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Impacts comportementaux, démographiques et fonctionnels des pesticides sur des annélides oligochètes du sol

Sylvain Bart

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Impacts comportementaux, démographiques et fonctionnels des pesticides sur des annélides oligochètes du sol

Thèse de doctorat de l'Université Paris-Saclay
préparée à AgroParisTech (l'Institut des
sciences et industries du vivant et de
l'environnement)

École doctorale n°581 agriculture, alimentation, biologie,
environnement et santé (ABIES).

Spécialité de doctorat: Sciences de l'environnement.

Thèse présentée et soutenue à Versailles, le 5 octobre 2018, par

Sylvain Bart

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Introduction générale

Depuis la première révolution industrielle, les activités humaines ne cessent d'engendrer des problèmes environnementaux, depuis la pollution des cours d'eau, des sols et de l'air, jusqu'à la destruction complète d'habitats. La survie de l'homme est pourtant directement liée à son environnement. Par conséquent, les pouvoirs publics ont pris en mains ces problématiques avec notamment la mise en place de l'évaluation des risques écologiques (ÉRE). Celle-ci s'intéresse aux effets des produits chimiques, ou toutes autres contaminations d'origine anthropique sur l'environnement. Dans certains domaines particuliers, tels que les pesticides, l'Autorité européenne de sécurité des aliments (EFSA) est tenue par la législation de l'Union européenne de réaliser une ÉRE. En France, c'est l'ANSES (Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'Environnement et du travail), créée le 1er juillet 2010, qui se doit de réaliser les ÉREs. L'évaluation du risque concerne tous les compartiments de l'environnement et notamment les sols qui ont été récemment reconnus comme patrimoine commun de la nation par la loi biodiversité 2016.

I. Le sol et sa faune

Le sol est issu de la transformation de la roche mère et correspond à la couche superficielle de la croûte terrestre. Il est constitué de particules minérales et organiques et contient également de l'eau et de l'air. C'est un compartiment clé des écosystèmes terrestres, source essentielle d'une grande diversité de services écosystémiques (MA, 2005). Ces services représentent les bénéfices offerts aux sociétés humaines par les écosystèmes. Le sol est tout d'abord le support des productions agricoles, sylvicoles et pastorales (services de production) qui sont possibles grâce au cycle des nutriments et à la production primaire (Wall, 2004). Il participe également au service de régulation du climat, en contrôlant les flux de gaz à effet de serre et en participant à la séquestration du carbone (Minasny et al., 2017). Le sol est ainsi essentiel pour l'homme et son environnement et il est considéré comme une ressource non renouvelable. Son fonctionnement est en grande partie assuré par la grande biodiversité qu'il héberge (Wolters, 2001) et parmi celle-ci, la faune édaphique a été largement reconnue comme essentielle à son fonctionnement (Lavelle, 1997; Hunt et Wall, 2002; Scheu et al., 2002; Fitter, 2005).

Cette faune est habituellement classée en fonction de la taille des individus (Tableau 1). Cette classification est largement acceptée par la communauté scientifique, bien qu'elle possède ses limites. En effet, plusieurs taxons peuvent par exemple appartenir à deux catégories de taille selon leur stade de développement. Cette classification se compose tout d'abord de la microfaune (< 0,2 mm) qui est représentée par les protistes et les nématodes. Ensuite, la mésofaune (0,2 à 2 mm) comprend les microarthropodes, dominés par les acariens, les collemboles, et les enchytréides (qui sont des annélides oligochètes). Enfin la macrofaune (2 à < 20 mm) est composée d'arthropodes, de mollusques et de vers de terre (qui sont eux aussi des annélides oligochètes). Ces derniers sont les organismes les plus abondants en termes de biomasse animale dans les écosystèmes terrestres tempérés (Lavelle et Spain, 2001; Pelosi, 2008).

Tableau 1. Répartition de la faune dans un sol de région tempérée (Bachelier, 1978).

Classe	Taille (∅)	Organismes	Densité (m ²)
Microfaune	< 0.2 mm	Protozoaire	10 ⁸ à 50 ⁸
		Nématodes	10 ⁶ à 20 ⁶
Mésofaune	0.2 à < 2 mm	Microarthropodes < 2 mm	
		-Collemboles	20 ³ à 500 ³
		-Acariens	20 ³ à 500 ³
		Enchytréides	10 ⁴ à 50 ⁴
Macrofaune	2 à < 20 mm	Lombriciens	50 à 400
		Mollusques	10 à 10 ²
		Arthropodes > 2 mm	10 ³ à 10 ⁴

II. Les vers de terre, des annélides oligochètes du sol

1. Classification systématique, écologique et rôle fonctionnel dans les sols

Parmi les organismes de la faune du sol, la classe des annélides oligochètes comprend 3 ordres : les Lumbriculidae, les Moniligastridae et les Haplotaxidae. Dans ce dernier ordre, on retrouve le sous ordre Lumbricinae, caractérisé par un clitellum (bourrelet tégumentaire présent chez les individus adultes) formé de multiples couches de cellules (les autres oligochètes ont un clitellum composé d'une seule couche de cellule) (Sims and Gerard, 1999). Ce sous-ordre, appelé aussi communément « vers de terre » représente 13 familles et

plus de 6000 espèces décrites à ce jour sur tous les continents, excepté l'Antarctique. La famille des lombricidés fait partie de ce sous-ordre, et comprend les principaux vers de terre de nos régions tempérées. Les vers de terre sont une composante majeure de la macrofaune des sols agricoles et en générale des écosystèmes terrestres. Ils représentent en effet une part de biomasse importante dans les sols, pouvant parfois atteindre plusieurs tonnes par hectare (Lavelle et Spain, 2001). On peut ainsi retrouver jusqu'à 700 individus par mètre carré en prairie. En France, le nombre d'espèces de lombriciens décrites est d'environ 180 (Bouché, 1972).

Bouché (1972) a également proposé une classification écologique des vers de terre, basée entre autres sur leur habitat, leur comportement et leurs ressources alimentaires. Cette classification comprend les vers de terre épigés, endogés et anéciques (Figure 1).

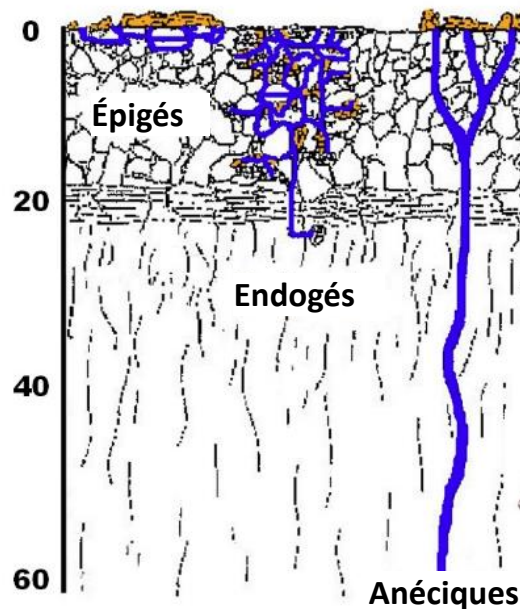


Figure 1. Classification écologique des vers de terre (<https://www.sciencelearn.org>.)

Les vers épigés (Photo 1. A) sont des vers pigmentés de petite taille (1-8 cm) qui vivent à la surface du sol et dans la litière. Ils se nourrissent de matières organiques peu décomposées. Ces espèces sont peu présentes en champs cultivés où la surface des sols est souvent pauvre en matière organique en décomposition.

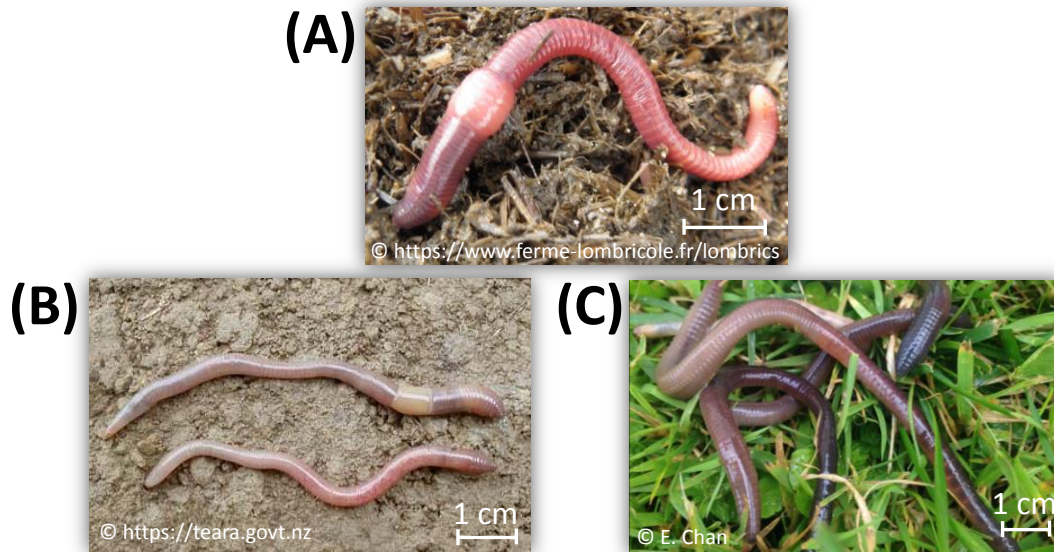


Photo 1. Exemple d'(A) espèce épigée (*Eisenia fetida*), (B) espèce endogée (*Aporrectodea caliginosa*) et (C) espèce anécique (*Aporrectodea giardi*).

