inhibit Ca^{2+} dependent phosphotriesterases). In both assays, the formation of 4-nitrophenol was monitored for 5 min at 405 nm, 25ºC and quantified using a 4-nitrophenol standard curve.

Blanks (reaction mixture free of sample) were checked for non-enzymatic hydrolysis of the substrates. All enzyme assays were run at least in triplicate and expressed as units per milligram of total protein (U mg^{-1} protein). One unit of enzyme activity was defined as one micromole of formed product per minute under the experimental conditions described above. Total protein content was determined by the Lowry method modified by Markwell et al. (1978), and using bovine serum albumin as the standard.

2.6. Acetylcholinesterase inhibition kinetics

In vitro inhibition kinetics were conducted to test for a direct interaction between ethyl-paraoxon and AChE activity. Tissue homogenates from unexposed earthworms (20 µl) were incubated individually with serial concentrations of ethyl-paraoxon (10^{-12} to 10^{-3} M final concentration) for 30 min at 25ºC. Inhibition assay was terminated by addition of the substrate (AcSCh), and the residual AChE activity was measured as described above.

2.7. Native polyacrylamide electrophoresis for esterase isoenzymes

Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was performed on a Bio-Rad Tetracell Electrophoresis Unit (Bio-Rad, USA). Homogenates (20 µL) were loaded on 4% stacking gel and 12.5% resolving gel for CbE analysis, or 8% resolving gel for AChE analysis, 0.75 mm polyacrylamide gel (25 mM Tris, 192 mM glycine, pH 8.3 as running buffer), and electrophoresed at an initial constant voltage of 30 V for 30 min, and afterwards 150 V until bromophenol blue tracking dye reached the bottom of the gel. The in-gel staining esterase activities were performed according to Manchenko (2002). Protein bands of CbE were visualized by incubation (2–3 min at room temperature) of the gels with a staining solution containing 100 mM Na-phosphate buffer (pH 6.4), 0.5 mg mL^{-1} 1-NA and 0.025g of Fast Blue RR salt, which was prepared and filtered immediately before use. Protein bands of AChE were visualized by incubation (12 hours at room temperature) of the gels with a staining solution containing 200 mM maleate buffer (pH 6.0), trisodium citrate 100 mM, copper sulphate 30 mM, 5 mM potassium ferricyanide and 150 mg AcSCh.

2.8. Statistical analysis

The Kruskal-Wallis test and the *post hoc* Mann-Whitney test were used to compare ranges of the esterase activities between treatments. Furthermore, we used the Jonckheere–Terpstra trend test (J-T trend test) to assess significant relationships between the median values of enzyme activities and the concentration of ethyl-parathion in soil. *In vitro* inhibition curves were fitted to the non-linear model (library of GraphPad Prism software, ver. 7.00, La Jolla California USA):

$$E = min + \frac{(max - min)}{1 + \left(\frac{I}{IC_{50}}\right)^{-Hillslope}}$$

where *E* is the percentage of residual enzyme activity relative to controls, *min* is the *E* response to the highest ethyl-paraoxon concentration, *max* is the *E* response to the lowest insecticide concentration, *I* is the logarithmic-transformed concentration of insecticide, the *Hillslope* coefficient describes the steepness of the dose–response curve, and IC_{50} is the median insecticide concentration that leads to a 50% reduction in the initial enzyme activity. Burrow length and cast production (both log-transformed) for each pesticide concentration were compared using a one-way ANOVA for each species separately.

3. Results

3.1. Effect of ethyl-parathion on earthworm body weight and behavior

The insecticide had a significant impact on earthworm body weight, which was species-specific (Fig. 16A). A significant decrease (12.6±0.8% to 21.58±1.04% compared with control values) was found in the body mass of *A. caliginosa* at 1 and 10 mg kg^{-1} (p<0.001), whereas *A. chlorotica* experienced an increase (8–10%) of body weight at the concentrations of 0.1 and 1 mg kg^{-1}, but such a weight gain was inhibited at the highest ethyl-parathion concentration (p=0.017). No dead were recorded during this toxicity bioassay.

The length of burrows produced by non-exposed individuals from both species was comparable (between 140 and 160 cm length) (Fig. 16B). Moreover, 0.1 and 1 mg kg^{-1} ethyl-parathion did not change in the burrow length formed by both species, although a significant shortening of burrow length was observed at the highest insecticide concentrations for *A. caliginosa* compared with its respective controls. However, cast production showed evidence of an insecticide effect on this behavior that was species-specific, but not dose-dependent (Fig. 16C).

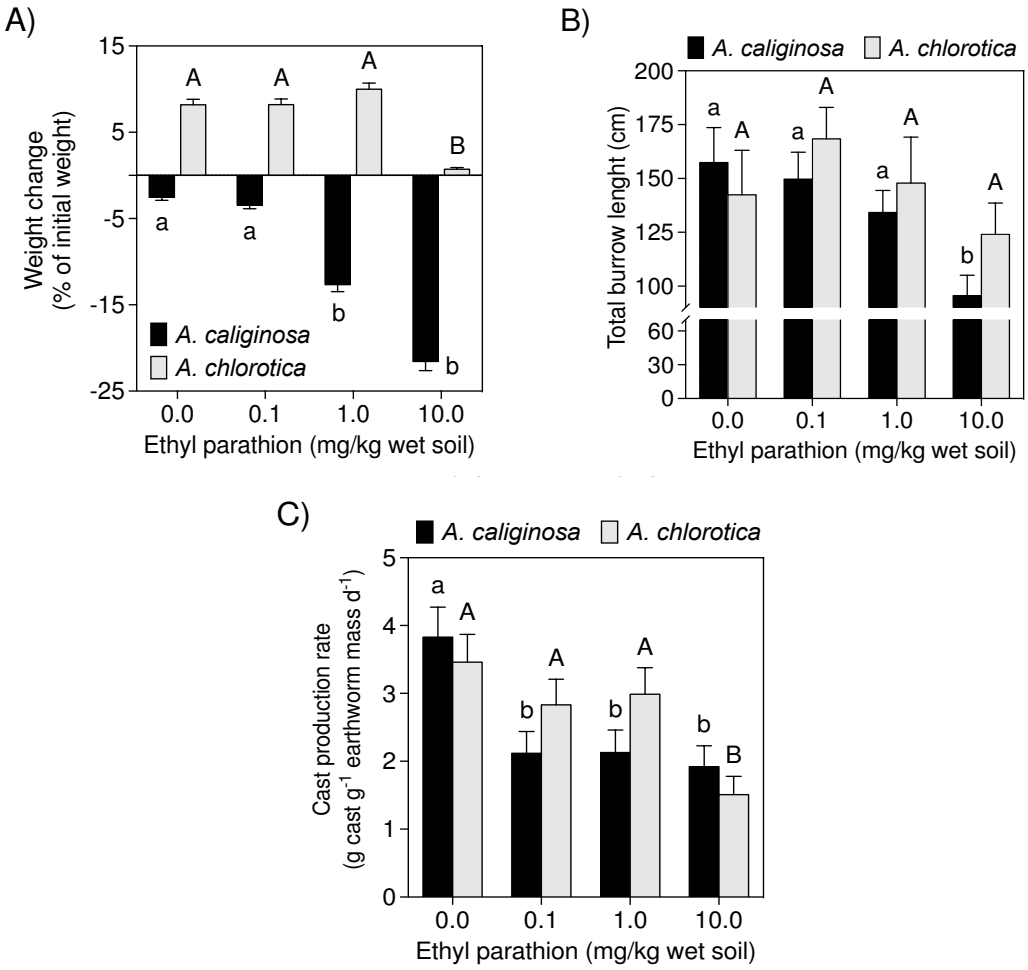


Figure 16. Effect of ethyl-parathion exposure (7 days) on earthworm weight (A), burrow length (B) and cast production (C). Bars represent the mean and the standard error of the mean (SEM). Different letters (lowercases for Aporrectodea caliginosa and uppercases for Allolobophora chlorotica) denote significant differences between treatments ($p<0.05$, Mann-Whitney).

Interestingly, both earthworm species exhibited the same cast production in pesticide-free soils (about 3.6 g of wet casts g^{-1} earthworm day^{-1}). Soils contaminated with 0.1 to 10 mg ethyl-parathion kg^{-1} caused a significant reduction in the cast production of *A. caliginosa*, whereas this behavioral response remained unchanged for *A. chlorotica*, except in the highest insecticide concentration.

3.2. Effect of ethyl-parathion on esterase activities

Basal levels of AChE and CbE activities for both earthworm species are summarized in Table 3. The activity of both esterases was two times higher in *A. caliginosa* than in *A. chlorotica*, and CbE activity was higher than AChE activity for both species. Exposure of earthworms to ethyl-parathion-contaminated soils caused a significant response of these esterases that was species-specific (H_3=52.6, p<0.001 for *A. caliginosa*, H_3=25.1, p<0.001 for *A. chlorotica*) (Fig. 17A). Whereas the mean AChE activity decreased in a dose-dependent manner (p<0.001, J-T trend test) in *A. caliginosa* (34%, 78% and 87% inhibition at the concentrations of 0.1, 1 and 10 mg kg^{-1}), this esterase activity remained unchanged in *A. chlorotica* exposed to 0.1 and 1 mg kg^{-1} ethyl-parathion, although was drastically inhibited (85% of controls) at the highest insecticide concentration (z=-4.36, p<0.0001, Mann-Whitney test).

Table 3. Specific esterase activities, expressed as U.mg-1 proteins (mean±SEM) are measured in homogenates from the whole body of the endogeic earthworms Aporrectodea caliginosa and Allolobophora chlorotica.

Species	Acetylcholinesterase ($U.mg^{-1}$)	Carboxylesterases ($U.mg^{-1}$)		
	AcSCh	4-NPA	4-NPV	1-NA
A. caliginosa	0.147±0.026	0.300±0.038	0.182±0.034	0.882±0.114
A. chlorotica	0.073±0.020	0.157±0.026	0.095±0.015	0.386±0.077

AcSCh=acetylthiocholine, 4-NPA=4-nitrophenyl acetate, 4-NPV=4-nitrophenyl valerate and 1-NA=1-naphthyl acetate.

Native PAGE corroborated these enzyme activity outcomes (Fig. 17A). In-gel staining AChE activity revealed two isoforms in the homogenates of both species. These protein bands may correspond to a membrane-isoform (the heaviest molecular weight one) and the soluble isoform of AChE. The staining intensity of these protein bands decreased in the ethyl-parathion-exposed earthworms accordingly with the inhibition of AChE activity found in the corresponding homogenates.

In vitro inhibition kinetics of homogenates incubated in the presence of ethyl-paraoxon generated typical sigmoid dose-dependent curves (Fig. 17B). The estimated IC_{50} values for the *A. caliginosa* AChE response were 1.81×10^{-7} M

(1.63×10^{-7}–2.03×10^{-7}, 95% confidential limits, r^2=0.99) and 1.12×10^{-6} M (8.16×10^{-7}–1.54×10^{-6}, r^2=0.98) for the *A. chlorotica* AChE response.

The response of CbE activity to ethyl-parathion was also species-specific, and depended on the substrate used in the enzyme assay. The hydrolysis rate of 1-NA was equally inhibited (24.5-38.5% of controls) in both earthworm species at the doses of 1 and 10 mg kg^{-1} ethyl-parathion (Fig. 18). However, the impact of ethyl-parathion exposure on the hydrolysis of nitrophenyl esters was markedly dependent on the species as evidenced by the CbE response at the lowest ethyl-parathion dose. The enzyme activity towards 4-NPV and 4-NPA was significantly depressed in *A. caliginosa* exposed to 0.1 mg kg^{-1} ethyl-parathion, whereas the hydrolysis rates of these substrates remained unchanged in *A. chlorotica* compared with its controls. Despite these interspecific differences, the J-T trend test showed a significant dose-dependent inhibition of CbE activity with ethyl-parathion concentration (p<0.0001).

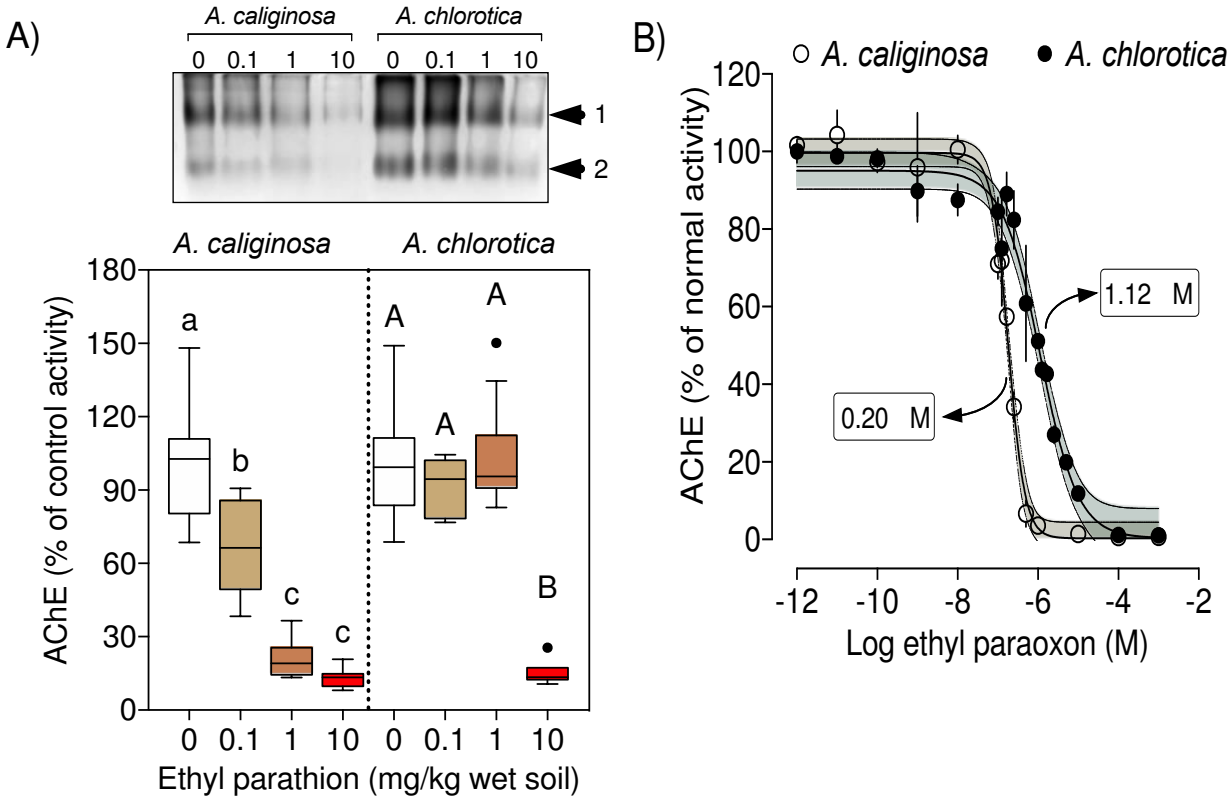


Figure 17: A) Response of acetylcholinesterase (AChE) activity in earthworms exposed to ethyl-parathion-contaminated soils for 7 days, and in-gel staining activity after native PAGE electrophoresis (protein charge=100–119 μg/lane for A. chlorotica, and 87–110 μg/lane for A. caliginosa). Tukey box plots indicate the median, the 25th and 75th percentiles (box edges), the range (whiskers) and outliers (black dots). Significant difference between treatments is indicated by different letters as in Fig. 1

(p<0.05, Mann-Whitney test). B) Dose-dependent inhibition curves for acetylcholinesterase (AChE) of Aporrectodea caliginosa and Allolobophora chlorotica in the presence of serial molar concentration of ethyl-paraoxon. Symbols are the mean±SEM, the bands show the 95% confidence intervals of the non-linear regression curves, and the arrows mark the IC_{50} values.

We also performed in-gel staining for CbE activity using the substrate 1-NA on post-electroforesed gels. These results showed multiple CbE isozymes, whose abundance was higher in *A. caliginosa* compared to *A. chlorotica* (Fig. 18). Moreover, the OP caused a reduction of staining intensity in those homogenates from the individuals exposed to the highest concentrations. These zymographic outcomes also confirmed the impact of ethyl-parathion exposure on the CbE activity of both earthworm species.

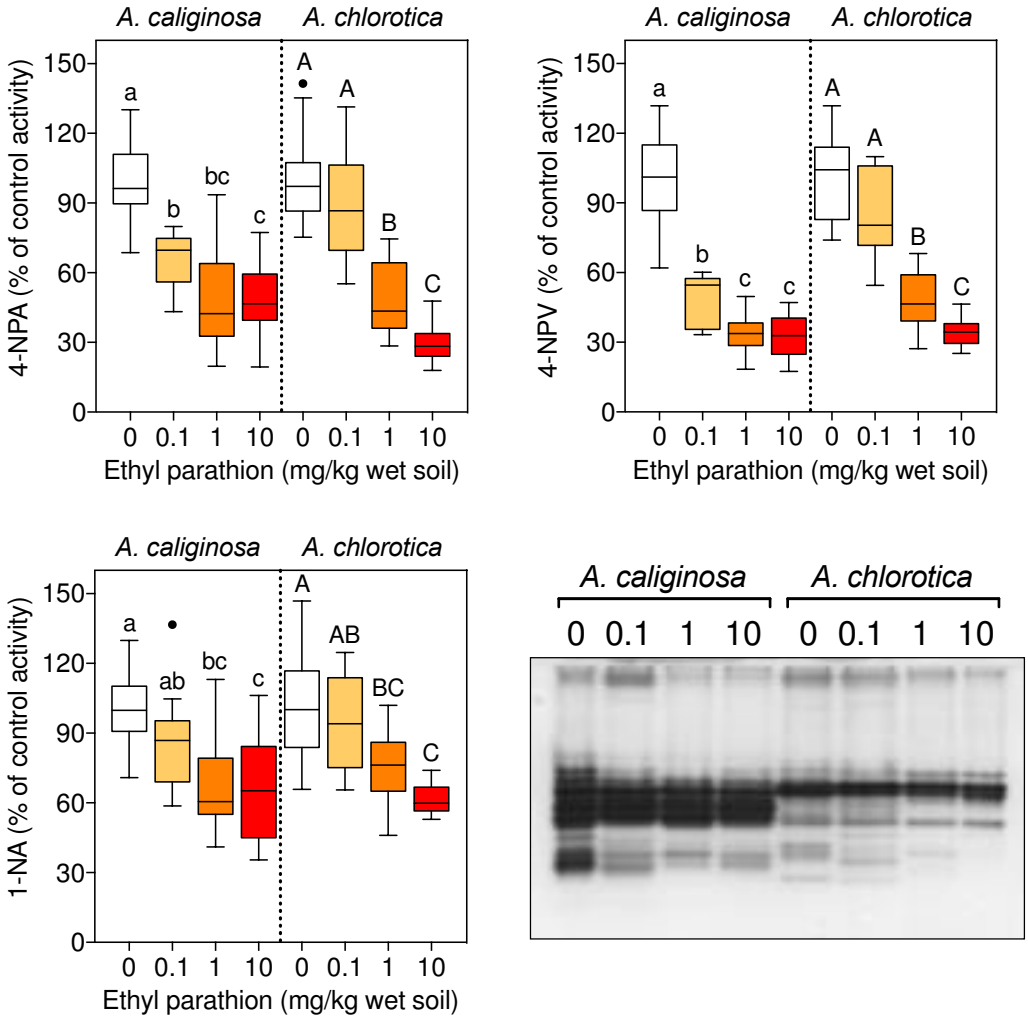


Figure 18: Response of carboxylesterase (CbE) activity in earthworms exposed to ethyl-parathion - contaminated soils for 7 days. The esterase activity was measured using the substrates 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV), and 1-naphthyl acetate (1-NA). In-gel staining activity after native PAGE electrophoresis was performed with homogenates (protein load=100–119 µg/lane for A. chlorotica, and 87–110 µg/lane for A. caliginosa) of all treatments. Tukey box plots indicate the median, the 25th and 75th percentiles (box edges), the range (whiskers) and outliers (black dots).

Significant difference between treatments is indicated by different letters as in Fig. 2 ($p<0.05$, Mann-Whitney test).

Correlations between AChE inhibition and behavioral biomarkers were tested. Although there was a linear relationship between the mean values of AChE inhibition and changes in body weight for both species ($r^2>0.94$, $p<0.05$) (Fig. 19A), the relationship between AChE inhibition and cast production was species-specific (Fig. 19B). A non-linear relationship was observed for *A. caliginosa*, by which low concentrations of ethyl-parathion caused a drastic inhibition of both biomarkers.

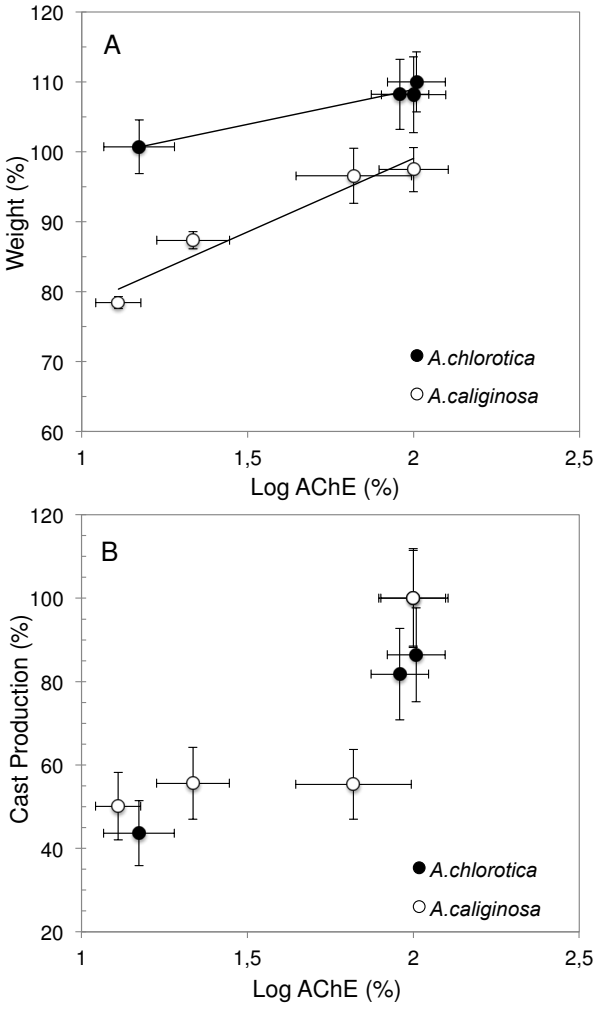


Figure 19: Relationship between acetylcholinesterase (AChE) activity and body weight change (A) or cast production (B) after exposure to ethyl-parathion (mean ±SEM). AChE activity is expressed as % of control values.