Les vers endogés (Photo 1. B) sont des vers non pigmentés, de taille moyenne (3-16 cm), vivant généralement dans les 15 premiers centimètres du sol où ils creusent des galeries subhorizontales (Figure 2). Les endogés se nourrissent d'un mélange de fraction minérale et de matière organique du sol. Ce sont les espèces que l'on retrouve le plus en milieu agricole.

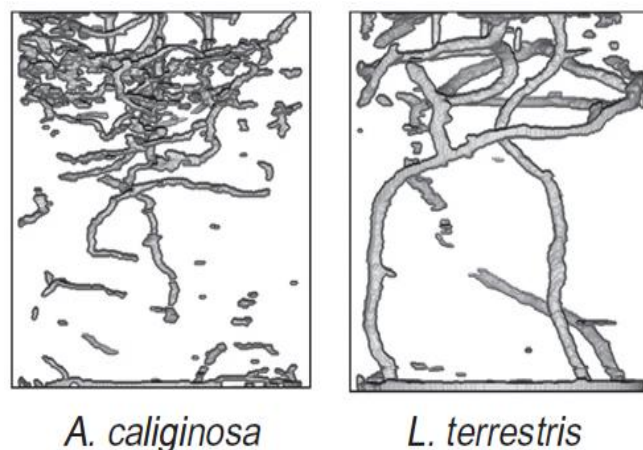


Figure 2. Exemple de réseaux de galeries d'une espèce endogée (*A. caliginosa*) à gauche, et d'une espèce anécique (*L. terrestris*) à droite. (Dittbrenner et al., 2011)

Les vers anéciques (Photo 1. C) sont des vers pigmentés, de grandes tailles (10-100 cm), vivant dans des galeries permanentes verticales et se nourrissent de matière organique à la surface du sol (Figure 2). Ces vers sont peu nombreux en champs cultivés de manière

intensive de par la faible teneur en matière organique en surface mais aussi de par leur forte sensibilité au travail du sol (charrue, labour...) qui détruit leurs galeries (Pelosi et al., 2016b).

Les vers de terre ont une grande influence sur les processus physiques, chimiques et biologiques du sol et sont considérés comme des ingénieurs de l'écosystème (Jones et al., 1994 ; Jouquet et al., 2006). La production de galeries par les vers de terre endogés et anéciques change la structure du sol en modifiant la macroporosité (Figure 2) (Jégou et al., 1998; Capowiez et al., 2001). Il a été montré que ces galeries ont un rôle majeur dans la stabilité du sol soumis à une compaction intentionnelle (Schrader et al., 2007). Les galeries jouent aussi un rôle clé dans la dynamique de l'eau, en améliorant son infiltration dans le sol. Ehlers (1975) a par exemple montré qu'après 10 ans d'inoculation de vers de terre, le taux d'infiltration de l'eau à travers le sol est passé de 15 à 27 mm heure⁻¹. De plus, en améliorant l'infiltration de l'eau, les galeries de vers de terre peuvent aider à réduire l'érosion du sol de 50% (Sharpley et al., 1979; Shuster et al., 2002). Elles vont aussi favoriser les échanges gazeux dans le sol (Figure 3) (Blouin et al., 2013) et la pénétration des racines des plantes (Figure 3) (Baker, 2004). Enfin, les galeries favorisent aussi la croissance des racines puisque ce sont des sites riches en éléments nutritifs (Canellas et al., 2002).

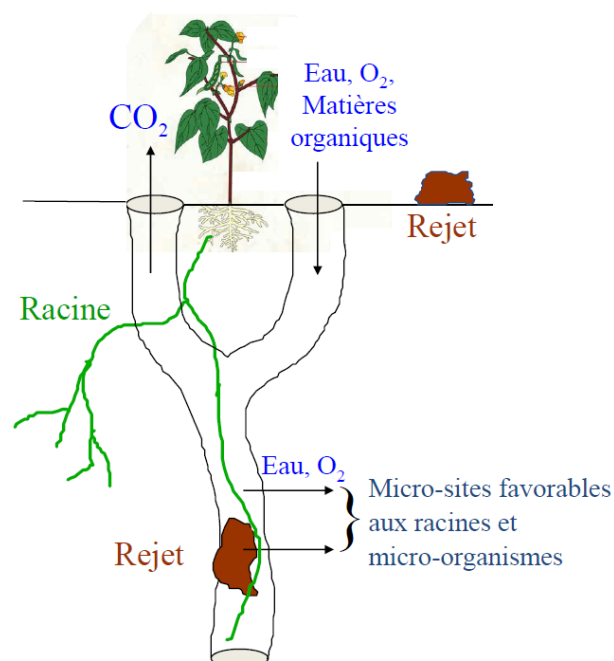


Figure 3. Rôles fonctionnels des galeries de vers de terre (©Yvan Capowiez)

Les vers de terre sont impliqués dans la dégradation de la matière organique, provenant majoritairement des résidus végétaux. Les vers épigés participent à la dégradation de la

matière organique de surface, principalement par fragmentation. Les vers anéciques participent en plus activement au phénomène de bioturbation en permettant l'incorporation dans le sol de la matière organique de surface qu'ils consomment. Les vers endogés (et anéciques) vont participer au brassage de la matière organique et du sol, et à sa dégradation. Après digestion, ils vont excréter des fèces appelés turricules, qui sont riches en éléments nutritifs directement assimilables par les plantes, ou sous forme de grosses molécules organiques, minéralisables par les microorganismes. Il a en effet été mis en évidence que les turricules de vers de terre sont des sites favorables pour la nitrification et la dénitrification microbiennes (Palmer et al., 2005; Costello et Lamberti, 2008). L'activité des microorganismes est donc fortement favorisée par la présence des vers de terre. Les activités enzymatiques sont plus fortes dans les parois des galeries que dans le sol environnant (Jégou et al., 2001). Celles-ci sont en effet des sites favorables aux microorganismes (Santruckova et al., 1993; Zhang et Schrader, 1993). Ceci résulte d'un effet direct du ver de terre qui enrichit les galeries par un enfouissement de la matière organique en profondeur (principalement les anéciques) et par une production de composés organiques (mucus) qui tapissent la galerie. Les vers de terre augmentent ainsi la teneur en azote du sol, mais aussi en phosphore (Suarez et al., 2004), en potassium, en calcium et en magnésium (Adejuyigbe et al., 2006).

L'ensemble des fonctions écologiques remplies par les vers de terre sont bénéfiques pour les cultures végétales et contribuent à la durabilité des systèmes agricoles (Bertrand et al., 2015).

2. Biologie et traits d'histoire de vie

Les vers de terre sont des invertébrés dits segmentés ou métamérisés. Ainsi, leur corps se présente sous la forme de segments circulaires consécutifs et le nombre de segments est relativement constant au sein d'une espèce. Le premier segment, le peristomium, entoure l'ouverture buccale et porte dorsalement un lobe charnu dirigé vers l'avant, le prostomium (Figure 4). C'est sur les segments suivants que l'on retrouve les différents organes, notamment les organes sexuels dont la localisation et la forme varient d'une espèce à l'autre et permettent l'identification au *genre* et à l'*espèce*.

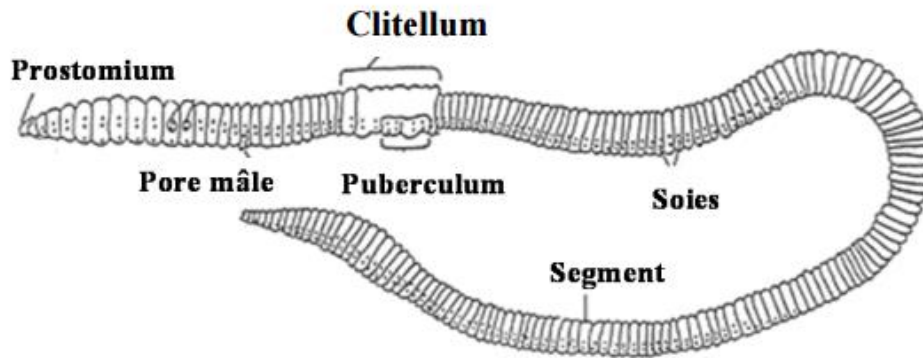


Figure 4. Représentation schématique d'un ver de terre

Le stade adulte est caractérisé par l'apparition du clitellum (Figure 4), qui est une partie renflée de l'épiderme formant une sorte d'anneau. Sur la partie ventrale du clitellum se trouve le puberculum, qui est un organe impliqué dans l'accouplement. Les vers de terre sont tous hermaphrodites (Sims and Gerard, 1998) et suite à un accouplement, une autofécondation, ou par parthénogénèse (selon les espèces), un cocon est déposé dans le sol *via* le clitellum. Le temps de maturation des cocons (photo 2) dépend de l'espèce, tout comme le nombre de juvéniles par cocons, mais également des conditions pédoclimatiques (i.e. température et humidité) (Jensen et al., 1998, Holmstrup et al., 1991).