4. Discussion

Short-term exposure to ethyl-parathion caused a marked interspecific difference in the response of both biochemical and behavioral biomarkers. According to these sublethal responses, the earthworm *A. chlorotica* was a more tolerant species to OP-contaminated soils than *A. caliginosa*. This assumption is supported by previous results that evidenced a progressive recovery of *A. chlorotica* AChE activity after 14-d exposure to ethyl-parathion (1 mg kg^{-1}), whereas this enzyme remained fully inhibited in *A. caliginosa* during more than 2 months of a recovery period in uncontaminated soil (Rault et al., 2008). On the other hand, if we assume that both species display a similar behavior in soil because they are endogeic and, therefore, exposure to the pesticide should occur at the same extent, then factors related to pesticide bioactivation and metabolism could contribute significantly to these species-specific differences in pesticide sublethal toxicity. In this line, many authors have suggested that CbEs play an important role as pesticide bioscavengers because of their higher sensitivity to OP inhibition compared with AChE sensitivity (Küster, 2005; Wheelock et al., 2008; Kristoff et al., 2010). Moreover, the number of CbE molecules, and therefore of binding sites, seems also an important factor in the OP toxicity, at least in mammals (Chanda et al., 1997). However, our results with this esterase activity indicated the following: i) basal CbE activity of un-exposed individuals of *A. caliginosa* was two-times higher than that of *A. chlorotica*, ii) the *in vivo* exposure trial revealed that CbE activity of *A. caliginosa* was more sensitive to inhibition by the insecticide than *A. chlorotica* CbE activity, and iii) the abundance of CbE isozymes was higher in *A. caliginosa* compared with that in *A. chlorotica*. According to these observations, the species *A. caliginosa* would be able to reduce the impact of an acute exposure to ethyl-parathion through its higher levels and higher sensitivity of CbE activity compared to *A. chlorotica*. However, changes in behavior biomarkers, particularly cast production, suggest the contrary. Clearly, other potential toxicodynamic and toxicokinetic factors contribute to the acute toxicity of OP pesticides. For example, enzymatic complexes such as the cytochrome P450-dependent monooxygenases are responsible for increasing OP toxicity through oxidative desulfuration of the parent compound to yield the oxon metabolite (Lee, 1998; Chambers et al., 2010; Hodgson, 2012; Dzul-Caamal et al., 2014). Other detoxifying enzymes such as phosphotriesterases (Vilanova and Sogorb, 1999) or glutathione *S*-transferases

(Jokanović, 2001) participate equally in ethyl-parathion (and metabolites) detoxification.

However, interspecific differences in OP susceptibility may also be explained by differences in the intrinsic AChE sensitivity to oxon metabolites of OPs (Chambers and Carr, 1995). Likewise, it is well known that gut motility of annelids is under cholinergic control (Fieber, 2017), so inhibition of AChE activity is expected to alter gastrointestinal transit of food with changes in cast production rate. Therefore, we performed *in vitro* inhibition trial with ethyl-paraoxon to elucidate whether the higher sensibility of *A. caliginosa* to the pesticide, in terms of behavioral responses, can be explained by ethyl-parathion-related disruption of cholinergic pathways. These results clearly revealed that the activity of *A. caliginosa* AChE was more sensitive to ethyl-paraoxon than that of *A. chlorotica*. Therefore, the intrinsic sensibility of AChE to this OP insecticide could be a significant cause for the interspecific differences in sublethal toxicity between these species. Nevertheless, we are aware that these results are still premature to make solid prediction about impact of OP exposure in natural populations of earthworms. Indeed, some studies show that applications of pesticides to agricultural lands cause a similar response in the abundance of *A. caliginosa* and *A. chlorotica* in France (Dinter et al., 2012), highlighting the importance of field monitoring studies in the predictions of pesticide impact at an earthworm population level.

One of the main scopes in the use of ecotoxicological biomarkers is to predict population-level adverse effects from sub-individual responses. Indeed, some studies have tried to link earthworm behavioral responses such as avoidance (Pereira et al., 2010; Jordaan et al., 2012; Martínez-Morcillo et al., 2013), burrowing activity (Capowiez et al., 2003) or cast production (Capowiez et al., 2010) to AChE inhibition. Current data support this cause-effect relationship with *A. caliginosa,* as the inhibition of AChE activity lead to either a decrease in the body weight, a strong threshold effect in the cast production, but no effect in the burrowing activity. It has long been demonstrated that the continue activity of feeding, burrowing and casting of endogeic and anecic earthworms has a strong influence on soil structure and function (Lavelle et al., 2006). Particularly, earthworm casts are significant hot spots of microbial proliferation and enzyme activities, that may contribute to mitigate the impact of environmental contaminants in soil (Lipiec et al., 2016; Sanchez-Hernandez et al.,

2014). Therefore, cast production is a behavioral biomarker of ecological relevance as early suggested by Capowiez et al. (2010), which has resulted very sensitive in soils contaminated by neonicotinoid insecticides (Pisa et al., 2015) or herbicides (Gaupp-Berghausen et al., 2015). In our study, the cast production and weight changes (an indirect indicator of feeding activity) were more sensitive markers to discriminate species-specific differences in pesticide toxicity compare with the burrowing activity.

5. Conclusions

Sublethal exposure to ethyl-parathion caused interspecific changes in the activity of target and detoxifying enzymes, as well as in the behavior of endogeic earthwoms. The species *A. caliginosa* was more impacted by the OP insecticide than *A. chlorotica* was. The dose-dependent inhibition of both AChE and CbE activities of *A. caliginosa* was reflected in a significant decrease of body weight (as an indicator of feeding activity) and cast production. Despite a lower number of CbEs isoenzymes and a lower total specific activity observed in *A. chlorotica* species, we confirmed that CbE activity could act as a detoxifying enzyme able to reduce the impact of ethyl-parathion on the target enzyme (AChE). This suggested that *A. chlorotica* CbE should be highly efficient in scavenging OP. Moreover, the *in vitro* outcomes revealed that the sensitivity of AChE to ethyl-paraoxon could be an additional mechanism in the species-specific differences observed in the selected toxicity endpoints. Our results suggest that these two species are highly recommended in the field monitoring of OP contamination, not only because they are among the most abundant species in the agricultural lands, but also because the high sensitivity of behavioral responses (e.g., cast production) that may have ecologically consequences for soil fertility and degradation.

Chapter 2 : In-vitro sensitivity of B-esterases and metabolic responses of two endogeic earthworms' species exposed to OP insecticides

1. Introduction

Organophosphates widely used to control harmful pests threat as well mammals and non-target organisms such as earthworms. They display a high acute toxicity by binding and inhibiting cholinesterase enzymes, in particular AChE (Pelosi et al. 2014). Besides, OP can be detoxified in the organisms through enzymatic hydrolysis. Carboxylesterases are known as potential bioscavenger of xenobiotics including OPs. This serine hydrolase is often more sensitive to inhibition by OP than AChE (Wheelock et al., 2008). Therefore, CbEs could detoxify the OPs before they reach targets in the nervous system. Previous study have shown that the *in vivo* and *in vitro* analysis of the AChE in two earthworm species, after exposure to an OP, showed a species-specific sensitivity (Jouni et al. 2018). Moreover, it is generally thought that exposure to pesticides initiates a metabolisation process. In eukaryotes, this usually occurs in two phases of which the second involves conjugation enzymes such as glutathione-*S*-transferase (GST), which attach a polar compound to the toxic molecules, promoting elimination by excretion (Sau et al. 2010).

The aim of this study was to characterize the location and properties of CbEs and GST. The *in vitro* sensitivity of CbEs to OP insecticides was investigated in both *A. caliginosa* and *A. chlorotica*. Moreover, *in vivo* response was also investigated using two different OP molecules in order to assess the potential role of these two metabolic pathways in the species-specific sensitivity in our model earthworms.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from Sigma–Aldrich (Saint Quentin-Fallavier, France): acetylthiocholine iodide (AcSCh), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV), 1-naphtyl acetate (1-NA), 1-naphtol, DTNB, Fast Garnet, 1-chloro-2,4-dinitro-benzene (CDNB), Reduced Glutathione (GSH), protease inhibitors, bovine serum albumin (BSA). The OP pure molecule ethyl-paraoxon **(O,O-Diethyl O-(4-nitrophenyl) phosphate ; PESTANAL®**) was purchased from Sigma-Aldrich and chlorpyrifos-oxon (O,O- diethyl O-3,5,6-trichloro-2-pyridyl phosphate) was

purchased from Cluzeau Info Lab (France). Commercial formulations of parathion (Oleobladan®; **93g.L^{-1}**) and chlorpyrifos-ethyl (Pyrinex®; **250g.L^{-1}**), two OP insecticides, were supplied by Bayer and Philagro respectively.

2.2. Soil and earthworms

Soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH 8.3) and healthy adults' specimens of *A. caliginosa* and *A. chloroctica* were collected from an apple orchard in Montfavet near Avignon, France. This orchard has received no pesticides treatment since 10 years. Worms were collected manually and were placed into plastic pots filled with fresh soil collected from the same orchard.

Soil was sieved at <2 mm and the water content was adjusted to 20-21% (approximately 81% of the maximum water holding capacity) with distilled water. Afterwards, wet soil samples (1 kg) were spiked with ethyl-parathion or chlorpyrifos solutions (40 ml) to yield the final concentrations of 1 mg and 1.3 mg active ingredient (a.i.) kg^{-1} wet soil respectively.

Both insecticide concentrations refer to the usual application rate and calculation of the Predicted Environmental Concentration (PEC). A control was prepared under the same conditions without pesticide application. The wet polluted or unpolluted soil was then split between containers (diameter 10 cm, height 3 cm) so that each contained 100 g soil. A single earthworm was placed in each Petri dish to limit interspecific interactions and to prevent cascade death (Sheppard and Evenden, 1992). Dishes were kept in a dark cold chamber (12±1°C) for the duration of the experiment. After 7 days of exposure, each worm was collected, weighed individually and frozen at -80°C until biochemical measurements (n=10 per treatment).

2.3. Homogenate preparation

Frozen worms were sliced in 1 cm length pieces and homogenized individually on ice with Ultra Turrax IKA T18 basic at 14000 rev min^{-1}, 4 times 10s with 1 min interval, in 20% (w/v) low-salt buffer containing 10mM Tris-HCl pH 7.3 10mM NaCl, supplemented with protease inhibitors (aprotinin, leupeptin and pepstatin, 5 µg mL^{-1},

antipain 1 µg mL^{-1}, and trypsin inhibitor 1 mg mL^{-1}. The homogenate was centrifuged (3000 × g, 10min, 4°C) and the supernatant obtained, called crude extract, was used for determining enzyme activities. In order to separate cytosolic or soluble fraction and microsomal fraction, the crude extract was centrifuged at 100,000g for 1h, at 4°C. All the different fractions (crude extract, cytosolic and microsomal ones) were kept at -80°C, supplemented with 10% glycerol as stabilizing agent, until biochemical analysis. Total protein content was measured by the method of Lowry modified according to Markwell et al. (1978), using bovine serum albumin as a standard.

2.4. Enzyme activity assays

Enzymes activities were monitored spectrophotometrically, at 25 °C using a microplate reader (Synergy HT, Bio-Tek®).

The activity of AChE was monitored at 412 nm and 25°C following the method by Ellman et al. (1961), and adapted by Rault et al. (2008), using a millimolar extinction coefficient (6,800 M^{-1} cm^{-1}) calculated according to a dithiotreitol-DTNB external calibration curve. The reaction medium (200 µL, final volume) contained 4 µL of homogenate, 0.375 mM DTNB and 3 mM of AcSCh in 0.1 M sodium phosphate buffer (pH 7).

Because the sensitivity of carboxylesterase activity to OP is highly dependent on the substrate, this activity was assayed using three different substrates, which differ in their carbon-ester chains: 1-naphtyl-acetate (1-NA), 4-nitrophenyl acetate (4-NPA) and 4-nitrophenyl valerate (4-NPV) to characterize multiple CbE isoenzymes following the microplate-scale assay by Thompson (1999). The hydrolysis of 1-NA was determined in a reaction medium (200 µL final volume) contained 25 mM Tris-HCl (pH 7.6), 1 mM $CaCl_2$ and 2 µL of diluted (1/20) homogenate. The reaction was initiated by adding 2 mM 1-Na (final conc.), and stopped after 10 min of incubation at 25ºC by the addition of 50 µL 2.5% SDS containing 0.1% Fast Garnet in 2.5% Triton X-100. The solutions were left to stand for 10 min at room temperature and dark, and the absorbance of the 1-naphthol-Fast Garnet complex was read at 530 nm. Enzyme activity was calculated from a standard curve (A_{530}=f([1-naphtol]) containing 1-naphthol in the same reaction mixture free of substrate. Hydrolysis of 4-NPV and 4-NPA was determined in a reaction mixture (250 µL, final volume including 2 µL of

diluted (1/5) enzyme extract) contained 1 mM 4-NPV and 50 mM Tris-HCl (pH 7.5), or 5 mM 4-NPA in 20 mM Tris-HCl (pH 8.0) and 1mM EDTA (to inhibit Ca^{2+} dependent phosphotriesterases). In both assays, the formation of 4-nitrophenol was monitored for 5 min at 405 nm, 25°C and quantified using a 4-nitrophenol standard curve.

GST activity was monitored at 340 nm and 25°C following the method described by Habig et al (1974) using a millimolar extinction coefficient (9,600 M^{-1} cm^{-1}). The reaction medium (200 µL, final volume) contained 2 µL of homogenate, 1 mM CDNB and 5 mM of GSH in 0.1 M potassium phosphate buffer (pH 7.4).

Blanks (reaction mixture free of sample) were checked for non-enzymatic hydrolysis of the substrates. All enzyme assays were run at least in triplicate and expressed as units per milligram of total protein (U mg^{-1} protein). One unit of enzyme activity was defined as one micromole of formed product per minute under the experimental conditions described above.

2.5. Enzyme characterization

Enzymes activities were characterized using the crude extract as enzyme source. The effect of substrate concentration was measured on the homogenate using varying concentrations of the corresponding substrate (0-10 mM) (except for CDNB substrate which was used from 0-1mM) final concentration. The general conditions for activity measurements were carried out as specified above.

2.6. *In vitro* B-esterase inhibition by pesticides

Sensitivity of both AChE and CbE activities to Chlorpyrifos-ethyl-oxon and ethyl-paraoxon (the pure OP molecules) in *A. caliginosa* and *A. chlorotica* were evaluated *in vitro*. The oxon forms of the OP were used for *in vitro* inhibition of B-esterases instead of their commercial formulation because *in vivo* OP pesticides are only activated after being metabolized.

Tissue homogenates from unexposed earthworms (20 µl) were incubated individually with serial concentrations of ethyl-paraoxon or chlorpyrifos-ethyl-oxon (10^{-12} to 10^{-3} M final concentration) for 30 min at 25ºC. Inhibition assay was terminated by addition of

the corresponding substrate, and the residual esterase activity was measured as described above. Controls (no pesticides) were run in the same conditions for the measurements of initial activity. Because (1) the target of OP pesticides is the AChE activity, (2) the paraoxon was a well known toxic compound, and (3) *A. caliginosa* has been pointed out as a sensitive earthworm species, we used the IC_{50} value obtained for *A. caliginosa*'s AChE in the presence of ethyl-paraoxon, as a reference. An esterase Susceptibility Ratio (SRe) was then calculated by dividing this IC_{50} reference-value by the IC_{50} value obtained from the other esterase activities either in the presence of ethyl-paraoxon or chlorpyrifos-ethyl-oxon, for both earthworm species. Beside, in order to compare the sensitivity of one given esterase activity between *A. caliginosa* and *A. chlorotica*, a species Susceptibility Ratio (SRs) was calculated by dividing the IC_{50} obtained from *A. caliginosa* by the IC_{50} obtained from *A. chlorotica*, for a considered activity and a given pesticide. The increase or decrease in sensitivity is indicated by SR values greater or less than one, respectively.

2.7. Data analysis

To estimate the apparent K_M and V_{max}, specific activity was plotted against substrate concentration and data were fitted to the Michaelis-Menten equation:

$$V_0 = (V_{max} \cdot [S]) / (K_M + [S]) \qquad \text{(eq. 1)}$$

where V_0 is the initial velocity and [S] is the substrate concentration.

The concentration of inhibitor inhibiting 50% of enzyme activity (IC_{50}) under our specific experimental conditions (pH, temperature, time, protein concentration) was calculated using the logistic curve from the library of non-linear regressions of the XLSTAT software:

$$y = E_0 \cdot e^{(-b \times [I])} + E_{res} \quad \text{(eq. 2)}$$

or

$$y = E_0 \cdot e^{(-b \times [I])} + E'_0 \cdot e^{(-b' \times [I])} + Eres \qquad \text{(eq. 3)}$$

where E_0 is the total activity of the control, E_{res} the percentage of residual enzyme activity relative to controls, $b = k_i \times t$ (k_i inhibition kinetic constant, t the time in min) and

IC_{50}=ln(2)/b. E'_0 and b' represent the intermediate parameters when different forms of enzymes sensitivity were applicable.

Comparisons of concentrations, differences between species, and differences between cytosolic and microsomal fractions were analyzed with one-way ANOVA followed by Tukey HSD post-hoc comparison test using XLSTAT software (version 2013.3.01).

3. Results

3.1. Cellular localization of enzymes

Taking into account the whole protein contents, we observed that a higher part is located in the cytosolic fraction for both *A. caliginosa* (77.4 3 ± 0.1 %) and *A. chlorotica* (71.6 ± 1.4 %) and only about 22% and 28% were located in the microsomal fraction respectively. For each earthworm species, AChE activity was mainly located in the microsomal fraction with a higher amount of membrane-bound enzyme for *A. caliginosa* (79.3 ± 4.7 %) compare to *A. chlorotica* (65.4 ± 4.4 %). In contrast, CbE activities were mainly found in the cytosolic fractions. For both species, this repartition follows the general distribution of the whole protein contents with more than 75% of soluble CbE for *A. caliginosa* and less than 70% of soluble CbE activities for *A. chlorotica* (Fig. 20). Among the different substrates, the CbE activity related to 4-NPA substrate was found to be almost equally distributed between both fractions with 59.5% of membrane-bound versus 40.5% as a soluble enzyme. The two other substrates 4-NPV and 1-Na exhibited 67.9% and 70.9% of membrane-bound form respectively. Concerning the GST activity, only one major soluble enzymatic form was detected in the cytosolic fraction with more than 97% of the total activity for both *A. caliginosa* and *A. chlorotica* (Fig.20).

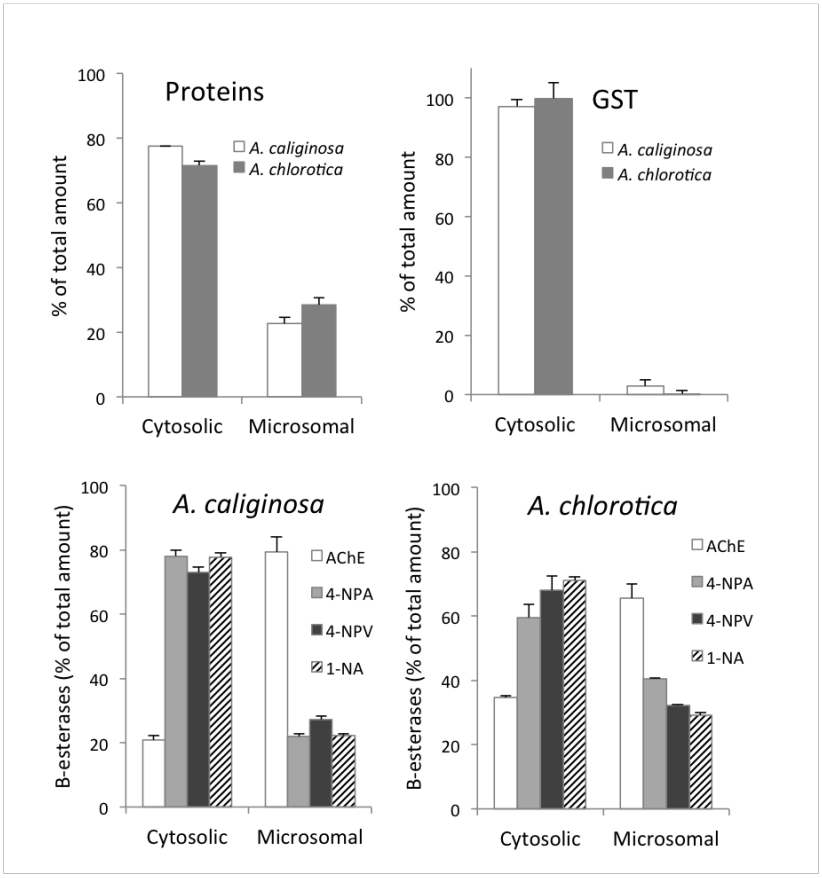


Figure 20: Cellular distribution of proteins, GST and B-esterases activities in A. caliginosa and A. chlorotica species. Protein and enzyme contents were measured in the cytosolic and microsomal fractions of the crude extract.

3.2. Characterization of enzymes using specific substrates

Acetylcholinesterase activity displayed a Michaelis–Menten kinetics within the selected range of substrate concentrations for both earthworm species (Fig. 21). The apparent Km and Vmax were 1.22 mM and 171.8 mU mg^{-1} protein, respectively, when using *A. caliginosa* species, whereas Km and Vmax were 2.90 mM and 131.6 mU mg protein, respectively, for AChE activity using *A. chlorotica*. No AChE inhibition for high substrate concentrations (up to 40 mM) was observed for *A. chlorotica*, while a decrease of 30% in AChE activity was obtained for *A. caliginosa* species (data not shown) as previously described by Rault et al. (2007). CbE activity showed a Michaelis-Menten kinetic regardless of substrate (Fig. 21). The substrates 4-NPA and 1-NA were more efficiently hydrolyzed by esterases using *A. caliginosa* extracts compared to *A. chlorotica* (V_{max} *A. caliginosa* higher than V_{max} *A. chlorotica*).

Concerning the affinity towards the different substrates, the K_M showed a close relationship with both species and for these three CbE substrates we observed a higher affinity than for AcSCh substrate (K_M for 4-NPA, 4-NPV and 1-NA were lower than K_M for AcSCh). Thus, the best catalytic efficiency estimated as the V_{max}/K_M ratio was obtained for CbE activity with 1-NA as substrate (Table 4). For both species, AChE exhibited a weak catalytic efficiency compare to CbE activity with a ten times and twenty times lower values for *A. caliginosa* and *A. chlorotica* respectively, compare to the catalytic efficiency obtained with 4-NPA and 4-NPV substrates. All of the kinetic parameters of AChE and CbE with the three substrates tested are shown in Table 1.

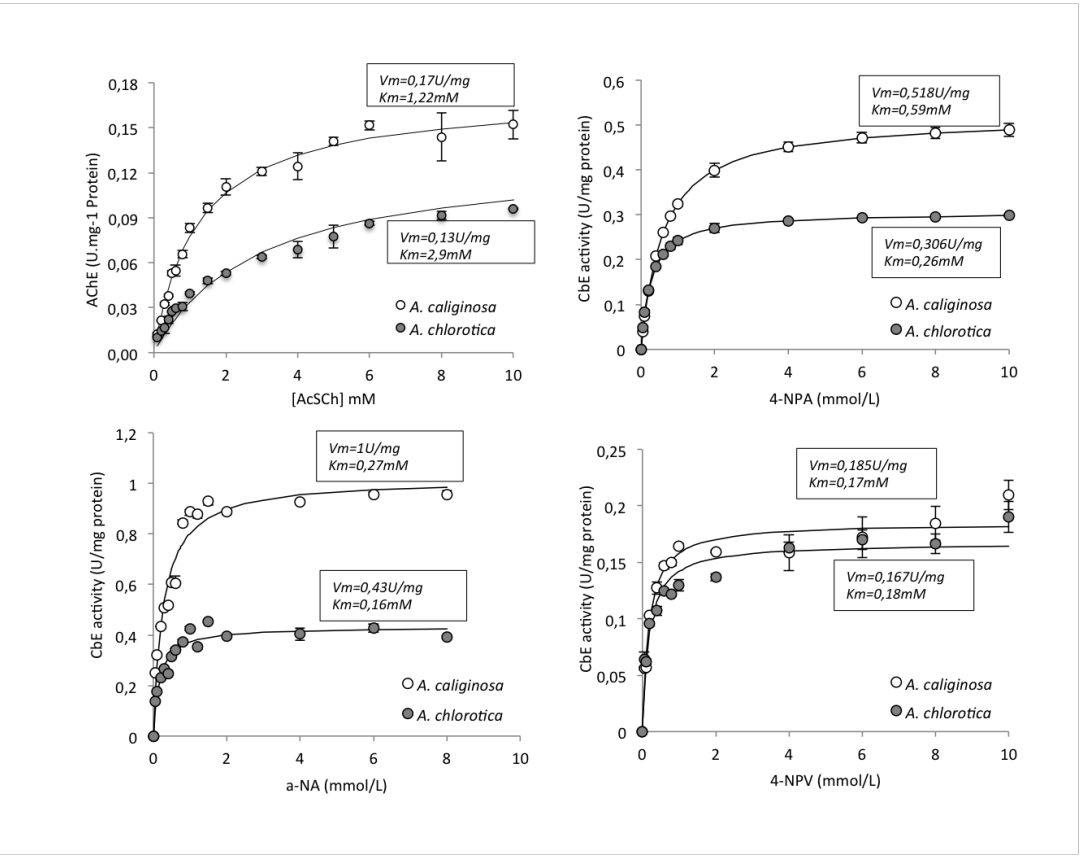


Figure 21: Determination of B-esterase kinetic parameters in both A. caliginosa and A. chlorotica species. Kinetic parameters (apparent V_m and KM) were estimated by fitting the data to the Michaelis-Menten equation. Each point corresponds to the mean of three independent assays (± standard deviation). Kinetic parameters are shown in Table 4.

Glutathione-S-Transferase activity displayed as well, a Michaelis–Menten kinetics within the selected range of substrate concentrations for both substrates and both earthworm species (Fig. 22). No significant differences were observed between species for either the affinity towards substrates or the efficiency of hydrolysis. Then,

the catalytic specificities of the GST enzyme were in the same range for both earthworm species (Table 4).

Table 4. Kinetic parameters for AChE, CbE and GST activities measured in A. caliginosa and A. chlorotica homogenate (mean ± SD).

Enzyme	Earthworm species	V_m (U.mg^{1-})	K_M (mM)	V_m/K_M (mL.min^{-1}.mg-1)
AChE	*A. caliginosa*	0.172 ± 0.003	1.216 ± 0.077	0.141± 0.003
	A. chlorotica	0.132 ± 0.004	2.904 ± 0.307	0.045 ± 0.001
1-NA	*A. caliginosa*	1.019 ± 0.035	0.270 ± 0.039	3.776 ± 0.131
	A. chlorotica	0.433 ± 0.016	0.164 ± 0.030	2.636 ± 0.095
4-NPA	*A. caliginosa*	0.518 ± 0.011	0.597 ± 0.051	0.869 ± 0.019
	A. chlorotica	0.306 ± 0.008	0.265 ± 0.035	1.153 ± 0.031
4-NPV	*A. caliginosa*	0.185 ± 0.006	0.172 ± 0.030	1.076 ± 0.035
	A. chlorotica	0.167 ± 0.007	0.178 ± 0.040	0.939 ± 0.039
GST-GSH	*A. caliginosa*	0.115 ± 0.000	0.514 ± 0.000	0.224 ± 0.000
	A. chlorotica	0.099 ± 0.000	0.468 ± 0.000	0.211 ± 0.000
GST-CDNB	*A. caliginosa*	0.314 ± 0.000	1 ± 0	0.314 ± 0.000
	A. chlorotica	0.278 ± 0.000	1 ± 0	0.278 ± 0.000

[a] Kinetic parameters were obtained by fitting the experimental data points to the Michaelis-Menten equation using XLSTAT software.

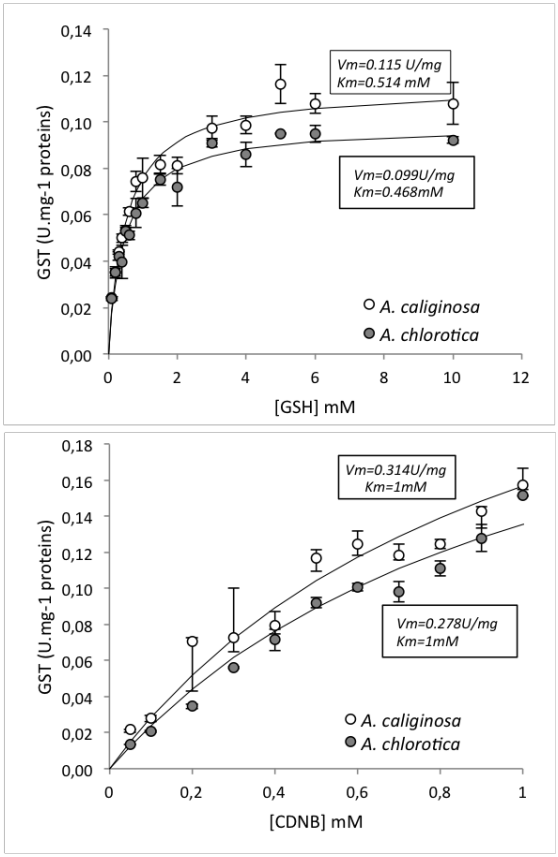


Figure 22: Determination of GST kinetic parameters in both A. caliginosa and A. chlorotica species. Kinetic parameters (apparent V_m and K_M) were estimated by fitting the data to the Michaelis-Menten equation. Each point corresponds to the mean of three independent assays (± standard deviation). Kinetic parameters are shown in Table 4.

3.3. *In vitro* inhibition of B-esterases

Inhibition of AChE and CbE activities of both earthworm species by OP pesticides followed a sigmoidal concentration response model (Fig. 23). All pesticides caused a strong inhibition of both enzyme activities with IC50s varying from 10^{-5} to 10^{-8} M (Table 5). We observed that the inhibition strongly depends on species and OP structure. *A. caliginosa* AChE was strongly inhibited by ethyl-paraoxon and appeared to be the highest sensitive B-esterase among the esterases of both earthworm species. *A. chlorotica* 4-NPA and 4-NPV CbEs were the only B-esterases that appeared more sensitive to ethyl-paraoxon than *A. caliginosa* AChE (5.6 and 1.5 times more sensitive respectively). In the presence of ethyl-paraoxon *A. chlorotica* 4-

NPA and 4-NPV were also 7 times and 2 times more sensitive respectively, than the corresponding CbEs in *A. caliginosa*. Moreover it is noteworthy that for *A. chlorotica* species, CbE activities using nitrophenyl substrates appeared highly sensitive compare to AChE with a susceptibility ratio 35 times higher for 4-NPA (5.665 vs 0.162) and 10 times higher for 4-NPV (1.585 vs 0.162) (Table 5).

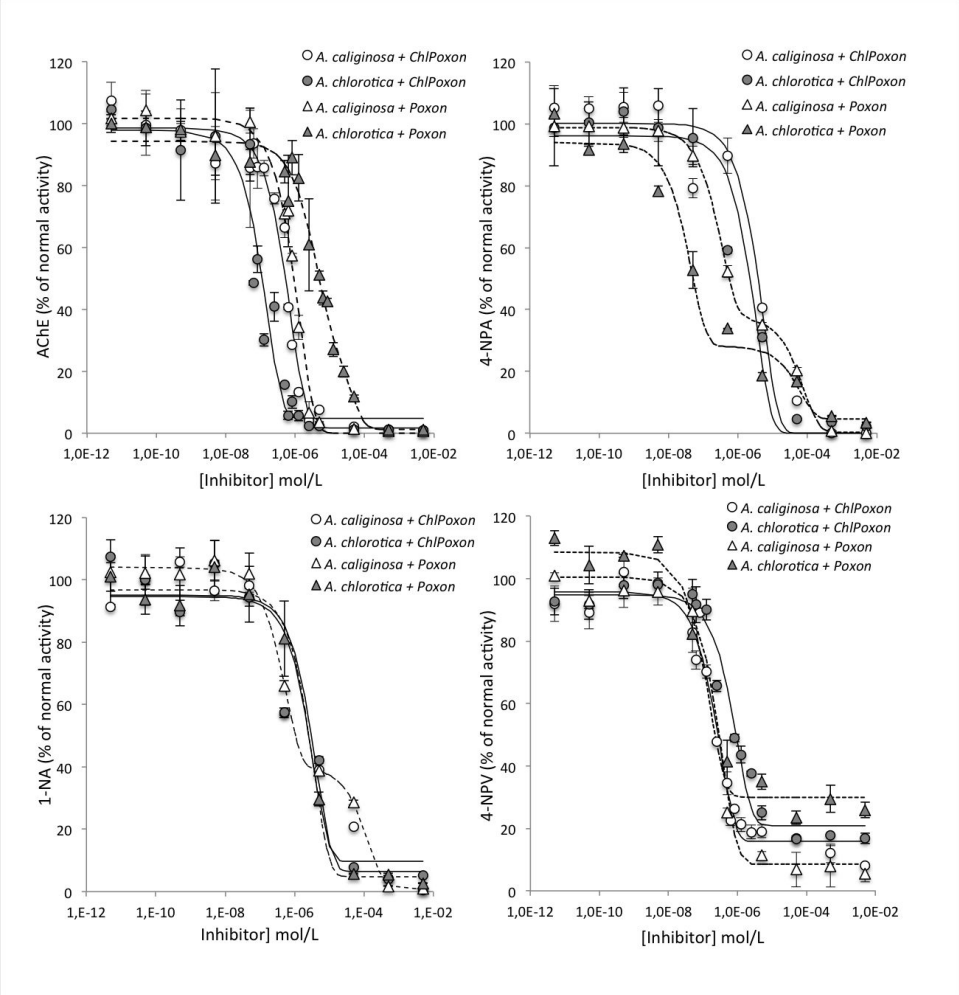


Figure 23: Effect of the organophosphate insecticides ethyl-paraoxon and chlorpyrifos-oxon on A. caliginosa and A.chlorotica B-esterases. Each point corresponds to the mean of three independent assays (± standard deviation). IC50 values are shown in Table 5.

Considering the inhibition induced by the presence of chlorpyrifos-ethyl-oxon, *A. chlorotica* AChE (IC50 = 1.06 x 10^{-7} M) appeared more sensitive than *A. caliginosa* AChE (IC50 = 5.27 x 10^{-7} M) with a species susceptibility ratio (SRs) 5 times higher. Except for *A. chlorotica* AChE, the inhibition induced by chlorpyrifos-ethyl-oxon on the others B-esterases was weak compare to those induced by ethyl-paraoxon.

Table 5: Molar concentrations of pesticides to yield in vitro 50 % of enzyme inhibition (IC50) of acetylcholinesterase (AChE) and carboxylesterase (CbE) activities of A. caliginosa and A. chlorotica and correlation coefficient (R2) for nonlinear regressions.

Esterase	Pesticides	Eartworm species	R^2	IC50 (M)	SRe	SRs
AChE	Paraoxon	*A. caliginosa*	*0.99*	$(1.81 \pm 0.00)\ 10^{-7}$	-	-
		A. chlorotica	*0.98*	$(1.12 \pm 0.00)\ 10^{-6}$	0.162	0.162
	Chlorpyrifos-oxon	*A. caliginosa*	*0.978*	$(5.27 \pm 0.60)\ 10^{-7}$	0.343	-
		A. chlorotica	*0.943*	$(1.24 \pm 0.21)\ 10^{-7}$	1.696	4.942
1-NA	Paraoxon	*A. caliginosa*	*0.998*	**$(4.01 \pm 0.00)\ 10^{-7}$**	**0.451**	-
				$(9.98 \pm 0.00)\ 10^{-5}$	0.002	-
		A. chlorotica	*0.993*	$(2.54 \pm 0.36)\ 10^{-6}$	0.071	**0.158** 39
	Chlorpyrifos-oxon	*A. caliginosa*	*0.926*	$(2.40 \pm 0.11)\ 10^{-6}$	0.075	-
		A. chlorotica	*0.932*	$(2.99 \pm 0.13)\ 10^{-6}$	0.060	0.800
4-NPA	Paraoxon	*A. caliginosa*	*1*	**$(2.42 \pm 0.00)\ 10^{-7}$**	**0.748**	-
				$(5.56 \pm 0.00)\ 10^{-5}$	0.003	-
		A. chlorotica	*0.98*	**$(3.19 \pm 0.00)\ 10^{-8}$**	**5.665**	**7.574**
				$(4.39 \pm 0.00)\ 10^{-5}$	0.004	1.265
	Chlorpyrifos-oxon	*A. caliginosa*	*0.969*	$(3.75 \pm 0.91)\ 10^{-6}$	0.048	-
		A. chlorotica	*0.96*	$(2.35\ 0.68)\ 10^{-6}$	0.077	1.591
4-NPV	Paraoxon	*A. caliginosa*	*0.981*	$(2.54 \pm 0.59)\ 10^{-7}$	0.713	-
		A. chlorotica	*0.983*	$(1.15 \pm 0.35)\ 10^{-7}$	1.585	2.223
	Chlorpyrifos-oxon	*A. caliginosa*	*0.976*	$(2.21 \pm 0.29)\ 10^{-7}$	0.817	-
		A. chlorotica	*0.973*	$(6.79 \pm 1.14)\ 10^{-7}$	0.266	0.326

[a] SRe corresponds to an esterase susceptibility ratio calculated by dividing IC50 value obtained for the AChE from *A. caliginosa* inhibited by ethyl-paraoxon (used as a reference) to the IC50 value of other esterase (from *A. caliginosa* or *A. chlorotica*) inhibited either by ethyl-paraoxon or chlorpyrifos-ethyl-oxon (SRe= $IC50_{(AChE\text{ -}A.caliginosa)}$/ $IC50_{(esterase)}$).

[b] SRs corresponds to a species susceptibility ratio calculated by dividing $IC50_{(A.\ caliginosa)}$/ $IC50_{(A.\ chlorotica)}$ for a given esterase.

A Susceptibility Ration >1 denotes an increase in sensitivity compare to the reference.

3.4. *In vivo* response of B-esterases and GST after OP exposure

Exposure of earthworms, either to ethyl-parathion or chlorpyrifos-ethyl using 1 mg and 1.3 mg active ingredient (a.i.) kg^{-1} wet soil respectively, did not induce significantly GST activity (Fig. 24). The response was similar for both species, and a slight but not significant increase in GST activity was observed with a stronger impact for *A. caliginosa* species. Concerning B-esterase activities, we have shown in previous study (Jouni *et al.* 2018), that a dose of 1mg.kg-1 of ethyl-parathion-contaminated soils, caused a significant response of these enzymes that was species-specific (Fig. 24). Whereas the mean of AChE activity decreased in *A. caliginosa* (78%; $p<0.0001$), this esterase activity remained unchanged in *A. chlorotica* exposed to ethyl-parathion. 4-NPA and 4-NPV activities were inhibited in both earthworms' species with 49% and 50% loss for 4-NPA activities for *A. caliginosa* and *A. chlorotica* respectively, 68% and 54% loss for 4-NPV activities for *A. caliginosa* and *A. chlorotica* respectively ($p<0.0001$) after exposure to ethyl-parathion. Chlorpyrifos-ethyl appeared less toxic to earthworms than ethyl-parathion. Except for the CbE activity measured with 1-NA (19% loss, $p<0.001$), no significant inhibition could be observed after exposure to this OP in *A. caliginosa* species. Concerning *A. chlorotica* species, we observed a 30% loss of AChE activity ($p<0.001$). CbE activities of *A. chlorotica* were not significantly inhibited after chlorpyrifos-ethyl exposure.

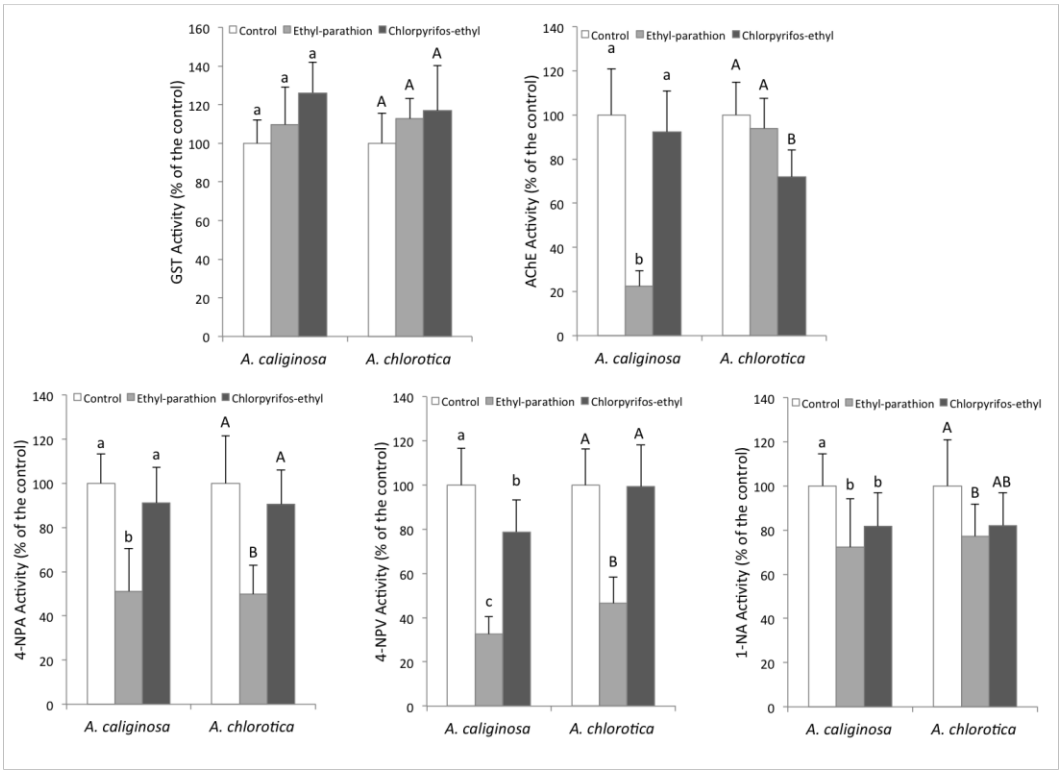


Figure 24: Response of GST and B-esterases (AChE and CbE) activities in earthworms exposed to ethyl-parathion or chlorpyrifos-ethyl contaminated soils for 7 days. The esterase activity was measured using the substrates 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV), and 1-naphtyl acetate (1-NA). Different letters indicates significant differences between treatments.