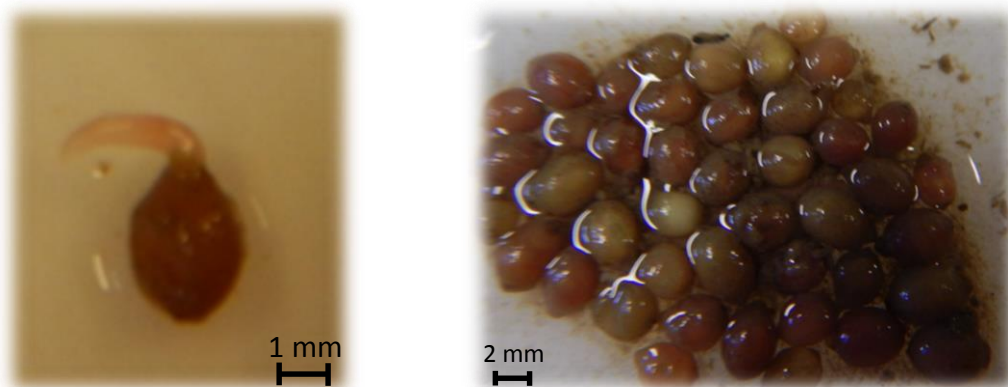


Photo 2. Cocons d'*Aporrectodea caliginosa*. A gauche, un ver juvénile sortant de son cocon. A droite, des cocons à différents stades de maturation, du jaune pour les cocons récemment déposés, au rouge foncé pour les cocons prêts à éclore. (Photo © Sylvain Bart)

La croissance des juvéniles dépend elle aussi des conditions pédoclimatiques et de l'espèce considérée. Les épigés ont une croissance plus rapide que les espèces des autres catégories écologiques (e.g. 5 à 6 semaines de l'éclosion jusqu'au stade adulte, à 20 °C pour *Eisenia fetida*) (Edwards et Bohlen, 1996; Edwards, 1998; Gunadi et al., 2002). En revanche, une espèce endogée comme *A. caliginosa* aura un temps de croissance, de l'éclosion

jusqu'au stade adulte, de l'ordre de 3-4 mois en laboratoire à 15 °C (Bart et al., 2018). Les espèces anéciques, quant à elles, mettent plus d'une année de l'éclosion jusqu'au stade adulte (Pelosi, 2008 pour *Lumbricus terrestris*). Au champ, les périodes d'activités des vers de terre se situent à l'automne et au printemps. A ces périodes, l'humidité et la température du sol (10 - 20 °C) sont propices à leur développement et leurs activités. Cependant, ces périodes coïncident avec celles de la mise en œuvre de certaines pratiques agricoles comme le travail mécanique du sol et l'utilisation de pesticides, qui peuvent perturber le cycle de vie des vers de terre.

III. Pesticides et vers de terre

1. Les pesticides et leur évaluation réglementaire

Les pesticides, produits phytopharmaceutiques (PPP) ou produits phytosanitaires sont définis par la directive européenne 91/414/CEE comme étant des substances actives et préparations contenant une ou plusieurs substances actives. Ces produits sont utilisés pour la prévention, le contrôle et l'élimination d'organismes jugés indésirables, qu'il s'agisse de plantes, d'animaux, de champignons ou de bactéries. Le terme pesticide regroupe les produits utilisés pour la protection de cultures végétales mais aussi les régulateurs de croissance des plantes, les défoliants, les agents de dessiccation ou les produits utilisés après la récolte pour protéger contre la détérioration durant le stockage et le transport. On retrouve aussi les pesticides à usage non agricole désormais appelés « produits biocides » au sens de la directive 98/8/CE. Ceux-ci sont destinés à des usages domestiques tels que la protection du bois contre les champignons ou les termites, la lutte contre les insectes ménagers ou contre les parasites (anti-acariens, antipuces).

Dans le cadre de la protection des cultures, les principales classes de pesticides sont les fongicides, les herbicides et les insecticides qui, à eux trois, représentaient plus de 88% des pesticides vendus en France métropolitaine en 2015 (Figure 5, UIPP 2016). Les pesticides sont vendus et utilisés sous forme de formulations (liquides, solides, voir gazeuses) afin de rendre leur utilisation la plus efficace possible. Ces formulations contiennent ainsi une ou plusieurs substances actives associées à différents adjuvants tels que des adhésifs, des agents antimoussants, des antigels, des liants, des agents dispersants ou des agents

d'appétence (pour attirer les nuisibles), pour ne citer qu'eux. Ces derniers sont inconnus dans la composition des formulations commerciales car il s'agit de « secrets de fabrication » des industriels.

L'utilisation des pesticides pour la protection des cultures remonte à l'antiquité, le soufre étant déjà utilisé 1000 ans avant J.C en viticulture. Le naturaliste romain Pline recommandait déjà au 1^{er} siècle l'utilisation de l'arsenic, comme insecticide. Au cours du 19^{ème} siècle, la chimie minérale s'est développée et a permis la production de nombreux pesticides minéraux à base de cuivre tel que la bouillie bordelaise utilisée pour prévenir les invasions fongiques comme le mildiou de la vigne. La chimie organique de synthèse s'est ensuite développée grâce aux recherches menées sur les armes chimiques durant la 1^{ère} guerre mondiale. Celles-ci ont abouti au développement des premiers pesticides de synthèse dans les années 1930. Les recherches menées lors de la seconde guerre mondiale ont aussi contribué à l'apparition de nouveaux produits tels que les pesticides de la famille des organophosphorés. Depuis, les progrès en chimie de synthèse ont permis le développement d'une vaste gamme de produits. Le recours systématique aux pesticides s'est généralisé après la seconde guerre mondiale pour répondre à une demande agricole forte. L'agriculture dite « conventionnelle » est aujourd'hui dépendante de l'usage de ces produits.

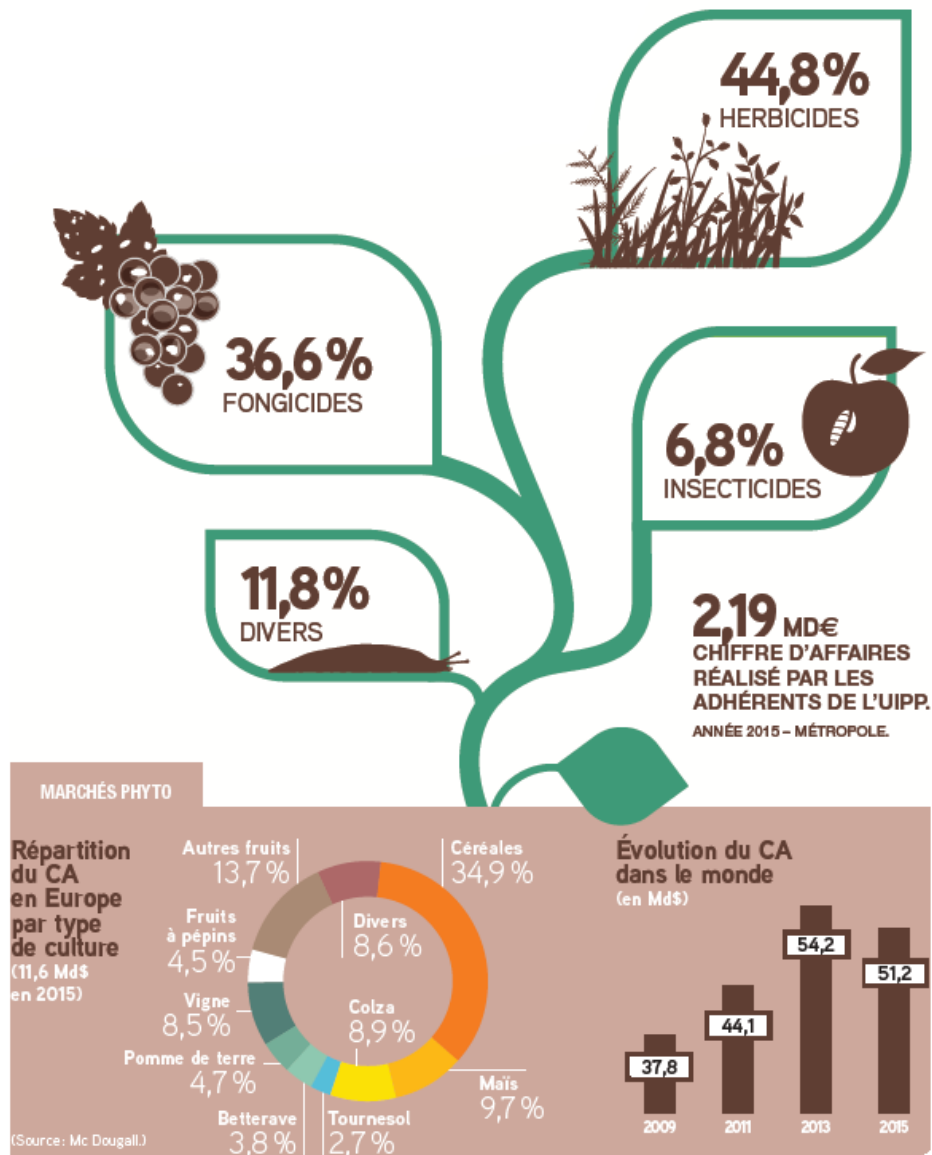


Figure 5. Le marché des pesticides en France et dans le monde (source : UIPP, Union des Industries de la Protection des Plantes. Repères 2016-2017. <http://uipp.org/flipbook/#page/1>)