4. Discussion

4.1. Characterization of B-esterases and GST activities

The use of B-esterase activities as biochemical biomarkers after exposure to OP insecticides has been extensively studied in different earthworm species (Booth and O'Hollaran 2001; Collange et al. 2010; Denoyelle et al. 2007; Hackenberger et al. 2008; Jordaan et al. 2012; Rault et al. 2007; Sanchez-Hernandez and Wheelock 2009; Schreck et al. 2008). Studies conducted in laboratory conditions led to the conclusion that *A. chlorotica* species was generally less sensitive than *A. caliginosa* (Rault et al 2008; Jouni et al 2018). Our results indicated that the catalytic efficiency of AChE is higher in *A. caliginosa* than *A. chlorotica*. It has been reported that AChEs

with reduced catalytic efficiency could be involved in xenobiotic defense (Kang et al., 2011b; Kim et al., 2012; Kim et al., 2014) as a bioscavenger, which is analogous to the function of butyrylcholinesterase (BuChE) in vertebrates (Lenz et al 2005). Soluble AChE had been shown, as well, to be involved in stress regulation in the honey bee *Apis mellifera* (Kim et al 2017). In the fruit fly having a single ace locus, the soluble form of AChE, was generated by alternative splicing and plays non-neuronal roles, such as chemical defense (Kim et al., 2014). Using earthworm species, previous studies reported that *A. chlorotica* exhibited multiple cholinesterase forms with an equal amount of AChE and BuChE activities (Rault et al. 2007). Native PAGE exhibited a higher part of soluble AChE in *A. chlorotica* than *A. caliginosa* (Jouni et al 2018) that was confirmed herein where 35% of the *A. chlorotica* activity appeared in the cytosolic fraction. These different AChE forms could account for a better capacity of *A. chlorotica* to cope with OP insecticide. Jouni et al (2018) clearly revealed that the activity of *A. caliginosa* AChE was more sensitive to ethyl-paraoxon than that of *A. chlorotica*. Comparing the AChE K_M values for both earthworm species, *A. chlorotica* exhibit a weak affinity for its substrate suggesting a poor accessibility to its active site. Taking into account, these different results strongly suggest that the intrinsic sensitivity of *A. chlorotica* AChE could be a significant cause for the lower sensitivity of this species towards OP insecticides. For *A. chlorotica*, the two AChE forms possessing different physiological functions could act as bioscavenger that can sequester OP insecticides, as previously described in some invertebrates (Kim and Lee 2018).

Despite the fact that a biochemical analysis of cholinesterase forms was investigated in different earthworm species (Rault et al 2007), no enzyme characterization was conducted on carboxylesterase activities in *A. caliginosa* or *A. chlorotica* species. CbEs could play an important role as bioscavengers because of their higher sensitivity to OP compare to AChE (Küster 2005; Wheelock et al 2008; Kristoff et al 2010). In the present study, for both *A. caliginosa* and *A. chlorotica,* AChE catalytic efficiency are about ten times below the catalytic efficiency obtained for the CbE activities, depending on the substrate employed (Table 4). The high V_m/K_M ratio obtained for CbEs support their important role in pesticide metabolism. Both earthworm species exhibited the same order of catalytic efficiency for CbEs, following the order 1-NA > 4-NPA or 4-NPV, and they were in the same order of magnitude as

V_m/K_M ratio obtained for the snail *Xeropicta derbentina* CbE activity for 1-NA (4.93 $ml.min^{-1}.mg^{-1}$) > 4-NPV (2.33 $ml.min^{-1}.mg^{-1}$) >4-NPA (0.19 $ml.min^{-1}.mg^{-1}$), (Laguerre et al 2008) or those found in the gill (0.18 $ml.min^{-1}.mg^{-1}$) and serum (7.66 $ml.min^{-1}.mg^{-1}$) CbE of the rainbow trout *Oncorhynchus mykiss* (Barron et al., 1999). The main location of CbEs in the cytosolic fraction in addition with multiple isoenzymes shown by Jouni et al (2018) occurred also in other species such as the earwig *F. auricularia* (Malagnoux et al 2014) or the crayfish *Procambarus clarkii* (Vioque-Fernández et al., 2007). However, in *A. chlorotica* the cellular distribution of B-esterases appeared more homogenous between cytosolic and microsomal fractions, with a decrease in the cytosolic part to the benefit of the membrane bound counterpart compare to *A. caliginosa*. Such cellular distribution probably enhances the role of CbE activities in the metabolic defense of the organism. Then, beside the fact that CbEs activities measured in *A. chlorotica* did not appear high enough to support an efficient mechanism against OP toxicity on their own, tissue-specific and cellular expression of CbE could play an important role depending on their specificity, to cope with exposure to OP.

GST activities were almost exclusively found in the cytosolic fraction of *A. chlorotica* and *A. caliginosa*, and kinetic parameters are very close related for both species. Affinity toward CDNB (1mM) or GSH (about 0.5 mM) were similar to K_M values described in zebrafish (Glisic et al 2015), and basal activities (115 $mU.mg^{-1}$) are in the same order than those previously measured in *A. caliginosa* and *A. chlorotica* (about 120 to 150 $mU.mg^{-1}$) (Givaudan et al. 2014) or in the wall muscle of *L. terrestris* (323 $mU.mg^{-1}$) (Sanchez-Hernandez and Wheelock 2009).

4.2. Sensitivity of B-esterase to OP pesticides

From the SRe and SRs ratios (Table 5) we found that CbEs from *A. chlorotica* appeared to be more sensitive to *in-vitro* inhibition by ethyl-paraoxon than *A. caliginosa* (SRs 2- to 7-times higher with CbE 4-NPV or CbE 4-NPA respectively). However, these differences between IC_{50} for CbEs inhibition are very weak compare to those found in the earwig *F. auricularia* (45 to 400-times higher depending on substrate) (Malagnoux et al., 2014). It is generally assumed that CbEs have the potential to sequester available OP pesticides, hence protecting AChE from inhibition

(Barata et al., 2004). Based on this hypothesis, the stronger inhibitory effect of ethyl-paraoxon on the CbE from *A. chlorotica* species would enhance its buffering capacity and could provide an efficient scavenger protecting effect of AChE from an irreversible inhibition. This assumption was confirmed by our results. Then, the irreversible inhibition of CbE activity by OP can be considered as an efficient detoxification pathway, because OP binds stoichiometrically to CbE, thereby reducing its impact on nervous system especially on AChE activity, the main target for acute OP toxicity (Maxwell and Brecht, 2001; Sogorb and Vilanova, 2002).

On the other hand, chlorpyrifos-ethyl-oxon had approximately the same *in-vitro* effect on CbE for both species, but induced a higher inhibition of *A. chlorotica* AChE activity. However, the response after exposure to chlorpyrifos (30% loss of AChE activity for *A. chlorotica*) was under the expected inhibition taking into account the high sensitivity of this enzyme measured after *in vitro* inhibition to chlorpyrifos-ethyl-oxon. As shown previously using native PAGE (Jouni et al., 2018), the abundance of multiple CbE isozymes was higher in *A. caliginosa* compared to *A. chlorotica*. Considering the weak differences observed between CbE sensitivity to chlorpyrifos-ethyl-oxon, it could be assumed that the capacity of CbE to protect AChE against chlorpyrifos-ethyl-oxon is not as effective as its protective effect against ethyl-paraoxon.

4.3. *In vivo* responses

GST activities have been shown to give positive responses to environmental disturbances for *E. fetida* and *Lumbricus rubellus* in previous studies (Brown et al., 2004; Ribeira et al., 2001). Schreck et al (2008) showed that a short-term exposure (3 days) of *A. caliginosa* to the insecticides chlorpyrifos increased GST activity, while long-term exposure induced a decrease of GST activity. This indicated that GST could participate in the detoxification of selected insecticides in a time-dependent manner. Velki and Hackenberger (2013) showed that the duration of exposure changed significantly the effects of pirimiphos-methyl (OP) and deltamethrin (pyrethroid) on *Eisenia Andrei* GST activity, with an increase on the first 3 days of exposure. In our study, 7 days exposure to OP insecticides did not induced GST activities. These results are consistent with previous observations made on *A.*

caliginosa and *A. chlorotica* species after exposure to either a fungicide, an herbicide or their mixture (Givaudan et al. 2014). These authors suggested that a pre-exposure to the pesticides in the fields was required for activation of the GST detoxication system.

5. Conclusion

The higher tolerance of *A. chlorotica* compare to *A. caliginoa* did not seem to depend on the catalytic properties of either *A. chlorotica* CbE or GST activities. Even if CbEs are well known to play an important role as bioscanvengers of OP, and despite the fact that the sensitivity of these enzymes *in-vitro* was higher than that for *A. caliginosa*, other toxicodynamic factors could contribute to the inter-specific toxicity of OP insecticides in those earthworm species. Different biological processes could induce a modification in the toxicity of a chemical compound while inside the organisms compare to *in-vitro* responses. It is particularly the case of metabolic pathways or defense mechanisms that could act very quickly after the exposure and modify the toxicodynamic of the insecticide. Moreover, beside defense mechanisms, the homogeneous distribution of AChE forms could be responsible for a complementary mechanism in the species-specific differences observed towards pesticides.

Chapter 3 : Interaction earthworms-microbiome: a taxonomic study of gut-symbiont in two endogeic species exposed to ethyl-parathion.

1. Introduction

The role of earthworms in ecosystems has received much attention since they are a dominant component of the soil fauna and considered key bioindicator of soil quality. They have determining effects on the overall soil structure, including organic matter turnover that make nutrients available for the earthworms and other living organisms. While burrowing and feeding, these ecosystem engineers push, ingest and change the soil structure, which produce galleries and casts (Lavelle 1988; Edwards & Bohlen 1996; Jégou et al. 2000).

Earthworms are in permanent interaction with soil particles and microbes through their cuticle and their digestive tract. Their activities thus affect the abundance, composition and dynamic of microbial communities as well as soil enzymatic activities (Drake & Horn 2007; Dempsey et al. 2011; Aira et al. 2015; Roubalovà et al. 2015). Earthworms build biogenic structures named the drilosphere and gather much investigation on earthworms-microorganisms interactions. The earthworms' gut has been characterized as a particular contrasted environment, consisting of an anaerobic reactor that flourishes in aerobic conditions. The earthworm's gut may act as a biological filter, detrimental to some microbes digested with the soil organic matter, while favorable to others due to suitable condition of the intestinal tract (i.e. mucus secretion that contain readily available organic compounds, neutral pH and high water content (Trigo & Lavelle 1995; Brown et al. 2000; Byzov et al. 2007). In this mutualistic relationship, microbial activities enhance the breakdown of organic material and cellulose, which improves nutrients assimilation by earthworms. Although, other microorganisms can be negatively influenced by the gut transit, presumably because they are of minor importance for earthworm's food, energy and activity, so their number decline and can also be eliminated (Edwards & Fletcher 1988; Wüst et al. 2011). Afterwards, refined soil with its new microbial composition will be ejected as casts (Mclean et al. 2006).

Besides changes in microbial composition of ingested soil, studies (Singleton et al. 2003) suggest the possible existence of a particular microbial group inhabiting the earthworms' gut. Previous studies that aimed to characterize the earthworms' gut microbiota have based their analysis on classical cultivation methods. However, emerging approaches based on environmental genomics and high throughput

sequencing methods provide the opportunity to identify both cultivatable and non-cultivatable microorganisms associated to earthworms' gut. Accordingly, it was reported that the ecological group is of primary importance in the selection of gut wall microbiome, followed by habitat and then species (Thakuria et al. 2010; Aira et al. 2015).

Organophosphate insecticides are commonly used worldwide, they cause damage to the enzyme acetylcholinesterase, which is critical for nervous signals transmission (Lionetto et al. 2013). Organophosphates are toxic to pests but also unintentionally threaten non-target organisms including human and earthworms. Toxicity assessments of such pesticide have been conducted on few earthworms' species that focused on behaviour, mortality, reproduction, enzymatic and cellular aspects (Dureja et al. 1999; Yasmin & D'Souza 2010; Dutta & Dutta 2016; Jouni et al. 2018). Although the gut microbiota contributes to the host physiology and health (Cabreiro & Gems 2013; Devkota & Chang 2013; Xie et al. 2016) and have abilities to degrade xenobiotic (Ma et al. 2017), very little is known on the relationship between earthworm gut microbiota and pesticides.

Here, we developed an experiment to investigate the effects of ethyl-parathion on the gut microbiota of two earthworm species and their relationships to ingested soil and ejected casts. To identify the structure and composition of both bacterial and fungal communities present in the three compartments, we used amplicon-sequencing approach.

2. Materials and methods

2.1. Soil and earthworms sampling

Two different experimental soils were sampled from two different orchards called Soil-K and Soil-G, situated in a 10km distance, in Montfavet near Avignon (France). Both orchards have received no pesticide treatments for the last 10 years. The first orchard (Soil-K) is a silt loamy soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH 8.3), dominated by the species *A.chlorotica.* The second orchard (Soil-G) is a silt-clay soil (38.3 % clay, 42.2% fine silt, 19.5% sand, 34 g kg^{-1} organic

matter, pH 8.5), dominated by the species *A.caliginosa.* Healthy earthworms from both species were collected manually from their original orchards, washed in tap water, blotted dried on filter paper and kept in the laboratory into plastic pots filled with fresh soil collected. Both earthworm species spent 5 days for cross-acclimation either in their own original soil or in the other soil where it was rare to find them. *A.chlorotica* individuals, highly abundant in the soil-K, were divided on two groups: one group was placed into plastic pots filled with fresh soil-K, the other group was placed into plastic pots filled with fresh soil-G. The same step of acclimation was done to *A.caliginosa,* which were separated in two groups either in their own soil-G or in soil-K. Afterwards, pots were kept in a dark cold chamber (12±1°C) for 5 days of acclimation.

Soils were sieved at <2 mm, and the water content was adjusted to 20-21% (approximately 81% of the maximum water holding capacity) with distilled water. Both wet soil samples (1 kg) were spiked with ethyl-parathion solutions (40 ml) to yield the final concentration of 1 mg active ingredient (a.i.) kg^{-1} wet soil. This ethyl-parathion concentration refers to the usual application rate and calculation of the Predicted Environmental Concentration (PEC). Control soils were prepared under the same conditions with a free-pesticide solution. The wet polluted or unpolluted soils were then split between containers (diameter 10 cm, height 3 cm) so that each contained 100 g soil.

2.2. Experimental setup and sample collection

After the acclimation period, earthworms were washed in tap water, blotted dried on filter paper and weighed. For each treatment, (2 different soils, control and 1 mg ethyl-parathion per soil), 32 earthworms of a same species were placed by 2 individuals in each Petri dish to allow enough amounts of casts. Dishes were kept in a dark cold chamber (12±1°C) for the duration of the experiment. Soils free of earthworms were used as controls (day 0). After 4 and 7 days of incubation, 5g of soil and casts sub-samples were taken from each replicate and treatment, and stored in plastic tubes at -80°C, until biochemical analysis. Then, earthworms from each container were placed in a moist filter paper for 5 days to collect their evacuated gut contents (fresh cast (day 12)).

Earthworms were dissected after 5 days of gut voiding. First, they have been washed in distilled water and then scarified by brief immersion in water at 50℃ (Singleton et al. 2003). Gut of each individual was removed and the guts of each biological replicats were pooled. All samples (soil, casts and guts) were stored at -80℃ until DNA extraction.

2.3. DNA Metabarcoding analysis :

2.3.1. DNA extraction

Microbial DNA of all samples: soil, casts and fresh casts, washes and the washed intestine (gut wall) was extracted using PowerSoil-htp 96 wells DNA Isolation Kit (MOBIO) according to the manufacturer's protocol. At the first step of DNA extraction, intestines were washed three time with the DNA extraction buffer through vortexing (1 minute) and short centrifugation (1 minute) steps. Washing suspensions were gathered and processed separately from the remaining gut material. The concentration and quality of the nucleic acids were measured using Nanodrop. DNA extracted samples were stored at -20℃ until PCR amplification and sequencing.

2.3.2. Quantitative PCR procedure

Quantitative real-time PCR (qPCR) was performed using a LightCycler 480 system (Roche Applied Science) to determine copy numbers of the bacterial 16S rDNA gene (Fierer et al 2005), using primer Eub338 (5'-ACTCCTACGGGAGGCAGCAG-3') and primer Eub518 (5'-ATTACCGCGGCTGCTGG-3') and copy numbers of the universal fungal ITS1 marker, using primer ITS1f (5'-TCCGTAGGTGAACCTGCGG-3') and primer ITS1r (5'-CGCTGCGTTCTTCATCG-3') (Fierer et al., 2005). Standard solutions of target genes were generated so to represent the naturally occurring mixture of each target gene across a random selection of 10 DNA samples obtained from leave mixtures (Hasset et al. 2009). Following gradient PCR tests in a Mastercycler Nexus Thermal Cycler (Eppendorf), annealing temperatures were determined at 55°C for each primer pair. PCR mixtures (20 µl) contained 200 µM dNTPs, 5 % (v:v) DMSO, 1 µM of each primer and 0.25 units FlexyTaq DNA-polymerase with the corresponding 5X PCR buffer (Promega). Reaction condition had an initial denaturation step of 10 minutes at 95°C, followed by 30 cycles of amplification consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C, 10 seconds extension at 72°C and a final extension step of 10 min at 72°C.

Amplification products were verified by using 2% Nusieve agarose gel electrophoresis (Lonza). PCR products were mixed following extraction from agarose gel (Nucleospin Gel and PCR clean up kit, Macherey Nagel). The DNA concentration of the standard solutions was determined using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.) and serial decimal dilutions were used as standards. For real time quantitative PCR, the mixtures contained 10 µl of SYBR green I master mix (Roche Applied Science), 1 µM each primer (final concentration), 2 µl of DNA template and ultrapure water to fill 20 µl. Reaction condition used the same program as for preparing standard solutions but with 45 cycles amplification and duration of denaturing, annealing and extension steps was reduced to 15s. The final step was set up to determine the melting temperature of the amplified product through slow increase of temperature (0.2°C.s^{-1}) between 50°C and 95°C. Quantification of each target gene was expressed as gene copy number per gram of dry leaf litter material.

2.3.3. Tagged amplicon sequencing

Bacterial amplicons have been obtained with primers Bakt_515F 5'-GTGYCAGCMGCCGCGGTA-3' and Bakt_928R 5'-CCCCGYCAATTCMTTTRAGT-3', which were designed to amplify the V4-V5 region of the 16S rDNA gene.

Fungal amplicons have been obtained with primers ITS1F_KYO2 5'-TAGAGGAAGTAAAAGTCGTAA-3' and ITS2R_KYO2 5'-GACTACHVGGGTATCTAATCC-3', which were designed to amplify the ITS1 region lying between the 18S and 5.8S ribosomal genes (Bokulich and Mills, 2013). The primers were complemented with illumina adapters CTTTCCCTACACGACGCTCTTCCGATCT, for Bakt_341F and ITS1F_KYO2 and GGAGTTCAGACGTGTGCTCTTCCGATCT, for Bakt_805R and ITS2R_KYO2. PCR amplification was performed in a reaction mixture of 25 µl containing 200 µM dNTPs, 5% (v: v) DMSO, 1 µM of each primer and 0.5U Pfu DNA polymerase with corresponding 10X PCR buffer (Invitrogen). Reaction condition had an initial denaturation step of 2 minutes at 95°C, followed by 35 cycles of amplification consisting of 40 seconds denaturation at 95°C, 45 seconds annealing at 60°C, 1 minute elongation at 72° and a final elongation step of 2 minutes at 72°C. Following verification of amplicons through 2% Nusieve agarose gel electrophoresis (Lonza)

and optimization of amplification condition when necessary, bacterial and fungal amplicons of the same sample were mixed together. Further amplicon purification, sample specific tagging and Illumina sequencing was achieved at the Plateforme Genomique Genotoul (Toulouse, France).

2.3.4. Bioinformatic analysis

The bioinformatic analysis uses recent tools such as *SWARM*, *CUTADAPT*, *VSEARCH* through reads matching, demultiplexing, sequence filtering, dereplication, clustering, chimer detection and taxonomic assignment steps (Martin 2011; Rognes 2016; Mahé 2017). The analysis was conducted by Juliette Chappat, a Master 2 student from Rennes University (Rapport de stage M2: Chappat, 2018).

2.4. Community analysis

Fungal and bacterial OTU abundance and taxonomic matrices were analyzed with the vegan package in R. The abundance value of each OTU was standardized by total abundance of the sample and by the total abundance of this OTU (double standardisation using 'wisconsin' transformation). Values were then log-transformed (Log *X+1*) before generating Bray-Curtis resemblance matrices. To visualize distance between samples, we used Principal coordinate ordination (PCO).

3. Results

3.1. Composition of the bacterial community of soil and casts depending on earthworms species and sampling time

We employed a combination of 16S rDNA and ITS gene sequencing and quantitative PCR in our analysis to study respectively the microbial biomass and community. Our results showed that microbial biomass and community vary between all sampled compartments. The compartments include first the soil sampled in the pots after 4 and 7 days of exposure to earthworms and pesticide or control pots (without worm

and pesticide). Second, casts sampled after 4 and 7 days (from pots exposed to pesticide and non-exposed pots) and casts collected on filter paper (5 days of gut voiding). Last, the earthworm gut obtained after dissection.

Statistical test (Adonis) showed that the microbial communities in the sampled compartments tend not to cluster together. In effect, two main clusters were identified for either bacterial (fig.25A) or fungal communities (fig.25B). The first cluster represents communities in the soil and casts, and the second one represents communities in the gut. Within these clusters, sub clusters vary according to the presence of earthworm species (*A.caliginosa* or *A.chlorotica*), the sampling time (4, 7 and 12) and the exposure to pesticide (dose 1 mg.kg^{-1}).

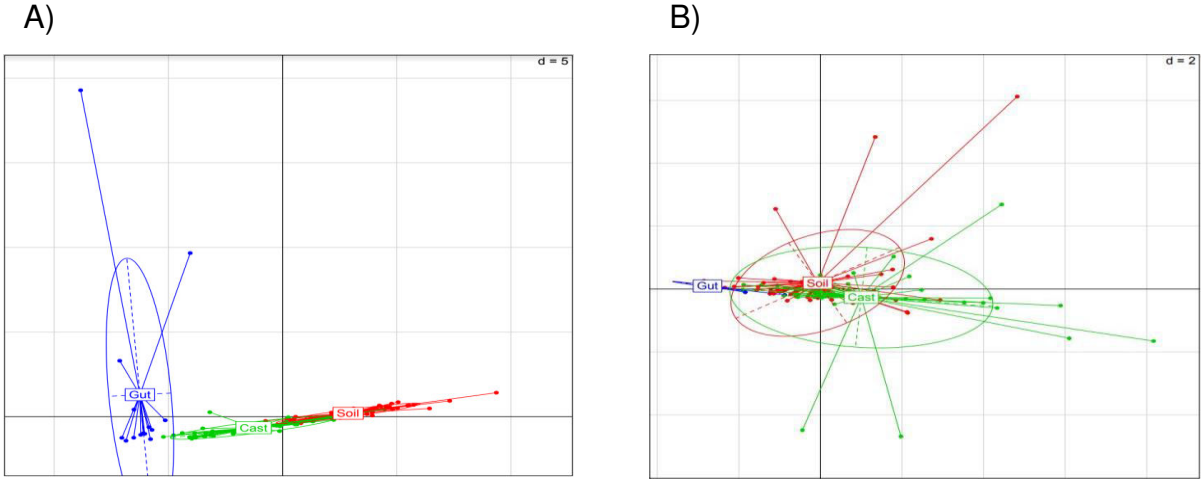


Figure 25: Community clusters (MDS) in the three sampled compartments (soil, cast and gut) at the phylum level for bacterial communities (A) and at the genus level for fungal communities (B) using a principal components analysis.

Moreover, microbial community in soil and casts are significantly different according to the statistical test (Adonis) (Fig. 26). However, despite the presence of two clusters one for soil and another for casts, some similarities were observed within both clusters especially for fungal communities (Fig. 26B). For bacterial communities similarities between soil and cast are less effective (Fig. 26A). Thus, fungal communities in casts recover close to the fungal communities in soil. At this level, there is no impact of the insecticide ethyl-parathion on the microbial community of soil and casts.

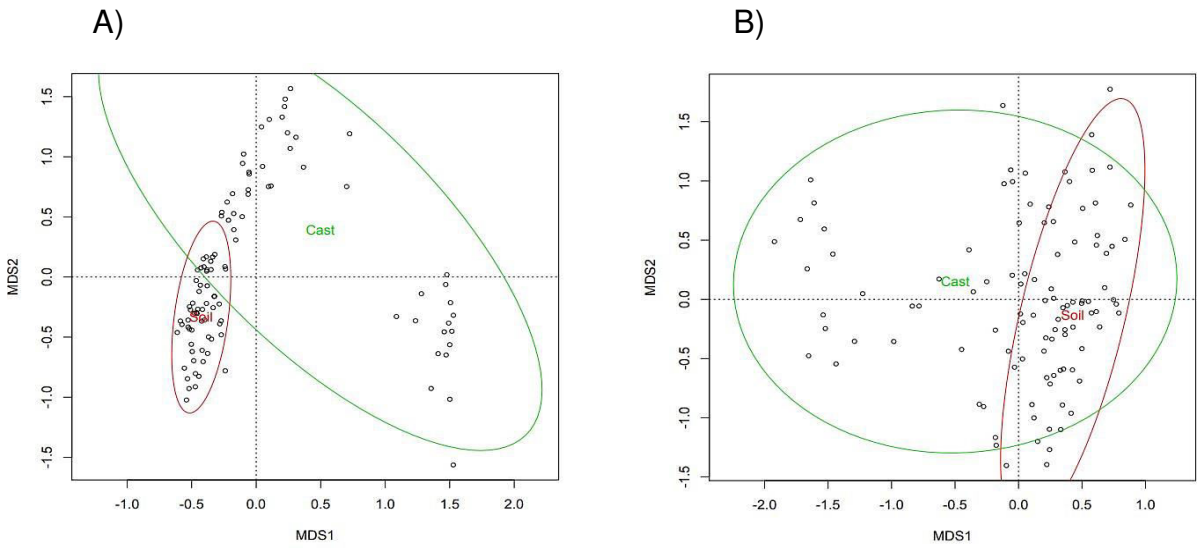


Figure 26: Community clusters (MDS) in soil and cast at the phylum level for bacterial communities (A) and at the genus level for fungal communities (B) using a principal components analysis.

Obviously, the effect of the sampling time on casts was prominent especially on bacterial communities. Microbial community in fresh casts collected on filter paper (days 12) exhibited significant differences from casts collected in plastic pots (Fig.27). However, the microbial composition of casts collected after 4 and 7 days, was more or less similar to the microbial composition of soil.

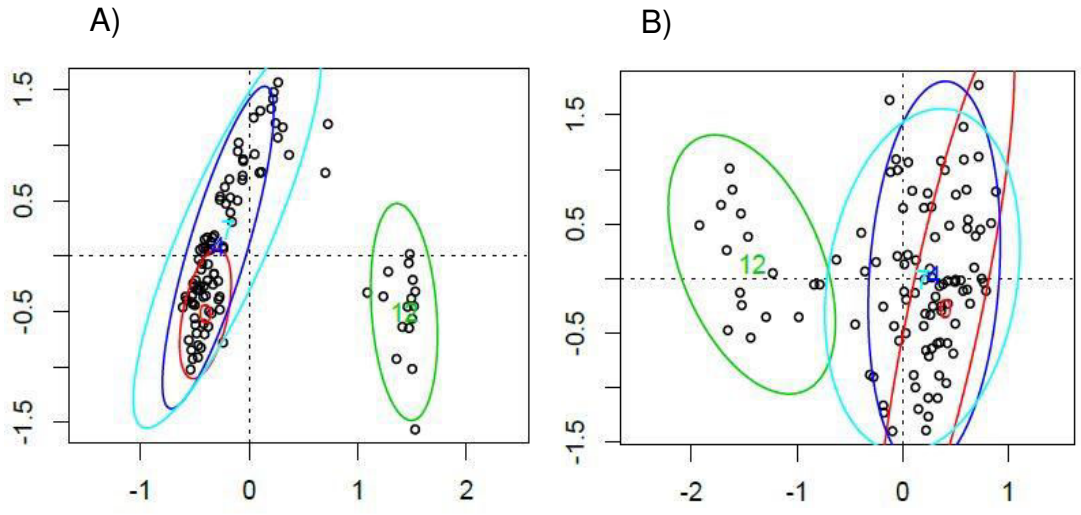


Figure 27: Community clusters (MDS) in soil and cast according to the three times of sampling (days: 0, 4, 7 and 12) at the phylum level for bacterial communities (A) and at the genus level for fungal communities (B) using a principal components analysis.

Matching preceding results, *Bacteroidetes*, *Proteobacteria* and *Acidobacteria* dominated the bacterial community of both the soil-G and the casts (Fig. 28A and 28B). However, their amount are different in comparison with soil samples and an increase of *Bacteroidetes* and a decrease in *Acidobacteria* were observed in the fresh cast compare to the soil and the casts collected after 4 and 7 days (Fig. 28).

In addition, *Ascomycota*, *Basidiomycota* and *Mortierellomycotina* dominated the fungal community of this soil (Fig. 28C). Similarly to soil fungal composition, casts were also dominated by three fungal phyla (Fig. 28D): *Ascomycota*, *Mortierellomycotina* and *Basidiomycota*. The sampling time has not any significant impact on fungal casts' composition comparing to bacterial community.

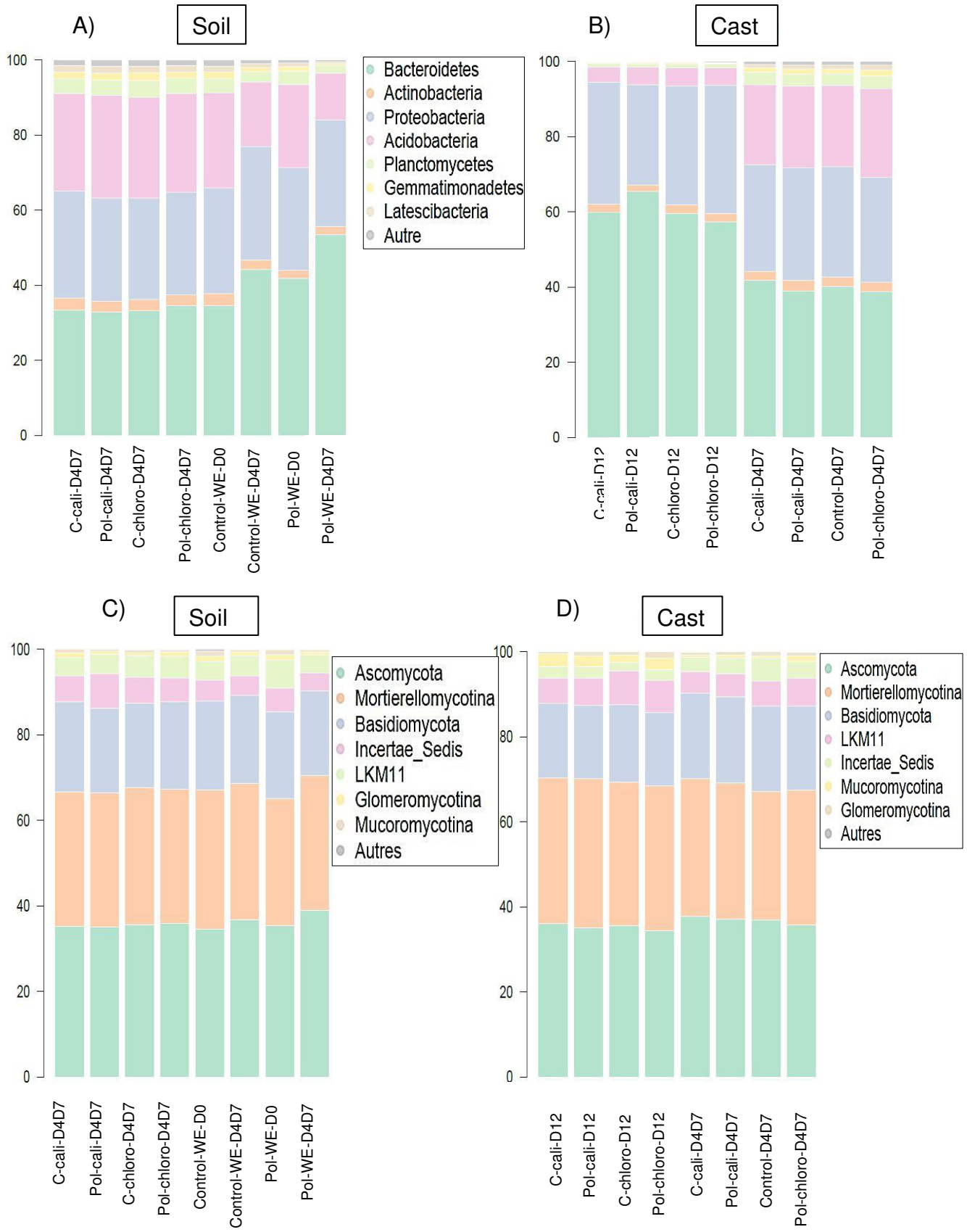


Figure 28: Relative abundances of bacterial (A,B) and fungal (C,D) phyla in soil and casts, based on the analyses of 16S rDNA and ITS genes. The most abundant bacterial phyla or fungal genus are displayed in the color of the respective phylum. C=control, WE=without earthworms Pol=polluted by ethyl-parathion; cali=A.caliginosa, chloro=A.chlorotica, D0=day 0, D4D7=days 4 and 7 (pooled), D12=days 12 (fresh casts collected on filter paper)

In addition, Ascomycota, Basidiomycota and Mortierellomycotina dominated the fungal community of this soil (Fig. 28C and 28D). Similarly to soil fungal composition, casts were also dominated by three fungal phyla: Ascomycota, Mortierellomycotina and Basidiomycota. The sampling time has not any significant impact on fungal casts' composition comparing to bacterial community.

3.2. Microbiome associated to gut wall

Changes of the microbial composition of ingested soil, observed in casts, suggested the presence of a main microbiome at the gut level of earthworms (Fig. 29). When comparing the guts of both species, our result showed a major variability of microbes in the gut of *A.caliginosa* than in the gut of *A.chlorotica*, but some similarities were noticed within both clusters.

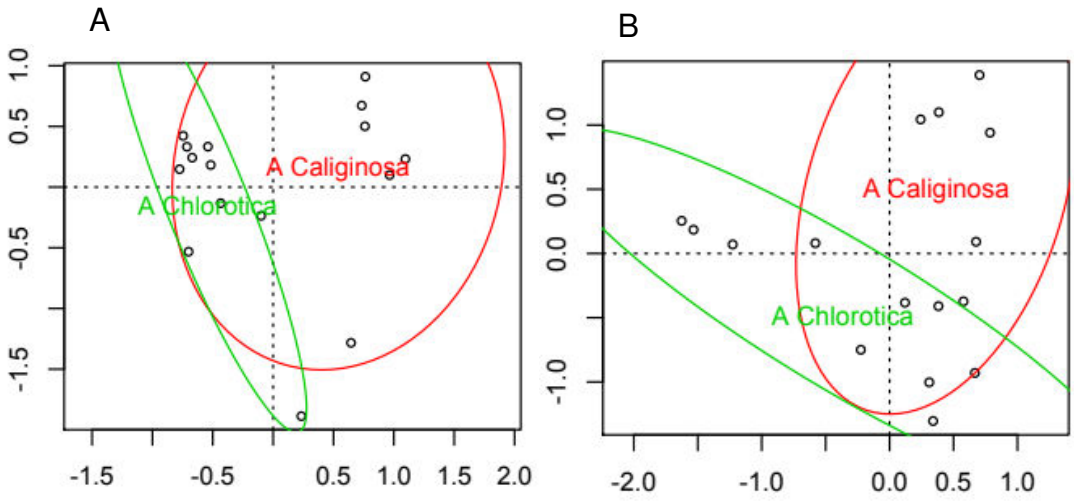


Figure 29: Community clusters (MDS) in earthworm's gut of both earthworms' species at the phylum level for bacterial communities (A) and at the genus level for fungal communities (B) using a principal components analysis.

Besides, the bacterial communities from the gut of *A. caliginosa* were dominated by two phyla *Actinobacteria*, *Proteobacteria*. However, the gut of *A.chlorotica* was dominated by one phylum *Actinobacteria* (Fig. 30A). Then (Fig. 30B), we identified the genus *rhodococcus* and *pseudoarthrobacter* from the phylum *Actinobacteria*.

While from the phylum *Proteobacteria*, we identified *Aeormonas* and *Verminephrobacter* genus. At this level, a minor effect of the insecticide ethyl-parathion is observed on bacterial community within the gut of *A.caliginosa.* The abundance of genus *rhodococcus* and *pseudoarthrobacter as well as Aeormonas* decreased in the presence of the pesticides, however, the abundance of other genus increase.

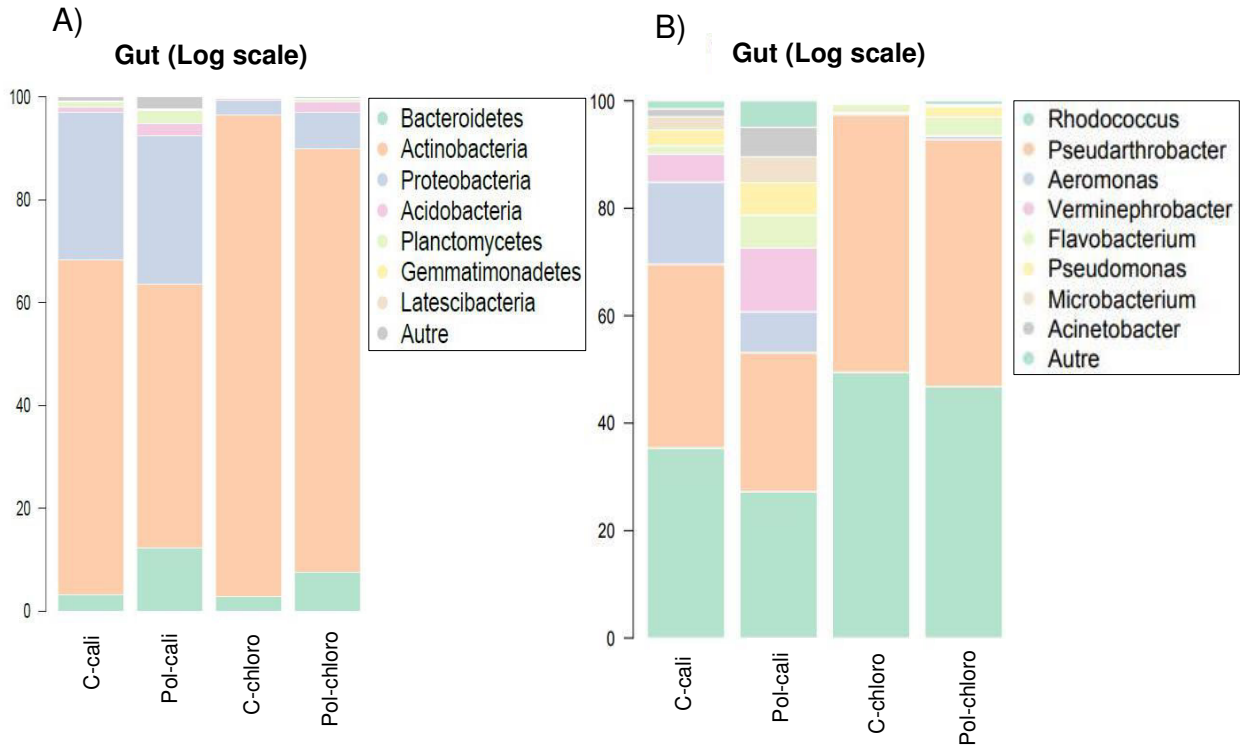


Figure 30: The relative abundances of bacterial phyla (A) and bacterial genus (B) in earthworms gut based on the analyses of 16S rDNA genes. The most abundant bacterial phyla are displayed in the color of the respective phylum and the most abundant bacterial genus are displayed in the color of the respective genus (We used a logarithmic scale to a better representation of less abundant phyla and genus). C=control, Pol=polluted by ethyl-parathion; cali=A.caliginosa, chloro=A.chlorotica

In Parallel, three fungal phyla dominated the fungal community of both species gut: *Ascomycota*, *Mortierellomycotina* and *Basidiomycota* but their amounts are different respective to the earthworm species (Fig. 31). A minor effect of ethyl-parathion was observed in the gut of *A.chlorotica,* where the abundance of *Mortierellomycotina* increased after exposure to this insecticide.

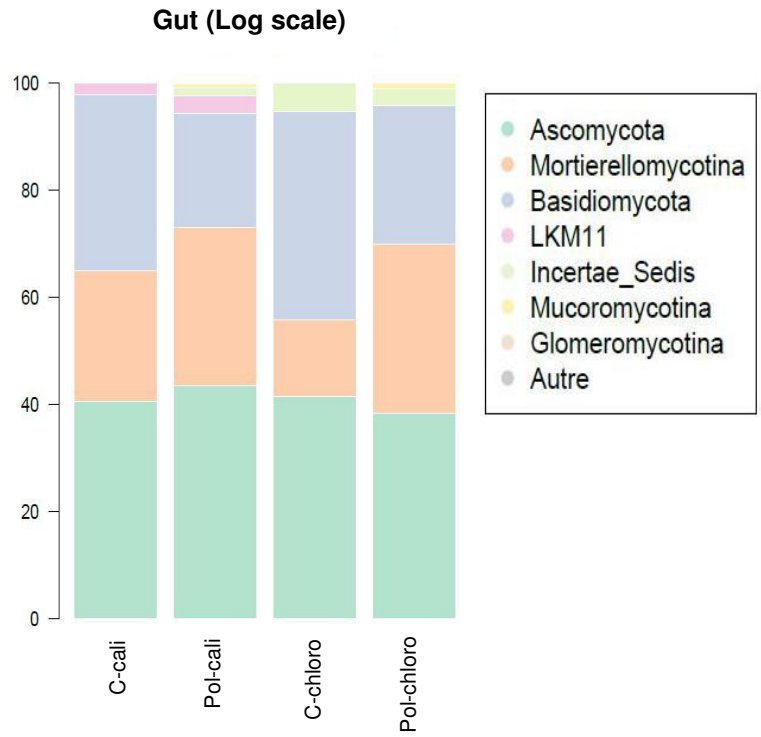


Figure 31: The relative abundances of fungal phyla in earthworms gut based on the analysis of ITS rDNA genes. The most abundant fungal phyla are displayed in the color of the respective phylum (We used a logarithmic scale to a better representation of less abundant phyla). C=control, Pol= polluted by ethyl-parathion; cali= A.caliginosa, chloro= A.chlorotica

4. Discussion

The aim of this study was to characterize the microbial taxa in the soil and earthworms gut for a better understanding of the interaction earthworm-microbiota in a pesticide-contaminated mesocosm. Actually, it is generally agreed that the soil is an unimaginable hotspot of microorganisms supporting their growth and activity. Due to the heterogeneity, diversity and temporal dynamics, the characterization of the soil metagenome remain an enormous challenge (Nesme et al. 2016). Microorganisms are potential vehicles underlying many services in soils, including nutrients transformation and cycling, organic matter decomposition and many other ecological functions. In this area, many studies reported that soil biochemical features and parameters as well as biotic and abiotic factors, influence and shape the microbial communities in soils, especially soil pH. All of the soil edaphic properties, geographical factors and climatic conditions are relevant to the microbial community and activity (Stromberg et al. 2002; Lynch et al. 2004; Gourmelon et al. 2016; Wang et al. 2017). In soil, earthworms interact with microorganisms and change the

microbial biomass and community via their activity of soil bioturbation. In particular, studies have documented the direct and indirect impact of earthworms on the composition, abundance and activity of soil microorganisms via their burrowing and casting activities (Lemtiri et al. 2014). This is constant with our findings proving that the microbial composition of fresh casts, relative to casts collected directly when evacuated, was specific and significantly different from cast collected in pots and from ingested bulk soil. Moreover, fresh casts interact with soil microbes once they are rejected, and recover the initial microbial composition of soil within few days. This is consistent with previous results showing that the variations of microbes in casts change in few hours (Parle 1963; Edwards and Bohlen1996). Thus, Following the timing of casts seems to be so important because, each sampling time corresponds to a newly casts microbial composition, which is introduced in the bulk soil.