L'utilisation des pesticides a permis un accroissement considérable de la production agricole durant les 60 dernières années mais dès les années 1960, la publication du livre de Rachel Carson, le « Printemps silencieux » (1962), a provoqué une prise de conscience des potentiels effets néfastes des pesticides sur la santé humaine et l'environnement. Cet ouvrage a été un facteur de l'émergence de l'écotoxicologie en tant que discipline scientifique. Le terme d'écotoxicologie fut proposé en 1969 lors d'une réunion d'un Comité *ad-hoc* de l' « *International Council of Scientific Unions* » par René Truhaut puis défini par lui-

même (Truhaut, 1975). Elle se définit comme la science qui étudie le comportement et les effets toxiques d'agents d'origine anthropique (tel que les pesticides) sur les écosystèmes, ou bien de produits d'origine naturelle mais dont l'homme a modifié la répartition dans les écosystèmes. L'écotoxicologie étudie les mécanismes et les conséquences écologiques de la pollution à l'échelle des populations, des communautés et de l'écosystème. Cette discipline recouvre aussi les études sur des organismes, voire au niveau cellulaire, dans la mesure où la compréhension des mécanismes à l'échelle individuelle aide à mieux aborder les effets sur les populations et les communautés qui composent les écosystèmes.

Ainsi, les premières études emblématiques sur les effets écotoxicologiques de l'utilisation des pesticides concernaient le DDT (dichlorodiphényltrichloroéthane). Cet insecticide a été commercialisé dès 1943 et a dominé le marché des insecticides jusqu'au début des années 1970. Cependant, des effets toxiques très importants ont été observés sur des populations d'oiseaux piscivores (mangeurs de poissons) et des oiseaux de proie, à travers un amincissement des coquilles d'œufs, réduisant le succès de reproduction (Hickey et al., 1968; Peakall et al., 1970; Ratcliffe, 1970). Ainsi, pour éviter tout risque pour l'homme et son environnement, l'utilisation des pesticides est aujourd'hui réglementée et les procédures d'évaluation du risque liée à leur utilisation sont en constante évolution.

La mise sur le marché des pesticides ne peut se faire qu'au travers la soumission d'un dossier d'autorisation de mise sur le marché (AMM) à l'EFSA (European Food Safety Authority) au niveau européen, et à l'ANSES (Agence Nationale de Sécurité Sanitaire de l'Alimentation) au niveau français. Ce dossier vise à évaluer l'efficacité sur les cultures cibles et l'innocuité des substances actives, ou des formulations commerciales, pour l'homme et l'environnement. Concernant la partie environnement, il doit, entre autres, faire mention de la toxicité du pesticide pour les invertébrés du sol, représentés par les vers de terre. Une façon pratique de caractériser la toxicité d'un pesticide consiste à estimer des paramètres couramment utilisés en toxicologie:

- La CL50 (Concentration Létale 50%) est la concentration qui cause la mort de 50 % des individus d'une population. Elle est utilisée pour caractériser une toxicité aiguë. Equivalent: DL50 (Dose létale 50%); LC50 (Lethal Concentration 50%).
- La CE50 (Concentration Efficace 50%) est la concentration qui induit 50% d'effet. Elle est utilisée pour caractériser une toxicité chronique et peut se calculer sur différentes

réponses à partir de la courbe dose-réponse (reproduction, comportement, croissance,..). Equivalent: EC50 (Effective Concentration 50%).

- La CSEO (Concentration Sans Effet Observable) est la plus forte concentration pour laquelle aucun effet n'a été observé. Equivalent: NOEC (No Observed Effect Concentration)

Pour déterminer ces paramètres sur les vers de terre, il existe des tests normalisés au niveau international (ISO et OCDE). Le test de toxicité aiguë de l'ISO 11 268-1 (2012) permet d'évaluer la toxicité aiguë sur 14 jours afin d'estimer la CL50. Le test de reproduction de l'ISO 11 268-2 (2012) ou de l'OCDE (2004) permet l'évaluer la reprotoxicité des produits *via* un test chronique de 28 jours qui permet d'estimer la CE50 reproduction. Il existe aussi des tests d'évitement (ISO 17512, 2008), qui permettent d'évaluer la capacité des vers de terre à choisir entre un sol contaminé et un sol contrôle (test à deux compartiments). Dans le cadre des dossiers d'AMM, seul le test sur la reproduction suivant l'OECD 2004 est aujourd'hui obligatoire (EFSA, 2017).

L'ensemble de ces tests standardisés recommandent l'utilisation de l'espèce *Eisenia fetida* (*fetida* ou *andrei*). L'élevage de cette espèce peut être relativement facile en conditions contrôlées et son cycle de développement est relativement court (8 semaines en laboratoire à 20 °C) (Venter et Reinecke, 1988). Toutefois, cette espèce épigée, souvent appelée « ver de compost », est rarement présente en champs cultivés en raison de la faible quantité de matière organique à la surface du sol. Sa représentativité par rapport à la réalité de l'exposition et des effets sur le terrain pose donc question. De plus, une récente méta-analyse réalisée par Pelosi et al. (2013) a mis en avant que l'espèce *E. fetida* était 2.5 et 3.5 fois moins sensible (basé sur les CL50) aux pesticides et métabolites que deux autres espèces très représentatives des champs cultivés que sont *Lumbricus terrestris* et *Aporrectodea caliginosa*.

2. Evaluation des effets des pesticides sur les vers de terre dans la littérature scientifique

Au-delà de ces aspects réglementaires, il existe dans la littérature scientifique de nombreuses études qui réalisent des évaluations du risque à *posteriori* (i.e. après l'autorisation de mise sur le marché). Ces études vont plus loin que les études réalisées *a priori* (dans le cadre des dossiers d'AMM). Tout d'abord, elles font appel à des espèces différentes, plus représentatives des conditions au champ. Ensuite ces études reposent sur des tests différents de ceux proposés par les normes ISO et OCDE, ou considèrent des niveaux d'organisations biologiques différents, de l'infra-individuel jusqu'à la communauté, tout en essayant de relier les effets observés à l'impact sur les fonctions écologiques remplies par les vers de terre.

Les études au niveau d'organisation infra-individuel se situent au niveau de l'expression des gènes, des dommages à l'ADN, de la physiologie ou des structures cellulaires. L'évaluation de ces effets a permis le développement biomarqueurs permettant d'approcher l'état de stress d'un organisme vis à vis d'une contamination (un pesticide par exemple) (Week, 1995). Le concept de biomarqueurs repose sur la relation entre l'exposition à une contamination chimique et tout changement biologique induit par celle-ci. Ce niveau d'organisation permet l'utilisation d'outils pour évaluer l'état de stress d'un organisme et comprendre les mécanismes sous-jacents de réponse des individus à ce stress. Cependant, à ce jour, Les liens entre ce qui se passe d'un point de vue écotoxicologique à ce niveau infra-individuel et ce qui se passe au niveau de l'individu ou de la population ne sont pas bien établis. Il est par conséquent difficile d'extrapoler les effets observés au niveau infra-individuel à des conséquences écotoxicologiques de l'épandage des pesticides pour les populations de vers de terre.