Microorganisms are an inevitable component of earthworms' natural diet, but there are some preferences and a substantial selection for feeding strategies regarding fungal and bacterial species, which was highlighted in many studies. However, the forage and selection for food resources by the worms remain unsolved (Zirbes et al. 2012). Effectively, the significant difference of the microbial composition observed between ingested soil and casts suggest the presence of certain conditions, as well as a core microbial community inside the earthworms gut. Despite many studies in this domain, however, whether earthworms' gut contains a specific core microbiome or not remains an open doubt. Indeed, the microbial composition of earthworm intestine has been supposed to reflect the constitution of the soil or ingested materials (Doube et al. 1997). Our results showed that the bacterial composition of *A.caliginosa*'s gut is dominated by *actinobacteria* and *proteobacteria* that dominate the bacterial community of ingested soil, but their abundance are significantly different. However, the gut of *A.chlorotica* is dominated by one phylum *actinobacteria*. In parallel, fungal community in the guts are similar to that of soil and casts. Many authors have shown that the number of *actinobacteria* contained in the ingested material, increased by transit through the gut of earthworms (Edwards & Fletcher 1988, Sinha et al. 2008). Crucially, earthworms are considered as natural bioreactors in which some microorganisms proliferate when coping with *in-situ* gut conditions. These conditions within the gut comprise a neutral pH, higher water content as well as a mucus production and organic matter. At the level of the gut,

depending on the responses of microorganisms, not all species survive the gut passage. Some modifications of soil microbial community occur and could be either a stimulation or a suppression under the influence of gut conditions. The microorganisms resistant to the environmental conditions in the gut could be involved in the breakdown of organic material and the biodegradation of pollutants (Trigo et al. 1999; Singleton 2003; Byzov 2007; Munnoli et al. 2010). Therefore, earthworms gut act as a selective ‘‘biological filter’’ for soil microorganisms.

Previous studies found small or no differences between microbial community in earthworms’ gut and community in soils and casts (Bassalik 1913; Egert et al. 2004). Besides, recent studies based on culture-based and molecular methods succeeded to isolate some specific microorganisms in earthworms gut (Byzov et al. 2009; Thakuria et al. 2010). These findings are not surprising since almost all animals contain intestinal microbial associates. Our results agreed with the fact the earthworms possess their own microbiome inside their gut, which differs between our both species and remained after three washes. According to our results, the gut of *A.caliginosa* harbors diverse genus including *Rhodococcus, pseudoarthrobacter, aeromonas*, *verminephrobacter, flavobacterium, pseudomonas, microbacterium* and *acinetobacter*. In parallel, *A.chlorotica*’s gut harbors only two genus *Rhodococcus* and *pseudoarthrobacter*. Furthermore, the genus *Rhodococcus* is known to be abundant in the gut walls of endogeic species that might intervenes in their ability to use more complex stabilized soil humic substances (Briones et al. 2005; Thakuria et al. 2010). In addition, the genus *verminephrobacter* identified in the species *A.caliginosa*, is known as an earthworm nephridia symbiotic bacteria, but the function of this symbiosis is still not known (Pinel et al. 2008; Lund et al. 2010).

Moreover, the organophosphate ethyl-parathion alters the bacterial community of *A.caliginosa* gut by increasing the abundance of some genus and decreasing others. Notably, microbial degradation of various pesticides and organophosphorus compounds has been well-documented (Kumar et al. 1996; Javaid et al. 2016). In particular, *Flavobacterium sp.* was the first isolated and identified microorganism that could degrade organophosphorus compounds then, *Pseudomonas sp.*, and several species of *Bacillus* and *Arthrobacter* have been also isolated and were capable of hydrolyzing organophosphate such as parathion. These isolates were documented for their capability of complete degradation of the organophosphate, and could utilize

it as sole source of carbon and nitrogen (Singh & Walker 2006). Efficiently, *Rhodococcus* is well documented for its capability to degrade pollutants such as organochlorine pesticides, and various organophosphorous pesticides without producing toxic metabolite (Verma et al. 2011; Sirotkina & Efremenko 2014). Therefore the higher abundance of *Rhodococcus* in the gut of *A.chlorotica* might intervene in the tolerance to ethyl-parathion. Then, we suggest that the major variability of microbes inside the gut of *A.caliginosa* increases the availability of ethyl-parathion metabolite making *A.caliginosa* a more sensitive species. A functional analysis of the microbiome associated to the gut of both species is needed in order to confirm our results. In addition, since microorganisms used enzymes to degrade contaminants, several enzymes including organophosphorus hydrolase (OPH), organophosphorus acid anhydrolase (OPAA) and methyl parathion hydrolase (MPH) were documented to intervene in the degradation of organophosphate (Singh 2009). In this study, we did not find these genes in microorganisms of soil, casts or gut after testing our designed primers corresponding to those enzymes. This suggests that since this soil has received no organophosphate treatment since long time, microorganisms have not developed such genes to detoxify organophosphates. However, it is of great interest to design primers corresponding to carboxylesterases bioscavengers, which are involved in pesticide detoxification.

5. Conclusion

The difference in microbial community in ingested soil, fresh cast and gut reflect the selective role of the earthworm gut in both earthworms' species. The bacteria associated to the gut of *A.caliginosa* are more diverse and fall into many genus, while the bacteria associated to gut of *A.chlorotica* fall into two genus. The insecticide ethyl-parathion has no effect on either the microbial community of soil or casts, but a minor effect at the gut level of *A.caliginosa*. The findings of this study suggest that the *Rhodococcus* genus, highly abundant in the gut of *A.chlorotica,* and known for its ability to degrade organophosphate intervenes in the tolerance of *A.chlorotica* to this insecticide. Future studies of the functional analysis of the gut microbiome of both earthworms, under the same conditions of this experiment, may be able to confirm our results.

Chapter 4: Soil enzyme activities: dynamic and responses in ethyl-parathion-treated soil under the influence of earthworms

1. Introduction

Pesticides usage is among the factors that contribute to a decline of the soil biological activity. Amongst pesticides, organophosphates insecticides (OP) are commonly used to control agricultural pests. However, they are known to threat environment and health. At microscopic level, pesticides may harm gravely microbes and their enzymatic activities (Baćmaga et al. 2015). Besides, at macroscopic level, non-target organisms as earthworms are as well negatively affected by pesticide application (Paoletti 1999). Collectively, earthworms and microbes are drivers of many crucial functions in soils, including organic inputs turnover, elements cycling and the development of soil structure. Thus, they are considered as biological indicator of soil quality and degradation.

In the ecosystems, earthworms interact with the soil microbiome in the drilosphere, at an external level due to their burrowing and casting activities, and at an internal level due to the direct contact of ingested soil with their gut content (Brown et al., 2000). Hence, earthworms alter the microbial properties in soils and change its composition after the gut transit, where the microbial selection occur (Gómez-Brandón et al., 2011). Adds, many studies have shown that the bacterial population in casts is much greater than of the ingested soil (Zaller et al., 2013).

Besides, microbial functions in soils are critically important through the activity of enzymes, which are of substantial importance in maintaining soil health. Mainly of microbial origin, these enzymes are correlated with the microbial activity and may function as intracellular, cell-associated or extracellular (Kiss et al., 1975; Nannipieri, et al.,1990; Utobo and Tewari 2015). Considering their pivotal role in many biochemical functions in soil, their rapid response to ecological disturbances and their facility of measurement, they are widespread used as an early indicator of soil quality and deterioration (Gil-Sotres et al. 2005; Panettieri et al. 2013) as well as a sensors for soil microbial and physio-chemical status (Srinivasa Rao et al. 2016). It has been demonstrated that soil enzymes are affected by the soil properties including pH (Dick et al. 2000) agricultural activities and land management (Medeiros et al. 2015) and display different responses in soil exposed to pesticides (Riah et al. 2014). Besides, Sanchez-Hernandez et al. (2017) showed that the organophosphate insecticide and its main metabolites are severely toxic to soil enzyme activities. However, the linking

between pesticides exposure and enzymes inhibition is complicated and results are still confusing (Sannino & Gianfreda 2001).

Earthworms are in direct contact with soil and are also affected by pesticides, which disturb their enzymatic and behavior activities (Jouni et al. 2018). Effectively, earthworms affect the microbial composition in soils mainly by the burrowing activity and the deposition of casts. For instance, casts constitutes a microbial hotspots due to their high concentration of carbon, and harbor higher enzymatic activities in comparison with the surrounding soil (Tao et al. 2009; Lipiec et al. 2016). Accordingly, biological interactions between earthworms, microorganisms and contaminants are complex and need a better understanding, in order to assess undesirable environmental effects.

The scope of this work is to examine the impact of *A.caliginosa* and *A.chlorotica* on the activity of soil enzymes during the degradation of ethyl-parathion in two different soils. Therefore, the aims were: 1) to determine whether *A.caliginosa* and *A.chlorotica* activity reduced the effect of ethyl-parathion, by comparing earthworm-free and earthworm-inoculated soils using one concentration of pesticide 2) to examine the dynamics of soil enzyme activities in soils and casts in either control soil or soil contaminated by pesticide. Our findings will help to understand the importance of earthworms in the maintenance of biological activities in soils under environmental disturbance.

2. Material and methods

2.1. Chemicals

The following chemicals were purchased from Sigma–Aldrich (Saint Quentin-Fallavier, France): 1-naphthyl butyrate (1-NB), 4-nitrophenyl butyrate (4-NPB), 1-naphthol, 4-nitrophenol, Fast Red ITR and Fast Blue RR salts, 4-nitrophenyl phosphate (4-NPP), 4-nitrophenyl glucopyranoside (4-NPG), sodium salicylate, sodium nitroprusside, sodium dichloroisocyanate. Urea and iodo nitrotetrazolium chloride were from ACROS Organics (France).

2.2. Earthworms and soil sampling

Two different experimental soils were sampled from two different orchards named Soil-K and Soil-G, situated in a 10km distance, in Montfavet near Avignon (France). Both orchards have received no pesticide treatments for the last 10 years. The first orchard (Soil-K) is a silt loamy soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH 8.3), dominated by the species *A.chlorotica.* The second orchard (Soil-G) is a silt-clay soil (38.3 % clay, 42.2% fine silt, 19.5% sand, 34 g kg^{-1} organic matter, pH 8.5), dominated by the species *A.caliginosa.* Healthy earthworms from both species were collected manually from their original orchards, washed in tap water, blotted dried on filter paper and kept in the laboratory into plastic pots filled with fresh soil collected. Both earthworm species spent 5 days for cross-acclimation either in their own original soil or in the other soil where it was rare to find them. *A.chlorotica* individuals, highly abundant in the soil-K, were divided on two groups: one group was placed into plastic pots filled with fresh soil-K, the other group was placed into plastic pots filled with fresh soil-G. The same step of acclimation was done to *A.caliginosa,* which were separated in two groups either in their own soil-G or in soil-K. Afterwards, pots were kept in a dark cold chamber (12±1°C) for 5 days of acclimation.

Soils were sieved at <2 mm, and the water content was adjusted to 20-21% (approximately 81% of the maximum water holding capacity) with distilled water. Both wet soil samples (1 kg) were spiked with ethyl-parathion solutions (40 ml) to yield the final concentration of 1 mg active ingredient (a.i.) kg^{-1} wet soil. This ethyl-parathion concentration refers to the usual application rate and calculation of the Predicted Environmental Concentration (PEC). Control soils were prepared under the same conditions with a free-pesticide solution. The wet polluted or unpolluted soils were then split between Petri dishes (diameter 10 cm, height 3 cm) so that each contained 100 g soil.

2.3. Collection of soil, cast

After the acclimation period, earthworms were washed in tap water, blotted dried on filter paper and weighed. For each treatment, (2 different soils, control and 1 mg

ethyl-parathion per soil), 32 earthworms of a same species were placed by 2 individuals in each Petri dish to allow enough amounts of casts. Dishes were kept in a dark cold chamber (12±1℃) for the duration of the experiment. Soils free of earthworms were used as controls. After 4 and 7 days of incubation, 5g of soil and casts sub-samples were taken from each replicate and treatment, and stored in plastic tubes at -80℃, until analysis.

2.4. Soil enzyme activities

Many studies have recommended the use of the most sensitive enzymes and to include more than one enzyme activity per biogeochemical cycle. Hence, we chose several extracellular enzymes activities known to be involved in the biogeochemical cycles of carbon (carboxylesterase and glucosidase), nitrogen (urease) and phosphorus (acid phosphatase) (Balota & Chaves 2010; Gougoulias et al. 2014; Lessard et al. 2014). These enzymes have shown different responses to pesticides exposure (Riah et al. 2014). Furthermore, we used dehydrogenase activitiy as a direct indicator of soil microbial activity (von Mersi & Schinner 1991). Enzyme activities were measured in 1:25 (w/v) soil-water suspensions according to Sanchez-Hernandez et al. (2015). One gram of wet soil and 25 ml of distilled water were homogenized for 30 min at room temperature (~20°C) in FalconTM tubes and using an orbital shaker (Elmi® Intelli-mixer RM-2L, C2 program, 25 rpm). This procedure was compatible with a high-throughput microplate-scale assays for each enzyme activity.

Carboxylesterase (EC 3.1.1.1) activity was measured using two different substrates, i.e., 1-naphthyl butyrate (1-NB) and 4- nitrophenyl butyrate (4-NPB), according to Sanchez-Hernandez et al. (2015). Two different carboxylic esters were used because of the existence of multiple enzyme isoforms with a marked substrate specific sensitivity to OPs (Sanchez-Hernandez et al., 2015). The reaction mixture consisted of 280 µl of Tris-HCl 0.1 M (pH = 8), 200 µl of soil-water suspension and 20 µl of substrate (5 mM, final conc.). After incubation (shaken for 20 min at 20℃ using a thermostatically controlled orbital shaker Elmi® Skyline DTS-2, 900 rpm), microplates were centrifuged (2500 rpm, 5℃ and 10 min), and 150 µl supernatants were transferred to new microplates. The product of naphthyl ester hydrolysis (1-naphthol) was revealed by adding 75 µl of a solution containing 2.5% (w/v) SDS in 0.1% Fast

Red ITR/2.5% Triton X-100, and the plate was then kept in dark for 20 min until complete color development. The absorbance of the naphthol–Fast Red ITR complex was read at 530 nm. In the case of 4-NPB hydrolysis, the formation of 4-nitrophenol was determined after addition of 75 µl of a solution containing 2% (w/v) SDS and 2% (w/v) Tris-base to the microplate containing the 150 µl supernatants, and the absorbance was immediately read at 405 nm. Enzyme activities were expressed as µmol of product per hour and gram of dry soil, using calibration curves made with 1-naphthol (1.5–100 $nmol.ml^{-1}$) and 4-nitrophenol (5–100 $nmol.ml^{-1}$). Calibration curves were made in the presence of soil-water suspensions to correct the adsorption of the chromogenic substances onto soil colloids. Controls (substrate-free) and blanks (soil-free) were used to correct background absorbance and non-enzymatic hydrolysis of the substrates, respectively.

Phosphatase (EC 3.1.3.2) and β-glucosidase (EC 3.2.1.21) activities were assayed according to Popova and Deng (2010) in a reaction medium containing 200 µl of soil-water suspension, 200 µl of sodium azide 2.5 mM and 100 µl of respective substrates (4-nitrophenyl phosphate or 4-nitrophenyl-d-glucanopyranoside, 5 mM final conc.) dissolved previously in 20 mM modified universal buffer (pH = 8). This pH allowed the determination of the total phosphatase activity, because both soils used in this study exhibited a pH close to 8. After a 90 min (phosphatase) or 60 min (β-glucosidase) incubation periods under continuous shaking at 20°C, microplates were centrifuged (2,500 x g, 10°C and 5 min), and 150 µl of supernatant were transferred to new microplates. The formed 4-nitrophenol was immediately (<1min) read at 405 nm after addition of 75 µl of 0.5 M NaOH per well to stop the reaction. Standard calibration curves were made with 4-nitrophenol (5–100 $nmol.ml^{-1}$).

Urease (EC 3.5.1.5) activity was measured according to the unbuffered method by Schinner et al. (1996). Hydrolytic reactions were performed in eppendorfs by mixing 200 µl 80 mM urea and 200 µl of soil-water suspension, and incubated (orbital shaking) for 2 hours at room temperature (~20°C). Reactions were terminated by addition of 400 µl of cold 1 M KCl containing 10 mM HCl. Tubes were agitated for additional 30 min to extract ammonium, and then centrifuged (4,500 g, 5 min, 10°C). Supernatants (150 µl) were poured in the wells of microplates, and ammonium was measured after addition of 75 µl of 1:1 (v:v) 0.3 M NaOH : 1.06 M sodium salycilate containing 4.6 mM sodium nitroprusside, followed by addition of 30 µl of 0.1% sodium

dichloroisocianide solution. Microplates were left for 20 min in dark for color development, and absorbance was read at 690 nm. Urease activity was expressed as g $NH4^+$-N h^{-1} g^{-1} dry soil using a calibration curve made with NH_4Cl (3.0–50 g $NH4^+$ ml^{-1}).

Dehydrogenase (E.C.1.1.) activity was measured according to von Mersi and Schinner (1991), using iodonitrotetrazolium chloride (INT) as the electron acceptor. The reaction was performed in eppendorf tubes by mixing for 2 hours at 40°C under agitation in the dark 0.2 g of soil-water suspension with 0.2 mL of 0.5% INT and 0.5 mL of Tris-HCl 1M, pH 7.0. Reaction was terminated by addition of 1 mL of 1:1 (v:v) pure ethanol : pure N-N'-dimethyl formamide (DMFO). Tubes were agitated for additional 60 min for color develpment, and then centrifuged (5000 g, 5 min). Supernatants (300 µl) were poured in the wells of microplates, and the formation of reduced iodonitrotetrazolium formazan (INTF) was determined spectrophotometrically at 464nm. The results were expressed as µmol INTF h^{-1} g^{-1} dry soil.

2.5. Data analysis

Data (enzyme activities) were first scaled and centered and then analysed with a PCA using the 'ade4' package in R. Differences between ellipses were further tested using a between-class analysis. Comparisons of enzyme activities between soils and pesticide treatments were analyzed with one-way ANOVA followed by Tukey HSD post-hoc comparison test using XLSTAT software (version 2013.3.01).

We used two numerical indexes to assess both the impact of ethyl-parathion, soil and earthworm species on soil enzyme activities: the GMean index (Hinojosa et al., 2004), and the *IBRv2* index which is a modified version by Sanchez et al. (2012) of the original *IBR* index (Beliaeff and Burgeot, 2002).

The GMean index was calculated as follow:

$$GMean = \left(\prod_{i=1}^{n} y_i \right)^{1/n}$$

where y_i is the enzyme activity and n is the total number of soil enzymes.

For IBRv2 calculations, in a first step, individual enzyme activities from ethyl-parathion -treated soils (X_i) were compared to mean values of enzyme activity from the reference soils (X_0), and log- transformed to reduce variance:

$$Y_i = Log\frac{X_i}{X_0}$$

In a second step, the general mean value (μ) and the standard deviation (σ) for each enzyme activity were used in the standardization of data (Z_i):

$$Z_i = \frac{(Y_i - \mu)}{\sigma}$$

Finally, a deviation index (A_i) was calculated for each enzyme activity using the standardized data (Zi) and the standardized mean of the reference soils (Z_0).

$$A_i = Z_i - Z_0$$

Positive A_i values indicate increase of soil enzyme activity compared to reference values, and negative A_i values indicate inhibition of enzyme activity. The A_i values were plotted in sunray plots for a visual inspection of all enzyme responses. The IBRv2 values represent, therefore, the sum of deviations between the reference and the ethyl-parathion treated soils, and it is calculated as follows:

$$IBRv2 = \sum_{i=1}^{n} |A_i|$$

3. Results

3.1. Effect of soil texture and ethyl-parathion on soil enzyme activities

Principal component analysis from control and ethyl-parathion treated soil were shown in (Fig. 32), after either 4 days of exposure (A) or 7 days of exposure (B). Significant differences could be observed between soil structures and between treatments. However, neither the presence of earthworms nor the earthworm species

present in the plastic pots induced significant differences in the enzyme activities responses (Fig. 32).