Le niveau d'organisation supérieur est celui de l'individu, intégrant les traits d'histoire de vie des individus (mortalité, croissance, reproduction) et leur comportement. L'étude des effets des pesticides à ce niveau d'organisation est réalisée à l'aide d'expérimentations de laboratoire, en conditions contrôlées. L'étude de la mortalité fut, dans les années 1980, le paramètre le plus étudié, permettant l'estimation de la CL50. Les CL50 sont utiles pour évaluer la toxicité aiguë d'un produit et comparer les différences de sensibilité entre taxons. Cependant, elles sont insuffisantes pour évaluer les conséquences écotoxicologiques à long terme de l'épandage des pesticides qui peut induire des effets drastiques à des

concentrations beaucoup plus faibles que les CL50 sur les traits d'histoire de vie des vers de terre. Neuhauser et al. (1985) explique ainsi que la reproduction peut être inhibée ou altérée à des concentrations bien en dessous des CL50 préalablement estimées. Il a en effet été mis en évidence que les CSEO (concentration sans effet observable) sont, en écotoxicologie terrestre, la plupart du temps bien plus faibles que les CL50 (van Gestel et al., 1992). Il est ainsi rapidement apparu que la reproduction et la croissance étaient des paramètres plus sensibles que la mortalité et écologiquement plus pertinents. En effet, un pesticide peut ne présenter aucune létalité à une concentration donnée, mais affecter les traits d'histoires de vie et donc la dynamique des populations et le bon fonctionnement du sol (Slimak, 1997; Luo et al., 1999; Lal et al., 2001).

Concernant les effets sur le comportement, il existe tout d'abord le phénomène d'évitement des zones contaminées, possible grâce à des récepteurs sensoriels (par exemple des chimiorécepteurs) présents chez les vers de terre (ISO 17512, 2008). Pour aller plus loin que la norme ISO 17512 (2008), précédemment évoquée, certains auteurs ont proposé des tests plus réalistes, avec l'exposition des vers de terre à des gradients de pollution (Lowe et al., 2016; Bami et al., 2017). Par ailleurs, des auteurs ont aussi proposé des expérimentations de laboratoire permettant d'évaluer les effets des pesticides sur la longueur et la densité des galeries produites par des espèces endogées et anéciques, ainsi que sur la production de turricules (rejets présents à la surface du sol produits par des vers de terre) (Capowiez et al., 2010; Dittbrenner et al., 2011). Ces études permettent de relier les effets écotoxicologiques, observés au niveau individuel, à des fonctions écologiques remplies par les vers de terre (e.g. aération du sol grâce aux galeries).

Au niveau de la population et de la communauté, les études sont généralement réalisées *in situ*, à l'échelle de la parcelle agricole. Ces approches permettent d'appréhender les conséquences écotoxicologiques de l'utilisation des pesticides sur les vers de terre ainsi que les conséquences écologiques associées (i.e. réduction des fonctions écologiques). Christensen et Mather (2004) ont, par exemple, mis en évidence que le fongicide benomyl augmentait significativement la migration de tous les vers de terre à partir du 2^{ème} jour après le traitement et que la proportion de juvénile, qui passait de 55% à 75% suite au traitement. Ces résultats indiquent que les populations ont été affectées, mais il est parfois difficile de trouver une explication quant aux effets observés. En effet, les résultats de ces

études sont dépendants de nombreux facteurs en interaction (e.g. conditions pédoclimatiques, relations interspécifiques). De plus, de par l'hétérogénéité des parcelles étudiées, les résultats présentent bien souvent une forte variabilité ce qui rend difficile l'analyse des données (Amossé et al., 2017). Pour ces raisons, Pelosi et al. (2014) expliquent que les effets observés au laboratoire sont souvent difficiles à mettre en lien avec ce qui est observé au champ en conditions réelles au niveau des populations voir des communautés. Enfin, ces études sont longues et coûteuses et il est donc impossible de multiplier les modalités (e.g. le nombre de pesticides et de concentrations étudiés). L'ensemble de ces contraintes font que ces études au niveau de la population et des communautés sont peu nombreuses dans la littérature scientifique ou en évaluation du risque.

Il apparaît ainsi que les études au niveau de l'individu permettent un bon compromis entre représentativité des conditions réelles d'exposition aux pesticides et évaluation/compréhension des effets observés. De plus, il existe des outils de modélisation qui permettent, à partir des données obtenus au niveau de l'individu, d'apporter une compréhension mécanistique des effets et une extrapolation dans le temps et à des niveaux d'organisation supérieurs.

IV. Apports de la modélisation pour analyser les effets des pesticides sur les individus

Les études pour évaluer la toxicité des pesticides, et plus généralement des toxiques (e.g. métaux lourds, produits chimiques industriels,...), pour les organismes vivants, sont majoritairement réalisées avec des démarches proches de celle de la toxicologie. C'est-à-dire qu'elles sont réalisées en laboratoire en étudiant l'effet des toxiques sur des espèces modèles. Les individus sont exposés à une gamme de concentrations et l'effet n'est recherché la plupart du temps que sur un unique paramètre biologique (survie, croissance ou reproduction) et sur une seule durée d'exposition (Isnard et al., 2001). Les résultats de ces essais ne permettent donc pas d'appréhender la complexité des écosystèmes où de nombreuses espèces, souvent éloignées des espèces modèles (cf partie III. 1.), sont exposées à différents pesticides ou toxiques. De plus les concentrations d'exposition varient au cours du temps, soit parce que les toxiques se dégradent, soit parce que leur disponibilité pour les

organismes évolue en fonction des conditions environnementales (Kaufman, 1967; Tang et al., 1999 ; Gevaio et al., 2000). Les expérimentations en laboratoire doivent donc être capables de prendre en compte ces aspects, tout comme les méthodes d'analyse des données produites.

L'outil qui permet de dépasser cette approche statistique est la modélisation. L'écotoxicologie se tourne de plus en plus vers cet outil lorsqu'il s'agit d'étudier l'effet des toxiques sur les organismes et les populations (Baird et al., 1996; Spromberg et al., 1998). La modélisation se définit comme la mise en équation mathématique de phénomènes physiques, chimiques ou biologiques, permettant une représentation théorique simplifiée d'une réalité qui ne peut pas être appréhendée dans son ensemble du fait de sa complexité (Devillers, 2017). Lorsqu'il s'agit de modéliser les effets d'un toxique sur le cycle de vie des individus (croissance, reproduction), les modèles d'intérêt en écotoxicologie sont les modèles mécanistiques puisque ceux-ci se basent sur des processus, des connaissances, plus ou moins précises, des mécanismes bio-physico-chimiques relatifs au fonctionnement du système considéré, ici l'individu. Ces modèles permettent d'intégrer mathématiquement des processus toxicologiques et biologiques à l'analyse des données d'écotoxicologie et d'étudier les interactions entre ces processus (Ashauer et al., 2006). La modélisation est également un outil de choix pour inférer des valeurs de paramètres non mesurables physiologiquement, émettre et tester différentes hypothèses, réaliser des prévisions (à une échelle de temps plus importante que le temps d'expérience), ou encore, extrapoler à d'autres conditions expérimentales ou concentrations d'exposition que celles qui ont permis de calibrer le modèle (Poggiale, 2009; Vindimian, 2000).

La variable d'effet choisie : l'énergie

L'énergie est une variable clef que les écologues ont placée depuis longtemps au cœur de la compréhension du cycle de vie des organismes et des populations. En effet, ce concept, proposé pour la première fois par Young (1807), est relatif en biologie à la faculté d'induire des réactions chimiques en opposition à l'évolution thermodynamique normale. Il permet d'intégrer sous une même fonction l'ensemble des molécules des organismes ayant cette faculté. Les écotoxicologues ont conceptualisé l'apport des méthodes de description de l'allocation de l'énergie, au niveau des individus, pour aboutir à une meilleure

compréhension des phénomènes de toxicité (Congdon et al., 2001). En effet, la quantité d'énergie par individu détermine sa survie, sa vitesse de croissance et sa fécondité. Le toxique est considéré comme perturbateur de l'utilisation de cette l'énergie, entraînant une déperdition en raison du stress subi (Calow, 1991), ou une mauvaise gestion de celle-ci vis-à-vis des fonctions vitales.

Dans un objectif d'analyse des effets des toxiques sur la gestion de l'énergie par un organisme, il faut tout d'abord calibrer un modèle permettant de décrire la dynamique de l'énergie chez celui-ci en l'absence de toxique. Jeong et al. (1990) ont construit un modèle de croissance chez *Bacillus subtilis* à plus de 200 paramètres rendant compte du rôle de chaque molécule présente dans chez cette espèce. Cependant, pour éviter des équations trop complexes qui rendent un modèle peu attractif, voire inutilisable en pratique, une approche plus simple a été proposée par Kooijman (1986). Dans celle-ci, l'énergie est utilisée pour trois grandes fonctions vitales : la croissance, la maintenance et la reproduction. Cette approche est appelée DEB pour Dynamic Energy Budget et peut être utilisée en écotoxicologie à l'aide des modèles qui en découlent, les modèles DEBtox, pour « Dyanmic Energy Budget and Toxicology » (Kooijman et Bedaux, 1996).