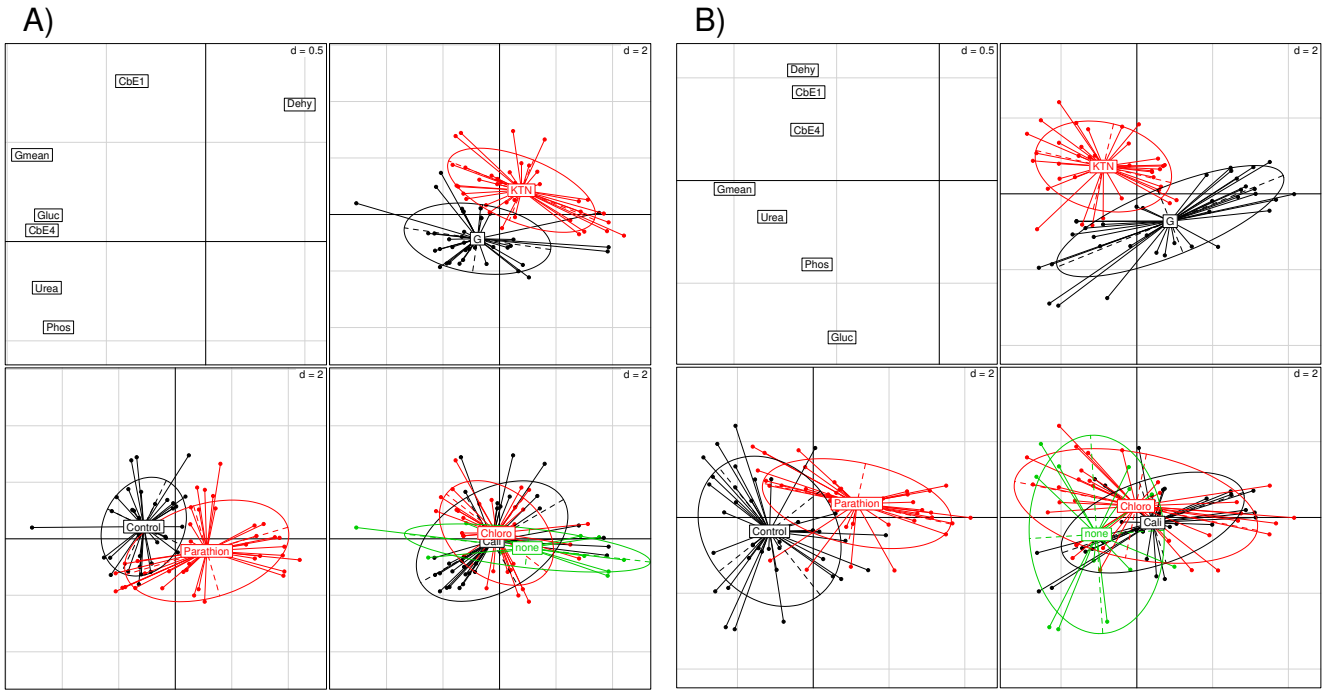


Figure 32: Principal component analysis of enzyme activities for soil G and soil K, and for control and Ethyl-parathion treated soils after A) 4 days or B) 7 days of exposure.

According to these observations, further analyses were conducted without discrimination of earthworm species. A global response of soil enzyme activities was then assessed by different enzymes-based indexes such as GMean index, T-SQI and IBRv2 indexes. GMean indexes are shown in (Fig. 33). In both soils, we observed a significant decreased in GMean index of control soils (Fig. 33A) when increasing the time of exposure. Despite the impact of time, it is noteworthy that the GMean index also decreased, compare to their respective control soils, after 4 and 7 days of exposure to ethyl-parathion. After 7 days of exposure, the Soil-G appeared more affected by ethyl-parathion than Soil-K (Fig. 33A).

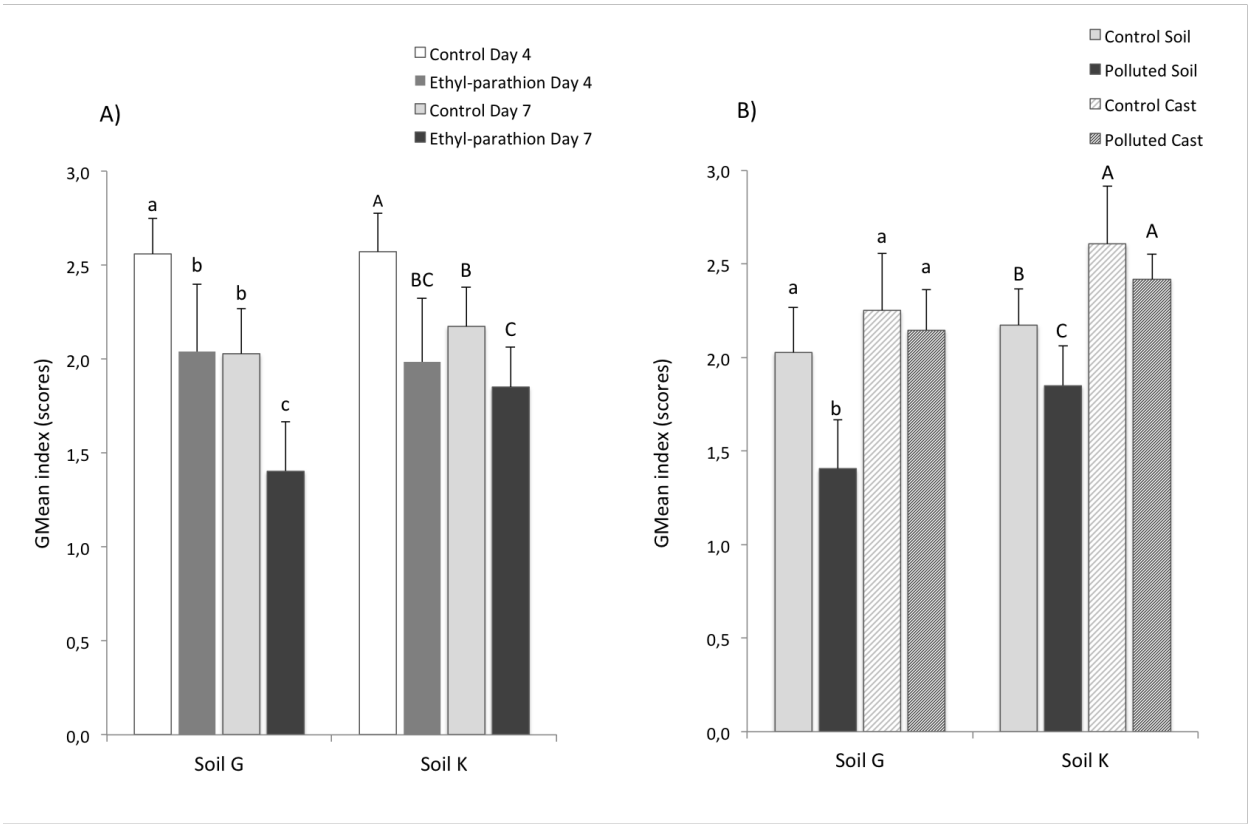


Figure 33: GMean index for enzyme activities from A) Control bulk soils and pesticide-treated bulk soils after 4 and 7 days of exposure and B) Bulk soils and casts collected in control and polluted microcosms after 7 days of exposure. Different letters denote significant differences between treatments ($p<0.05$).

When considering the enzyme activities measured in cast, we first observed that the GMean values are higher for cast collected in control soil than the corresponding bulk control soil with a significant effect for Soil-K ($p<0.0001$). After 7 days of exposure, the cast did not exhibited any significant decrease in GMean indexes compare to cast control and they appeared to be higher than in the corresponding bulk polluted soil ($p<0.0001$). Then in order to show the effect of ethyl-parathion after 4 days of exposure, mean (± SD, n=8) enzyme activities of control and ethyl-parathion polluted soils are presented in (Table 6). Both soil exhibited similar enzyme activities except for phosphatase and dehydrogenase activities that were lower or higher respectively, in the silt-loamy soil-K. 4 days of exposure to ethyl-parathion had a significant effect on most of the enzyme activities except for urease activities. An inhibition of carboxylesterase and phosphatase activities was observed. Moreover, depending on soil structure two enzymes exhibited different responses. Dehydrogenase activity was higher in the silt-loamy soil K and exhibited an increase after exposure, while a lower activity was recorded in the silt-clay soil G following by a non-significant

decrease after exposure. Response in β-glucosidase activity depended on soil structure, and a decrease was noticed after ethyl-parathion exposure in soil-K (Table 6).

Table 6: Variation in soil enzyme activities (mean ± SD, n=19) in soil after 4 days of exposure to ethyl-parathion at the dose of 1mg a.i. kg-1 dry soil.

		Time of exposure			Statistics
Enzyme activities	Soil	Control Day 4	Ethyl-parathion Day 4	*Inhibition after 4 days (%)*	P-value
Carboxylesterase 4-NPB	G	15.11 ± 2.52 (a)	11.24 ± 3.80 (b)	*-25.6*	<0.0001
(µmol h^{-1} g^{-1} dry soil)	K	14.40 ± 4.06 (a)	8.17 ± 2.80 (b)	*-43.3*	<0.0001
Carboxylesterases 1-NB	G	1.92 ± 0.76 (a)	1.20 ± 0.28 (b)	*-37.4*	<0.0001
(µmol h^{-1} g^{-1} dry soil)	K	2.55 ± 0.62 (a)	1.56 ± 0.64 (b)	*-37.2*	<0.0001
Phosphatase	G	1.61 ± 0.38 (a)	1.25 ± 0.49 (b)	*-22.4*	0,009
(µmol h^{-1} g^{-1} dry soil)	K	0.90 ± 0.12 (a)	0.68 ± 0.12 (b)	*-24.6*	<0.0001
β-Glucosidase	G	1.34 ± 0.19	1.34 ± 0.35	*0*	1
(µmol h^{-1} g^{-1} dry soil)	K	1.40 ± 0.12 (a)	1.00 ± 0.29 (b)	*-28.4*	<0.0001
Dehydrogenase	G	76.5 ± 14.4	61.7 ± 18.7	*-19.3*	0.136
(nmol INTF h^{-1} g^{-1} dry soil)	K	110.7 ± 27.6 (a)	141.2 ± 24.0 (b)	*+21.6%*	0.0005
Urease	G	66.42 ± 5.45	61.63 ± 8.51	*-7.2*	0.398
(µg $NH4^{+}$-N h^{-1} g^{-1} dry soil)	K	62.12 ± 6.06	55.14 ± 7.72	*-11.2*	0.046

3.2. Enzymatic indexes of soil quality

To go further in the effect of ethyl-parathion exposure, T-SQI values were calculated for each enzyme activities. 4-NPB and 1-NB carboxylesterase activities were considered as a single activity to point out the impact on ethyl-parathion on each class of enzyme activities. As shown above, most of the enzymes are inhibited after 4 days of exposure. Sunray plots of residual enzyme activities respect to those of control soils, provided a visual illustration on the overall effect of ethyl-parathion (Fig. 34). They clearly showed an inhibition of all activities (values below the dotted lines which represent the control) after 4 days of exposure except for the dehydrogenase activity in Soil-K. After 7 days of exposure, both carboxylesterase and phosphatase activities were fully recovered, while a partial recovery of β-glucosidase activity was observed in Soil-K. Beside, a 7 days exposure continued to inhibit enzyme activities

measured in the silt-clay Soil-G, except the dehydrogenase activity, which fully recovered its initial activity (Fig.34).

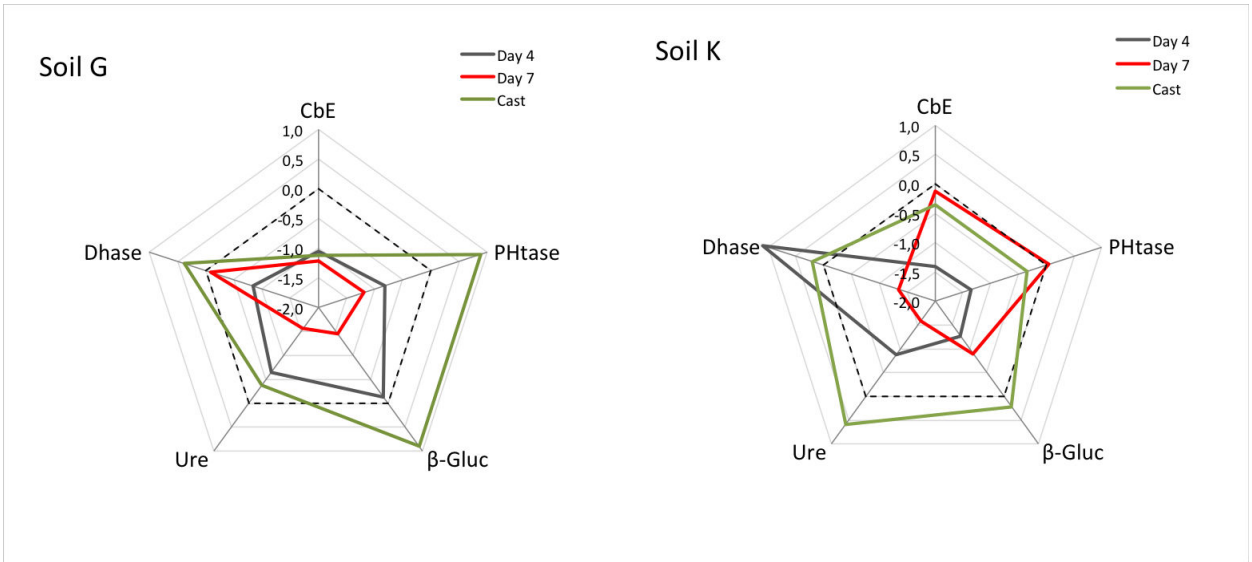


Figure 34: Sunray plots showing distribution of T-SQI scores calculated for each enzyme activity measured in bulk soils after 4 and 7 days of exposure and in cast collected after 7 days of exposure compare to their respective control values (doted lines in both plots).

Considering the enzyme activities measured in cast collected after 7 days of exposure, the values of T-SQI are close to the reference (dotted lines) when collected in Soil-K. Cast collected in Soil-G are mainly close to the reference, however a decrease in CbE activities was observed while phosphatase and β-glucosidase activities were enhanced.

IBRv2 index allowed a quick visualization of the impact of ethyl-parathion on soil enzyme activities. Higher is the absolute value of this index, higher is the deviation from the control soil. As shown in (Fig.35), the IBRv2 score increased with increasing time of exposure in Soil-G from 3.5 to 5.5 after 4 and 7 days of exposure respectively, while no difference were observed for the Soil-K which reached a IBRv2 score close to 3.8 for both dates. IBRv2 scores calculated from cast collected after 7 days of exposure were low compare to the bulk soil with values ranging from 0.67 to 0.31 when collected in Soil-G and Soil-K respectively.

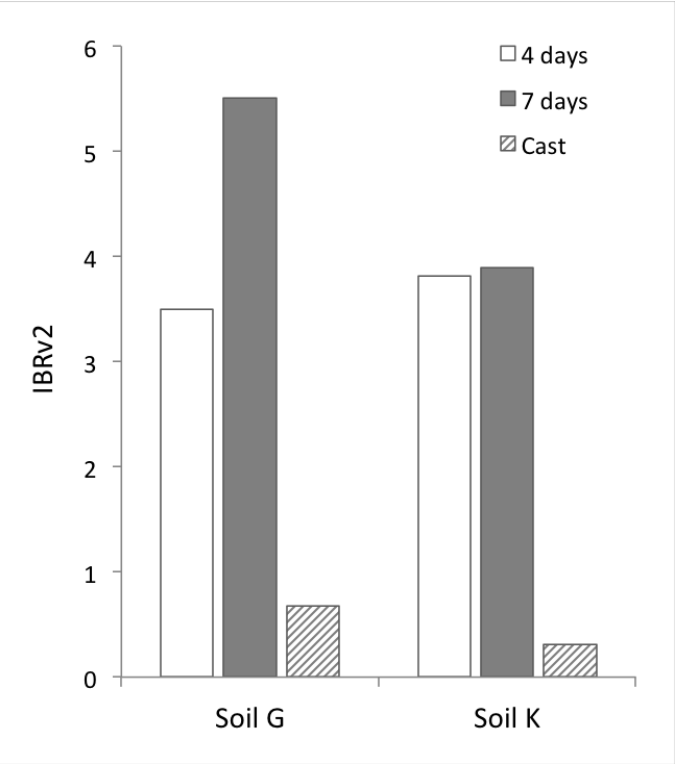


Figure 35 : IBRv2 scores calculated for each enzyme activity measured in bulk soils after 4 and 7 days of exposure and in cast collected after 7 days of exposure.

4. Discussion

4.1. Impact of soil texture and Ethyl-parathion on soil enzyme activities

This study showed that short-term exposure to ethyl-parathion caused a significant inhibition of soil enzyme activities such as carboxylesterases, phosphatase and β-glucosidase. Because these enzymes are mainly correlated to extracellular activities, their activities provide a significant view of the total enzyme activity of soil associated to soil organo-mineral complexes (Dick et al., 1997; Shaw and Burns, 2006; Nannipieri, 2006). These results are consistent with previous studies showing that both carboxylesterase and phosphatase could be used as potential indicators of OP contaminated-soils. The activity of β-glucosidase measured in control soils (about 1.38 $\mu mol.h^{-1}.g^{-1}$ dry soil) was close related to previous work (Turner at al. 2002; Sanchez-Hernandez et al. 2017). However, the inhibitory effect of ethyl-parathion on this enzyme activity depends on soil structure with a significant decrease in the silt-

loamy soil compare to the silt-clay soil. Previous studies have found that β-glucosidase activity could provide an early indication of changes in organic matter status (Monreal and Bergstrom 2000), but clay content appeared to be the key factor exhibiting a positive relationship with β-glucosidase activity (Turner et al. 2002). Thus in our study, the higher clay content in Soil-G could allow the β-glucosidase to be maintained at its control value, despite the presence of ethyl-parathion. Finally, N-cycling enzyme activity such as urease was not affected by ethyl-parathion which correlate with previous study showing that exposure to chlorpyrifos, another OP insecticide, did not modify the urease activity (Sanchez-Hernandez et al 2017). However, the hydrolytic activity in soils is associated to both the production of enzymes by active organisms in the presence of substrate, and the activity of extracellular enzymes associated to organic matter and clays. This latter association could either prevent or enhance the interaction between pesticide and soil enzymes (Nannipieri et al 2002; Gianfreda and Rao 2008). Other studies have shown that urease activity could be positively correlated to living organisms (then to dehydrogenase activity) instead of extracellular enzymes (Bello et al 2013). Dehydrogenase activity, which is known to reflect viable cells, is mostly used as an indicator of soil microbial activity (von Mersi and Schinner, 1991; Shaw and Burns, 2006). In the present study, this enzyme clearly discriminates between the two different soils with a higher activity found in the silt-loamy soil (compare to the silt-clay soil), followed by an increase after 4 days of exposure. The higher activity found in the silt-loamy soil is in agreement with previous studies showing a negative correlation between dehydrogenase activity and soil water content (Wolinska & Stepniewska 2012). However, the opposite responses of dehydrogenase observed in the present study are not easy to correlate depending on soil structure. Relationships between soil dehydrogenase and the total microbial activity is not always obvious, especially in the case of complex systems like soils, where the microorganisms and processes involved in the degradation of the organic compounds are highly diverse (Wolinska & Stepniewska 2012). Then, microrespirometry analysis could be a usefull and complementary tool in order to assess microbial biomass in both soils under ethyl-parathion exposure, and to correlate this microbial biomass with the response of soil enzyme activities. Considering the microbial community of cast, it is admitted that casts are enriched in available nutrients and harbour greater microbial populations and biomass than surrounding soil (Sheehan et al. 2008; Huang and Xia

2018). In the collected cast studied herein, all the enzyme activities are similar to those measured in the bulk control soils, except the β-glucosidase and dehydrogenase that exhibited respectively a decreased and an increase in cast for both soils. Significant decrease in cellulose content following breakdown of organic matter during the ingestion of soil, could explain a decrease in β-glucosidase in cast. On another way, earthworms' mucus facilitated the mineralization and humification of organics and led to the greatest increases of microbial activity (Huang and Xia 2018). Then, we could assume that the increase of dehydrogenase activity reflects the increase of microbial activity in cast, due to the high contact with mucus during the intestinal passage.

Regarding the effect of ethyl-parathion, only CbE activities were inhibited in the cast collected in the soil G. As specified above, the higher content of clay in soil G could enhance the contact between insecticide and CbE extracellular enzymes, within the gut transit in both earthworm species. When ingested, the soil is fragmented, which increase the surface area for microbial activities and contact. Interestingly, in this study, the response of soil enzyme activities occurred very quickly after 4 days of exposure. Previous works have shown that pesticide induced changes in soil enzyme activities after longer exposure to chlorpyrifos such as 2 weeks (Sanchez-Hernandez et al. 2017) or even 6 and 12 weeks (Tejada et al. 2011). As an example, dehydrogenase, phosphatase and urease exhibited a significant decrease after 45 days of chlorpyrifos exposure, while no effect was observed after 3 days of exposure (Tejada et al. 2011). These observed differences could lie on the toxicity of the two OP insecticides that vary depending on their mammalian LD50 (2000 and 6.8 $mg.kg^{-1}$), water solubility (1.18 to 13 at 20°C) and half-life hydrolysis in water (2118 and 302) for chlorpyrifos and Parathion respectively. Moreover, their half-life in soil (30.5 and 21-58) is governed by both soil organisms able to break down the pesticide, and by the type of soil (e.g. sand, loam and clay), pH and temperature (Kumar et al. 2018).