Modélisation des effets sur les individus

Pour modéliser les effets d'un toxique, la première étape est de chercher un descripteur quantitatif, de la présence de la substance toxique auquel relier cet effet. La concentration dans les tissus de l'organisme est la solution retenue dans les modèles d'effet (Flammarion et Péry, 2004). Cependant, la dynamique de la concentration d'une substance dans un organisme au cours du temps est difficile à mesurer alors que la concentration dans le milieu est plus accessible. Le modèle doit donc, dans un premier temps, relier la concentration dans le milieu et la concentration dans les tissus. Dans le cadre de cette modélisation toxicocinétique (TK), le modèle utilisé est le modèle dit « modèle linéaire à un compartiment » (Widmark et Tanberg, 1924). Ce modèle toxicocinétique considère l'organisme comme un seul compartiment, et repose sur l'hypothèse que l'absorption d'un composé est proportionnelle à sa concentration en solution, et son élimination proportionnelle à sa concentration interne. Ce modèle est décrit avec l'équation suivante :

$$\frac{dC_i}{dt} = K_a \times C_e(t) - K_e \times C_i(t)$$

où C_i est la concentration interne du toxique, C_e la concentration externe du toxique dans le milieu (le sol ou l'eau par exemple), K_a et K_e sont respectivement les taux d'absorption et d'élimination du toxique par l'organisme considéré. Cette cinétique peut être modifiée par la croissance de l'organisme lors de l'exposition (Kooijman, 2010) pour tenir compte à la fois de la dilution par la croissance (le volume augmentant, la concentration diminue mécaniquement) et du fait qu'intrinséquement, plus l'individu est petit plus la cinétique est rapide.

Dans un second temps, il s'agit de relier la concentration interne et l'effet (sur la gestion de l'énergie). Cette description de la dynamique de l'effet du toxique sur l'organisme est la toxicodynamique (TD). Les modèles de type DEBtox considèrent que lorsque la concentration interne d'un toxique excède une valeur seuil, appelée Concentration Sans Effet (CSE) (calibrée par le modèle), l'effet sur un paramètre explicatif du cycle de vie (en lien avec la gestion de l'énergie) est proportionnel au dépassement de cette CSE (Kooijman et Bedaux, 1996).

Ainsi, le couplage toxicocinétique-toxicodynamique (TKTD) avec un modèle bioénergétique basé sur la connaissance de la biologie de l'espèce, permet de relier des mesures d'effets à des temps d'exposition différents avec un même jeu de paramètres et de fournir des seuils d'effet (CSE) sur les organismes exposés ainsi qu'une compréhension mécanistique des effets observés (le mode d'action du toxique correspond au paramètre du modèle bioénergétique affecté). Pour aboutir à ce genre de modèle, il est donc tout d'abord nécessaire de calibrer un modèle bioénergétique qui servira à analyser les données écotoxicologiques au niveau individuel, sur le cycle de vie.

Modèles bioénergétiques pour l'analyse des effets au niveau individuel chez les vers de terre.

Pour analyser des données d'écotoxicité à l'aide d'un modèle TKTD, il est nécessaire en premier lieu de calibrer un modèle basé sur la gestion de l'énergie au niveau individuel qui tient compte de manière précise des caractéristiques de l'espèce utilisée (e.g. forme des

courbes de croissances, allocation de l'énergie au stade adulte entre croissance et reproduction).

Il existe dans la littérature des modèles de type DEB pour les espèces de vers de terre épigées, *Dendrobaena octaedra* (Jager et klok, 2010), *Eisenia fetida* (Jager et al., 2006) et *Lumbricus rubellus* (Hobbelen et van Gestel, 2007; Klok, 2008). Cependant, ces espèces épigées (cf partie II, 1) ne sont pas dominantes en champs cultivés ou les pesticides sont épandus, et il serait plus pertinent d'utiliser une espèce endogée, plus représentative des vers de terre présents au champ. Cependant, il n'est pas possible d'utiliser ces modèles pour une autre espèce puisque chaque espèce présente une gestion différente de l'énergie, avec des taux de croissance et de reproduction différents. Johnston et al. (2014) ont proposé un modèle basé sur la gestion de l'énergie pour l'espèce endogée *Aporrectodea caliginosa*, très commune dans les agroécosystèmes (Boström et Lofs-Holmin, 1996; Boag et al., 1997; Curry et al., 2008; Perez-Losada et al., 2009; Decaëns et al., 2011). Le modèle proposé présente une bonne généralité (e.g calibré pour tout type de nourriture) et a été calibré dans un objectif d'extrapolation à l'échelle de la population. Cependant pour l'analyse de données écotoxicologiques au niveau de l'individu, leur modèle comporte certaines limites, (et les mêmes limites existent pour les modèles sur les espèces épigées précédemment citées). Tout d'abord, leur modèle est basé sur l'hypothèse de coûts de maintenance énergétique élevé conduisant à une courbe de croissance en taille de type von Bertalanffy, ce qui ne correspond pas aux courbes de croissance des vers de terre (Lofs-Holmin, 1983) qui sont de type linéaire en taille. De plus, l'équation de croissance est imposée et ne laisse aucune souplesse pour prendre en compte, par exemple, une diminution de la quantité de nourriture fournie au cours du temps ou une modification de la gestion de l'énergie par l'organisme.

V. Objectifs et démarche de la thèse

Cette introduction a été l'occasion de mettre en avant l'importance des vers de terre dans les sols agricoles, leurs intérêts écologiques, et le risque que représente l'utilisation des pesticides pour ces organismes. Actuellement, l'évaluation du risque telle qu'elle est pratiquée est imparfaite pour plusieurs raisons. D'une part parce que l'espèce utilisée est

peu représentative des agroécosystèmes et d'autre part, parce que les tests réalisés sont peu réalistes et l'analyse statistique de type *ad hoc* qui en est faite ne permet aucune extrapolation dans le temps ou à des niveaux d'organisation supérieurs. Il existe cependant des outils de modélisation qui pourraient permettre une meilleure analyse des données, permettant notamment de décrire les effets au cours du temps sur l'ensemble des paramètres du cycle de vie d'un individu. Ces réflexions ont précisé les différents objectifs de ma thèse.

Le premier objectif de ce travail de thèse est de proposer une espèce modèle de ver de terre pertinente d'un point de vue écologique, pour l'évaluation du risque liée à l'utilisation des pesticides. Ensuite, afin de calibrer un modèle bioénergétique qui aura pour vocation l'analyse des effets des pesticides sur cette espèce, il a été nécessaire d'approfondir les connaissances sur son cycle de vie en laboratoire. Ainsi, dans la Partie 1, j'ai (i) réalisé un travail de synthèse bibliographique sur l'espèce modèle choisie, *Aporrectodea caliginosa* (chapitre 1) et (ii) étudié son cycle de vie en laboratoire en réalisant différentes expérimentations, afin de et calibrer un modèle bioénergétique (chapitre 2).

Le second objectif est d'évaluer les effets des pesticides sur le cycle de vie de l'espèce choisie *via* la mise en place d'expérimentations de laboratoire, plus réalistes que celles utilisées dans le cadre de l'évaluation du risque (en termes de conditions d'exposition). Ainsi, dans la Partie 2, j'ai dans un premier temps évalué les effets des pesticides sur la mortalité et la capacité d'évitement de l'espèce modèle (chapitre 1). Ensuite, les chapitres 2 et 3 traitent respectivement des effets des pesticides sur la reproduction et la croissance, dans lesquels les données ont été analysées avec des outils statistiques, mais aussi avec des outils de modélisation *via* le couplage du modèle bioénergétique (précédemment calibré) à un modèle toxico-cinétique. Enfin, je me suis fixé l'objectif d'évaluer l'effet des pesticides sur les fonctions écologiques fournies par les vers de terre *via* l'utilisation de notre espèce modèle (chapitre 4).

Ce manuscrit de thèse se termine par une discussion de l'ensemble des résultats. Des pistes pour la suite des recherches à mener sont explicitées.

Partie 1 - Le modèle biologique et son fonctionnement bioénergétique

Chapitre 1 – Le modèle biologique

Ce chapitre correspond à un article intitulé « *Aporrectodea caliginosa*, a relevant earthworm species for a posteriori pesticide risk assessment: Current knowledge and recommendations for culture and experimental design », publié dans la revue *Environmental Science and Pollution Research*. <https://doi.org/10.1007/s11356-018-2579-9>. (Un matériel supplémentaire est disponible en Annexe 2)

En évaluation du risque, il est courant de s'appuyer sur des espèces modèles pour représenter un taxon ou un groupe fonctionnel. Parmi les vers de terre, l'espèce modèle est *Eisenia fetida*. Cette espèce, préconisée dans les normes ISO et OCDE est largement utilisée en écotoxicologie et pour l'évaluation du risque liée à l'utilisation des pesticides. Cependant, nous avons mis en évidence que pour augmenter la pertinence de l'évaluation du risque et dans la compréhension des conséquences écotoxicologiques de l'utilisation des pesticides dans les agroécosystèmes il était nécessaire d'utiliser une autre espèce, à la place ou en complément d'*E. fetida*. Cette espèce doit notamment être dominante au champ, c'est à dire dans les zones potentielles d'utilisation des pesticides. C'est ainsi qu'après une lecture approfondie de la littérature scientifique, le choix de l'espèce modèle s'est tourné vers l'espèce endogée *Aporrectodea caliginosa* (Savigny, 1826). J'ai réalisé une revue bibliographique focalisée sur les effets des pesticides sur le cycle de vie de cette espèce et son comportement lors d'expérimentation en laboratoire. Cette revue a aussi été l'occasion de proposer une méthode d'élevage basée sur la littérature et enrichie de premières expériences au laboratoire. L'élevage en laboratoire est un élément clé pour une espèce modèle, puisqu'il permet l'approvisionnement en individus pour les expérimentations.

Résumé : L'utilisation des vers de terre dans les tests écotoxicologiques est largement utilisé et certains tests sont obligatoires pour l'évaluation du risque liée à l'utilisation des pesticides, dans le cadre de leur homologation. L'espèce modèle actuelle pour ces tests standardisés est *Eisenia fetida*. Cependant, cette espèce est rarement retrouvée dans les sols agricoles et souvent moins sensible aux pesticides que les autres espèces trouvées dans ces

sols. Pour faire progresser l'évaluation des effets des pesticides sur les organismes non ciblés, il est nécessaire d'effectuer des tests *a posteriori* en utilisant des espèces plus pertinentes. L'espèce endogée *Aporrectodea caliginosa* est représentative des champs cultivés dans les régions tempérées et est suggérée comme une espèce modèle. Après avoir présenté des informations sur sa taxonomie, sa biologie et son écologie, nous avons passé en revue les connaissances actuelles concernant sa sensibilité aux pesticides et mis en avant les lacunes de la recherche et les perspectives prometteuses. Enfin, des conseils et des recommandations sont donnés pour la mise en place d'un élevage en laboratoire, et d'expérimentations faisant appel à cette espèce.

***Aporrectodea caliginosa* s.s, a relevant earthworm species for a posteriori pesticide risk assessment: Current knowledge and recommendations for culture and experimental design**

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Abstract

Ecotoxicological tests with earthworms are widely used and are mandatory for the risk assessment of pesticides prior to registration and commercial use. The current model species for standardized tests is *Eisenia fetida*. However, this species is absent from agricultural soils and often less sensitive to pesticides than other earthworm species found in mineral soils. To move towards a better assessment of pesticide effects on non-target organisms, there is a need to perform a posteriori tests using relevant species. The endogeic species *Aporrectodea caliginosa* s.s is representative of cultivated fields in temperate regions and is suggested as a relevant model test species. After providing information on its taxonomy, biology and ecology, we reviewed current knowledge concerning its sensitivity towards pesticides and highlighted research gaps and promising perspectives. Finally, advice and recommendations are given for the establishment of laboratory cultures and experiments using this soil dwelling species of earthworm.

Keywords: Plant protection products, soil ecotoxicology, agroecotoxicology, Lumbricidae, agroecosystems, breeding.

I. Introduction

The use of pesticides may harm soil fauna, which are involved in key soil functions and related ecosystem services (McLaughlin and Mineau 1995; Blouin et al. 2013; Bertrand et al. 2015). Earthworms are ecosystem engineers (Jones et al. 1994) representing the most important living biomass in terrestrial ecosystems, often up to one ton per hectare in hardwood forests and in pastures (Lavelle and Spain 2001). They modify soil structure and improve water regulation, nutrient cycling and primary production (Lavelle et al. 2004; Seeber et al. 2008; Bottinelli et al. 2010; Blouin et al. 2013). Moreover, they are recognized as indicators of soil biological activity (Paoletti 1999) and have been used as model organisms in soil ecotoxicology for more than thirty years (OECD 207 1984; Spurgeon et al. 2003).

During the 1980s, standardized acute tests with earthworms were developed to assess the effects of pollutants (OECD 207 1984). Subsequently other standardized tests, assessing earthworm survival, reproduction and behaviour, were approved by the International Organization for Standardization (ISO) (ISO 17512-1 2008; ISO 11268-1 2012; ISO 11268-2 2012). These tests are regularly updated and some of them are used in the risk assessment process associated with the registration of new pesticides. *Eisenia fetida* (Savigny) or *Eisenia andrei* (Bouché 1972) are recommended as a test species in standardised tests and is widely used in ecotoxicological studies because they are relatively easy to breed and have a short generation time (OECD 1984), allowing for quick and cost effective tests. Despite these advantages, it does not generally inhabit mineral soils (Lowe and Butt 2007) and therefore is rarely found in cultivated fields where pesticides are applied. Furthermore, Pelosi et al. (2013) highlighted that LC50 values for *Lumbricus terrestris* (Linnaeus) and *Aporrectodea caliginosa* (Savigny), two common soil-dwelling species in cultivated fields, were significantly lower than LC50 values for *E. fetida*. Consequently, these authors recommended *A. caliginosa* to be used in ecotoxicological tests. Similarly, van Capelle et al. (2016) proposed *A. caliginosa* and *L. terrestris* as non-target soil organisms for environmental risk assessment of genetically modified plants. Klobucar et al. (2011) also proposed *A. caliginosa* for genotoxicity field surveys using biomarkers. The use of *A. caliginosa* species to test the effects of pesticides is thus increasingly argued in the scientific literature (Booth and

O'Halloran 2001; Spurgeon et al. 2003). Other temperate soil-dwelling species such as *Lumbricus rubellus* or *Octolasion cyaneum* have also been advocated for ecotoxicological tests (Lowe and Butt 2007). However, *L. rubellus* being epi-endogeic is rarely found in conventionally cultivated fields due to the lack of surface litter and Pelosi et al. (2013) showed that the sensitivity of *L. rubellus* to pesticides was lower than that of *E. fetida*. The endogeic species *O. cyaneum* is widespread but not common in all cultivated fields because it prefers moist habitats such as wet sands and reproduces parthenogenetically (Sims and Gerard 1999).



Figure 1. World distribution map of *A. caliginosa* based on the literature review. Each number represents an area or a country where the species *Aporrectodea caliginosa* was found (See Appendix, Table S1 for the list of countries or regions and references).

The endogeic species *A. caliginosa* is ubiquitous and data obtained from the literature review was used to map its global geographical distribution (Fig. 1). We recorded all the countries or regions where *A. caliginosa* individuals were sampled and the study purpose (i.e. ecology, ecotoxicology or biodiversity studies). *A. caliginosa* has been found in all temperate zones (i.e. in Europe, America, Asia, Oceania and South Africa, see Fig. 1). Moreover, it is one of the most abundant species in most soils (and in particular cultivated soils) of temperate zones (Boström and Lofs-Holmin 1996; Boag et al. 1997; Curry et al. 2008; Perez-Losada et al. 2009; Decaëns et al. 2011). It lives in the first 15 cm of soil and is

highly representative of agricultural soils, which is one of the two most relevant criteria for test organisms (i.e., the representativeness of the ecosystem to protect) according to the European Food Safety Authority (EFSA 2015). *A. caliginosa* displays high ecological plasticity and adaptability in agroecosystems (Bouché 1972; Sims and Gerard 1999), especially to agricultural practices such as soil tillage (Crittenden et al. 2014). In addition, *A. caliginosa* is able to survive in soils with low organic matter (i.e. 1.4% organic carbon, McDaniel et al., 2013) and moisture content (i.e. at least three weeks under drought conditions, McDaniel et al. 2013). *A. caliginosa* plays several key ecological roles such as nutrient cycling, i.e. increasing nitrogen flux, lowering the C/N ratio (Sandor and Schrader 2007; McDaniel et al. 2013) and enhancing nutrient availability for plants and microorganisms (Sharpley and Syers 1976; Sharpley and Syers 1977). *A. caliginosa* can also increase microbial biomass (Svensson and Friberg 2007) and its relatively high burrowing activity can have a positive impact on water infiltration /discharge (Ernst et al. 2009; McDaniel et al. 2015) and on soil aeration (Francis and Fraser 1998). Nevertheless, *Eisenia* sp. remains the most frequently used species in assessing the impact of applied pesticides on earthworms (Fig. 2). In 2016, 76 references involving pesticide effects on *E. fetida* were found compared with only 6 for *A. caliginosa* (Fig. 2), probably due to technical difficulties involved in obtaining a sufficient number of individuals for experiments. Moreover, it is recognized that *A. caliginosa* has wide ranging morphological variation as it is a complex of species (see section 3). This issue, addressed in this paper, can hinder the use of this species as a laboratory test organism. In this paper, we focused on *Aporrectodea caliginosa* s.s, commonly recognized as a separate species (Sims and Gerard 1999) and often referred to in current scientific literature as *A. caliginosa*.