Another aim of our study was to assess the role of two endogeic species of earthworms on soil enzyme activities after ethyl-parathion exposure. Our results have shown that the presence of earthworms did not modify the impact of ethyl-parathion on soil enzyme activities. Soil texture clearly appeared to be the main factor governing the impact of ethyl-parathion on soil enzymes. This result is consistent with previous studies showing that, the impact of soil type is higher than that of earthworm

species on casts (Clause et al. 2014). Moreover, depending on earthworm species, higher impact of *L. terrestris* compare to *A. chlorotica* or *A. rosea* was observed on cast properties (Clause et al. 2014). Other earthworm species, such as *Lumbricus terrestris and Lumbricus rubellus* (Dempsey et al. 2013; Sanchez-Hernandez et al. 2018), or *Metaphire guillelmi* (Tao et al. 2009), have shown to improve soil enzyme activities.

4.2. Enzyme-based indexes for soil quality

The simple enzyme-based indexes of soil quality used in this study allowed to assess differences between control and ethyl-parathion treated soils. The GMean index provided a quick indicator of soil pollution, despite the fact that some enzyme activities (urease for both soils and for β-glucosidase in soil-G) were not affected by ethyl-parathion. In particular, we could differentiate the impact of pollution depending on soil structure directly from the GMean response. A direct impact was observed on soil-G after 4 days of exposure, with an increasing effect after 7 days. The impact of pollution on the quality of soil-K was assessed after 4 days of exposure, and then no other modification occurred when increasing the exposure period. These latter results suggested that the soil quality of soil-K remained at the same level whatever the time of exposure. Other studies have used the GMean as a suitable indicator for soil pollution in the presence of chlorpyrifos (Sanchez-Hernandez et al. 2017).

The analysis using T-SQI index was proposed by Mijangos et al. (2010) as an integrative enzymatic index of soil pollution. This index measures the magnitude and direction (increase or inhibition) of changes induced by an environmental stressor on soil enzyme activities compare to those of a reference. Our results are consistent with previous work in which different pesticide decreased T-SQI values on treated soils (Munos-Leoz et al. 2013; Sanchez-Hernandez et al. 2017). Moreover the IBRv2 index, which confirms the response of T-SQI values, integrates the global response of several biomarkers (soil enzyme activities in our case) to evaluate the soil quality. This index needs reference value from non-treated soil, then, high IBRv2 values reflect high impact of the insecticide on the global quality of the soil. According to this statement, our findings clearly highlighted that the silt-loamy soil-K (either bulk soil and cast) remained in a better healthy status than silt-clay soil-G after exposure to

ethyl-parathion. Moreover, based on T-SQI values, soil-K exhibited a recovery of the carboxylesterase activities after 7 days of exposure that could induce a restoration of its global quality compare to soil-G, by scavenging the OP molecules. In addition, soil quality of cast collected in both contaminated soils remained very close to their control references. According to the soil structure, ethyl-parathion induced higher disturbances in cast collected in soil-G compare to soil-K.

5. Conclusion

Our study has shown that impact of ethyl-parathion on soil enzyme activities mainly depends on soil texture rather than the presence and/or the species of earthworms. In particular, enzyme activities measured in silt-clay soil appeared to be more impacted than enzymes measured in a silt-loamy soil. It seems that silt-loamy soil are able to restore faster their initial quality than silt-clay soil. Moreover, our results provide evidence that the impact of ethyl-parathion on cast, as well as the microbial communities of casts do not depend on earthworm species. This result confirms that the impact of soil type is higher than earthworm species on both cast and bulk soil. By providing physical protection for organic matter, microorganisms, nutrients, exogenous compounds, and enzymes, clay content could enhance the contact between soil components at different scale.

General discussion and perspectives

Pesticides usage has been a fundamental part of many programs to protect plants from pests, weeds or diseases and humans from vector-borne diseases, as well as to increase agricultural production to satisfy the high demand of food (Popp et al. 2013; Glover-Armengor & Tetth 2009; Aktar et al. 2009; Nicolopoulou-Stamati et al. 2016). Grievously, pesticides are assessed for risks posed to ecosystems and for toxicity toward living organisms by interfering with specific biochemical systems. The extensively and sometimes improper application of pesticides, has led to pesticides losses in the environment that reached many compartments including soil, air and water. Thus, the dispersion of their residues in the environment has threat mammals, soil biota, bees, fish, birds and plants (Carvalho 2017; Paoli et al. 2015; Köhler & Triebskorn 2013). In the past 25 years, a noticeable research effort was developed to discern the behavior of these chemicals in the environment, including their cycling and fate along with their toxicity to living organisms. In soils, pesticides alter strongly soil functions and properties and affect the soil micoflora and its activity, thereby disturbing soil health (Prashar & Shah 2016). To assess soil quality and deterioration, various parameters have to be characterized and often biological indicators are employed (Bünemann et al. 2018). Earthworms are considered as potential bioindicators of soil quality for plenty of reasons. They are largely distributed and frequent in different soil types and horizons and are easy to collect. Likewise, they are in direct contact with soil and contaminants through their skin and when burrowing and feeding in soil (Fründ et al. 2011).

OPs represent one of the most important classes of insecticides, which exert toxic effects on the enzyme AChE. Since several years, there has been a decline in the amount of OPs pesticides in use, especially in developing countries, along with a market switch to other compounds acting in other targets than ChEs. Despite this fact, studies suggest that OPs will remain at the head of the line, since they are effective and inexpensive (Pattanapairoj & Chetchotsak 2010; Eddleston & Chowdhury 2015). In our experiments, we used the organophosphate insecticide “ethyl-parathion”. This insecticide is no more used in France, however, it is still used in other countries and in laboratory as a toxic model molecule.

This thesis focused upon the effect of ethyl-parathion on two endogeic earthworms' species, using an integrative approach to link ecotoxicology and microbial ecology. Very little is known about the functional correlation between soil type, earthworm species and microbial communities on pesticide fate in soils (Sanchez-Hernandez et al 2018). The following scheme resumes the experimental design of my project (Fig. 36).

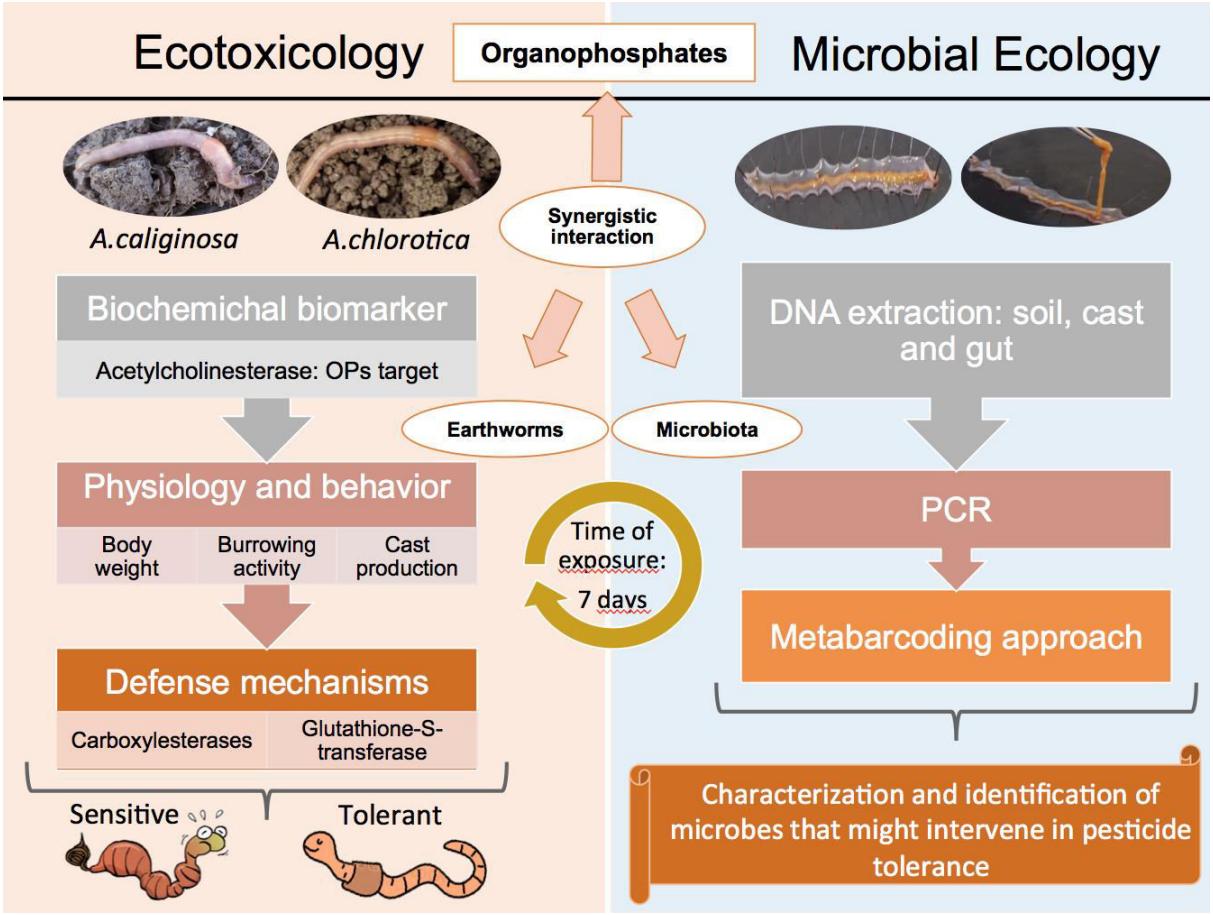


Figure 36 : Graphical abstract illustrating ecotoxicological and microbial experimental design.

In the first part, we investigated the response of earthworms using biomarkers at physiological, biochemical and behavioral levels. Accordingly, we confirmed that the species *A.chlorotica* is more tolerant to the organophosphate insecticide ethyl-parathion than *A. caliginosa*. Our results showed that a short-term exposure to ethyl-parathion (7 days) caused a marked interspecific difference in the response of both biochemical and behavioral biomarkers. *A.caliginosa* was shown to be sensitive to ethyl-parathion exposure (Rault at al. 2018; Jouni et al. 2018). This study pointed out that even in the same ecological group of earthworms, which display the same

behavior, mechanisms related to pesticide bioactivation and metabolism could contribute significantly to species-specific differences in responses to pesticide sublethal toxicity. Therefore, we suggested that the inclusion of more than one species from the same ecological group is relevant, because of the different responses of species to pesticide exposure.

Then, we hypothesized that CbEs enzymes could intervene in *A.chlorotica* tolerance to ethyl-parathion. Effectively, CbEs were reported to play an important role as pesticides bioscavengers, due to their higher sensitivity to OP inhibition compared to AChE sensitivity. Their numbers in addition to their catalytic mechanism constitute an important factor in the OP toxicity (Wheelock et al. 2005; Kristoff et al. 2010; Gong et al. 2017). Surprisingly, the basal CbE activity of unexposed individuals of *A. caliginosa* was two-times higher than that of *A. chlorotica,* and the abundance of CbE isozymes was higher in *A. caliginosa* compared with that in *A. chlorotica.* Added, the *in-vivo* exposure trial revealed that CbE activity of *A. caliginosa* was more sensitive to inhibition by the insecticide than *A. chlorotica*. In this occurrence, we expected that *A. caliginosa* would be able to reduce the impact of ethyl-parathion through its higher levels and sensitivity of CbE activity. Nevertheless, changes in behavior biomarkers, particularly cast production, and weight suggested the contrary. Therefore, we established a cause-effect relationship between AChE inhibition and the alteration of other mechanisms such as a loss in the body weight and a strong threshold effect in the cast production (Fig. 37). This was particularly observed in *A. caliginosa* species. It was documented that the gut motility of annelids is known to be under cholinergic control. Then AChE inhibition is expected to alter gastrointestinal transit of food, which induce modification in the cast production rate (Fieber 2017). In our experiments, we confirmed that changes in body weight and cast production could be linked to the inhibition of AChE activity. These alterations induced a disruption of advantageous functions of earthworms in soil bioturbation, via their feeding and castings abilities, which can influence other soil component since earthworms are crucial candidates in the soil food web.

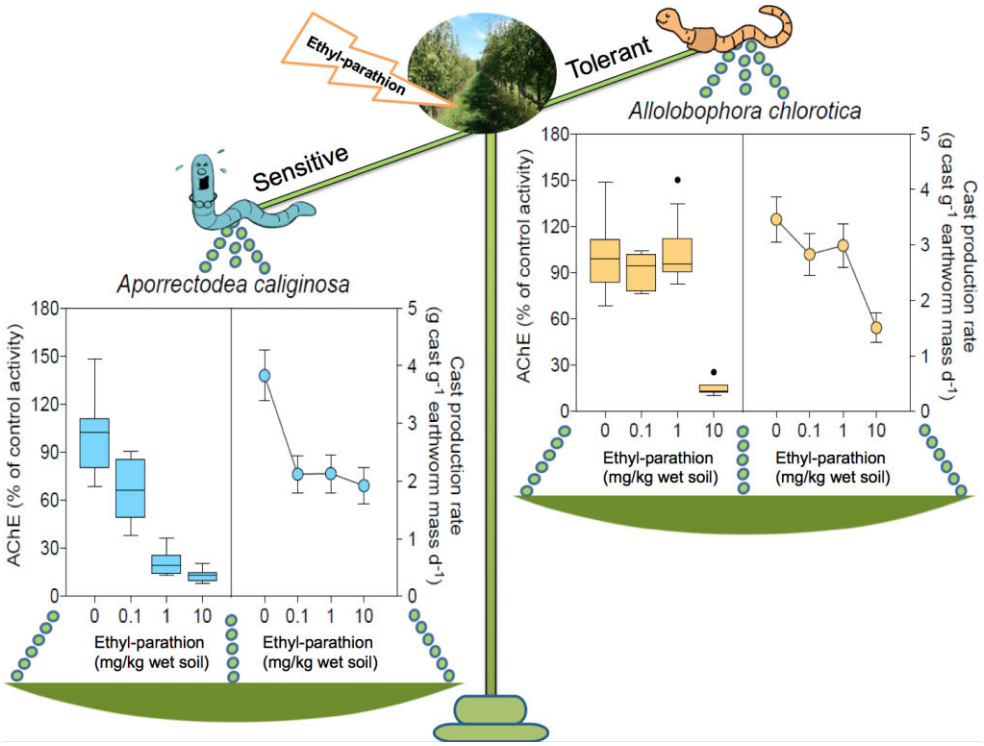


Figure 37 : Graphical abstract on biochemical and behavioral responses

From an ecotoxicological point of view, Chambers and Carr (1995) reported that the interspecific differences in OP susceptibility might also be explained by differences in the intrinsic AChE sensitivity to oxon metabolites of OPs. To test this hypothesis, we conducted an *in-vitro* experiment trial to study the intrinsic AChE sensitivity to ethyl-paraoxon. Our results revealed that the activity of *A. caliginosa* AChE was more sensitive to ethyl-paraoxon than that of *A. chlorotica*. Therefore, the intrinsic sensibility of AChE of *A.caliginosa* to this OP insecticide could be a significant cause for interspecific differences in sublethal toxicity between these species. However, the same *in-vitro* trial conducted using chlorpyrifos-ethyl, another OP, showed that the activity AChE of the species *A.chlorotica* was significantly inhibited. Thus, the intrinsic sensitivity of AChE is dependent on the species as well as the type of the OP molecule. This clearly shows that other mechanisms of metabolization intervene in the transformation and detoxification of OPs, then in the toxicity of the molecule inside the organisms, for example the carboxylesterases and GST.

To follow previous results, we characterized the *in-vitro* responses of both carboxylesterases and glutathione-S-transferase, in both earthworm species. Our

results showed that difference in the tolerance of both species to OPs did not seem to depend on the catalytic properties of CbEs and GST. The responses of the CbEs and GST to the OP exposure was more or less the same in both species. Finally, studies reported an important mechanism involved in metabolism and detoxification of both internally generated compounds and xenobiotic, called CYP450 enzymes (Chakrapani et al. 2008; Sanchez-Hernandez et al. 2014). We are aware that the study of these enzymes could improve our understanding about the toxicity of both OP in both species. Added, this could also elucidate the possible implication of these enzymes in the species-specific differences of both worms according to studies suggesting that CYP450 protein levels varied by species (Lu et al. 2017).

- In perspective, the biochemical characterization of the CYP450 enzymes could complete our understanding to toxicological interactions and mechanisms of action of this insecticide. These enzymes are essential for the detoxification of chemicals and the metabolism of drugs (Li et al. 2018).

Above all, our results in the first part did not fully explain the mechanisms that helped *A.chlorotica* to tolerate this OP. Therefore, we employed conjointly a DNA metabarcoding approach, for a better understanding of the complex interactions earthworms-microbiota that might intervene in insecticide tolerance and detoxification, and we followed the soil enzyme activities in two different soil compartments: bulk soil and cast.

The application of pesticides tends to have long persistence in the soil. They influence soil organisms and disturb soil health. In addition, they alter soil functions and properties like nutrient content, soil organic carbon as well as soil microflora and activities of enzymes. However, such effects depend on many biotic and abiotic factors ranging from soil features to the ability of some organisms to degrade pesticides. Crucially, a number of pesticides are transformed or degraded by soil microbes with their appropriate enzymes, then, the pesticides can be used as a source of energy or nutrient (Prashar & Shah 2016). In this context, it was of critical importance to investigate the implication of the earthworm-gut microbiome in the activation of microorganisms involved in the biotransformation of pesticides. In this second part, we focused on the identification of microbes present in the digestive

tract of the earthworms, as well as microbes in ingested soil and casts, after exposure to ethyl-parathion.

In this part we aimed to (1) compare the microbial communities of ingested soil, cast and gut in order to apprehend if the earthworm-gut harbors a specific microbiome; (2) characterize the gut-microbiome of both earthworms' species in order to link the identified microbes to some functions involved in the biodegradation of pesticides; (3) assess the role of earthworm on soil enzyme activities in the presence or absence of an OP insecticide.

Microorganisms are important items in the earthworm diet (Lemtiri et al. 2014), but the strongest evidence to date is that the gut of earthworms act as a biological filter for ingested soil (Singleton et al. 2003; Drake & Horn 2007; Byzov et al. 2007). On the basis of these collective observations, studies showed the occurrence of a specific gut microbiome in the gut of earthworms (Thakuria et al. 2010). Our results agreed with these findings, as we showed that the microbial community between ingested soil, gut and cast were significantly different. It is true that the gut-microbiota and cast of earthworm are dominated by the microbiota of preingested material, but we showed that their abundances are significantly different, some phylum as acidobacteria dominated in soil but not in gut and the gut of both species were dominated by actinobacteria, which is different from soil. Indeed, in spite of their belonging to the same ecological group, microbial community in the gut of both species was significantly different. Thakuria et al. (2010) showed that the strongest determinant selection of bacteria associated with the gut wall of earthworms is ecological group followed by habitat and then, species. Our findings confirmed this hypothesis showing that even in the same ecological group, the gut microbiome varied between the species.

In the last two decades, the isolation and characterization of microbial strains able of degrading pesticides and their residues has received much attention. Bacteria and fungi were the potential candidates in pesticides biodegradation.

Inside the gut of earthworms, the digestion of soil organic matter takes place through a mutualistic relationship between ingested microflora and earthworm (Trigo & Lavelle 1993; Lemtiri et al. 2014). In this area, a direct contact occurs between soil, microorganisms and pollutants. In effect, the bioturbation of soil by earthworms affect

also the fate of pesticides. For the herbicide atrazine, it was reported that earthworm increases it persistence (Binet et al. 2006). Otherwise, earthworms' activity was also reported to impact microorganisms activity that enhance the mineralization of this herbicide (Kersanté et al. 2006). In addition, other studies documented that *A. caliginosa nocturna* participate in the metabolization of pesticides (Schreck et al. 2008). However, none of these studies have considered conjointly the biochemical and microbial mechanisms for pesticides degradation in earthworms.

Concerning soil enzyme activities, our results showed a significant impact of the soil texture rather than an impact of earthworms' species. In parallel, we used the IBRv2 index that allows a quick visualization of the impact of ethyl-parathion on soil enzymes. Our findings showed a low score of this index in the earthworms' casts. Since, casts represent the soil that passed through the gut; we can suggest that the pesticide was detoxified or degradated at the gut level. In this line, Azadeh and Zarabi 2014 reported that most biotransformation and biodegradation of chemicals are done in the earthworm gut.

In our study, the outcomes of the first part (ecotoxicology) have elucidated some of the responses of both earthworms' species to ethyl-parathion exposure, at the biochemical and behavioral levels. In the second part (microbial ecology), we identified and characterized microbial community in soil, gut and cast. The relevant issue of this characterization at the level of gut microbiota, is the presence of the *Rhodococcus* strain, which is capable of degrading endosulfan as well as organophophates (Verma et al. 2011; Sirotkina & Efremenko 2014; Singh et al. 2015). Our results showed a higher abundance of this genus within the gut of *A.chlorotica,* which could be correlated to its tolerance to ethyl-parathion.

- In perspective, an isolation and functional analysis of the microbes inside the earthworm gut (bacteria and fungi) is needed to confirm our results. At this level, it is important to understand the microbial metabolism that intervene in the degradation of the OP, and the eventual metabolites formed after the degradation process. This can be used in bioremediation that offer an efficient and less expensive option for decontamination of damaged habitats.

Finally, the results of this thesis pointed out the need to develop a functional analysis at the level of the earthworm itself and the microbial communities. This could be

evaluated using toxicogenomic techniques focused on selected physiological functions. As well, metaproteomics and metabolomics approaches could help to investigate the network of metabolic interactions between earthworms and its bacterial symbionts.

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