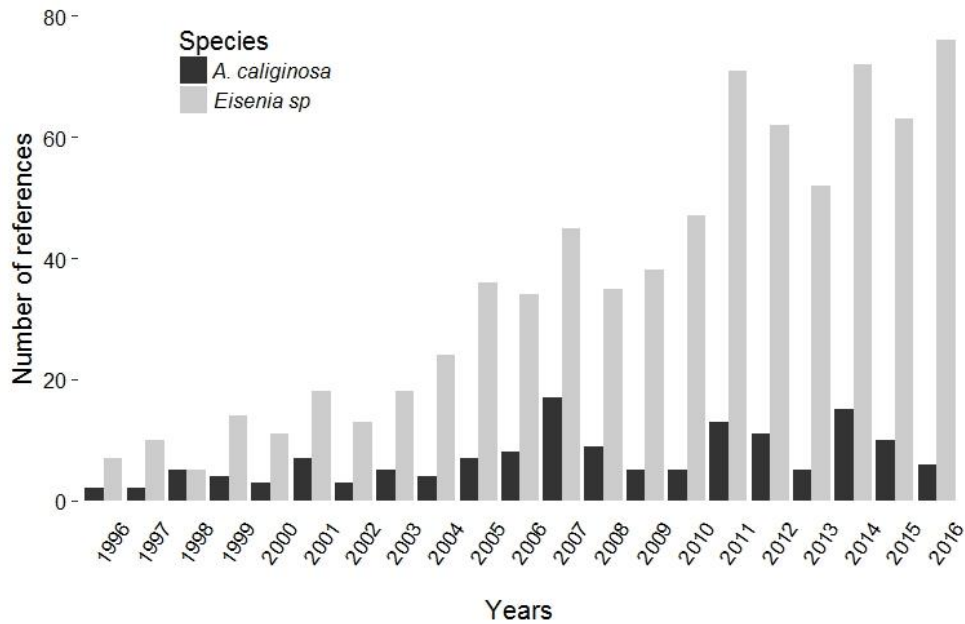


Figure 2. Number of references dealing with *Aporrectodea caliginosa* and *Eisenia sp.* and pesticides over the last twenty years. (Source: ISI Web of Knowledge, using “All Databases” option, with the formula (in Topic): (pesticid* OR herbicid* OR fungicid* OR molluscicid* OR nematocid* OR insecticid* OR plant protection product* OR crop protection product*) AND (eisenia OR fetida OR foetida OR andrei) for *Eisenia sp.* or (caliginosa* AND earthworm*) for *A. caliginosa*.

We aimed to review current knowledge on *A. caliginosa* s.s in order to assess its suitability as a species for a posteriori pesticide risk assessment, and to give recommendations for laboratory culture and experimental design. General information on the biology and the ecology of this species i.e., taxonomic aspects, morphological description, geographical distribution, and life history traits are first given. The effects of pesticides on its life history traits and behaviour are then reviewed. Finally, based on published information and on our own experience, advice for the establishment of laboratory cultures and experiments are provided. The study highlights knowledge gaps, further research opportunities and perspectives to promote the use of *A. caliginosa* s.s as a suitable species in a posteriori pesticide risk assessment procedures and soil ecotoxicological studies.

II. Search method

A systematic literature review was conducted based on keywords in the ISI Web of Knowledge, using the “All Databases” option, with the following formula: caliginos* AND

earthworm* in Topics. 1195 references were obtained. This review was focused on the endogeic *A. caliginosa* s.s which could be found in literature under different names (see section 3.). We thus excluded references dealing with *Aporrectodea tuberculata*, *Aporrectodea nocturna* and *Aporrectodea trapezoides*. References related with *A. caliginosa* s.s biology and ecology as well as the impacts of pesticide and metal contaminants on life history traits and behaviour were selected. Literature associated with impacts of heavy metals on *A. caliginosa* under laboratory conditions are presented in Appendix (Table S2) but they have not been incorporated into this review because they were not directly associated with pesticide risk assessment. For the purposes of this study, pesticides are defined as chemicals used against “harmful” organisms in agroecosystems. The titles were first read, then a second selection was made based on the abstracts and finally on full texts. Thus 27 publications related to the impact of organic pesticides on life history traits and behaviour of *A. caliginosa* s.s were selected (see section 5.). Finally, based on the abstracts, selected references on the biology and ecology of *A. caliginosa* were used to inform other parts of this review (see sections 4. and 6.).

III. Taxonomic considerations

Aporrectodea caliginosa (Savigny 1826) belongs to Annelida: Oligochaeta: Lumbricidae and its classification has changed several times and is considered a species complex. Bouché (1972) divided *A. caliginosa* into two species with another genus name: *Nicodrilus caliginosus* composed of three subspecies (i.e. *N. c. caliginosus*, *N. c. alternisetosus* and *N. c. meridionalis*) and *Nicodrilus nocturnus*. Sims and Gerard (1985) reported that four morphs were commonly recognized as separate species: *Aporrectodea caliginosa* s.s (syn. *turgida*), *Aporrectodea tuberculata*, *Aporrectodea nocturna* and *Aporrectodea trapezoides*. The variations in these four species are now considered to be mostly phenotypic. In this paper, we refer exclusively to the *A. caliginosa* s.s morph which can be considered as a “morphospecies” (i.e. a species distinguished from others only by its morphology). More recently, Briones (1996) proposed two subspecies: *A. caliginosa caliginosa* and *A. caliginosa trapezoides*. To summarize, information on *Aporrectodea caliginosa* s.s presented in this review was recorded under different names in the scientific literature: *Aporrectodea*

caliginosa s.s., *Aporrectodea caliginosa caliginosa* (or *Allolobophora caliginosa caliginosa*) and *Nicodrilus caliginosus caliginosus*. This endogeic species is currently named *Aporrectodea caliginosa*, and is referred to as such in the rest of this paper.

Diaz Cosin et al. (2011) explained that comparing published data on species that belong to a complex of species could be hazardous, as the authors may have incorrectly identified the species. This is the case for *A. caliginosa* but it could be the same for other species e.g. Cunha et al. (2014) revealed the existence of cryptic lineages (morphospecies) within *Pontoscolex corethrurus* using molecular markers. Even *E. fetida* and *E. andrei*, used as model species in ecotoxicology, are part of a species complex (Latif et al. 2017).

In this situation it is proposed that a full detailed description of individuals along with geographic origin should be provided in any associated publications. Moreover, when possible, individuals should be DNA barcoded to verify if they belong to *A. caliginosa*.

IV. Morphology and life history traits

Adults of *A. caliginosa* are typically composed of 120-150 segments. They are between 60 and 85 millimeters in length, with a biomass between 200 to 1200 mg (Sims and Gerard 1999). Individuals lack any significant pigmentation but the anterior segments are pale pink in coloration (Sims and Gerard 1999; Bouché, 1972).

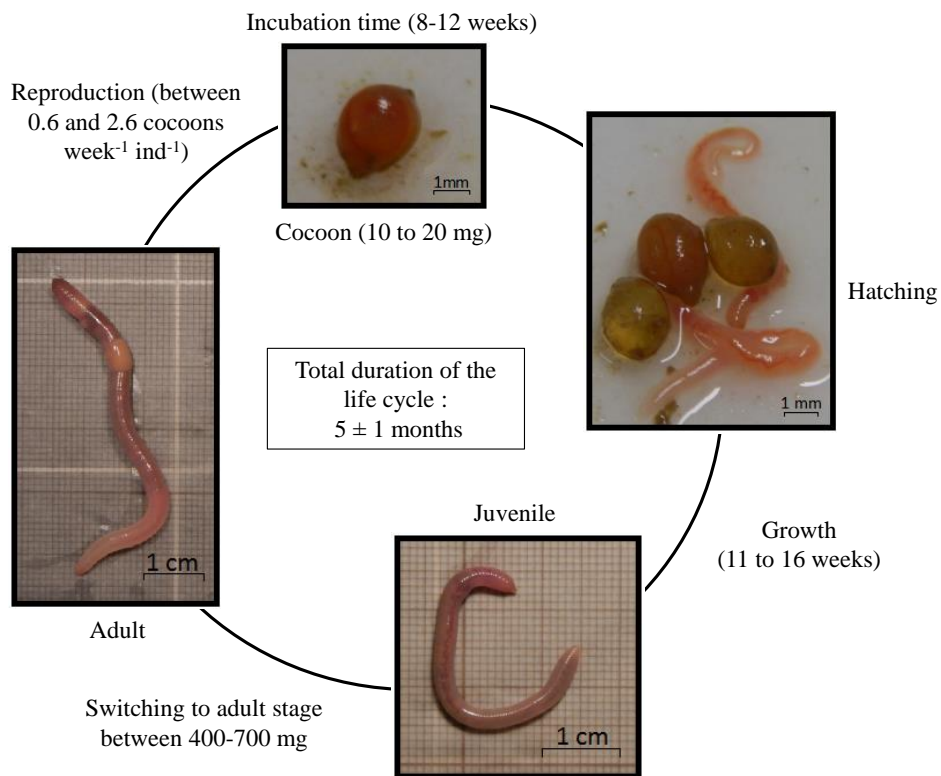


Figure 3. Life cycle of *A. caliginosa* under laboratory conditions in a field loamy soil supplemented with horse dung milled at 1 mm as food, 15 °C, 60-70% of the soil water holding capacity (personal observations, photos ©Sylvain Bart).

The life cycle of *A. caliginosa* under laboratory conditions is described in Fig. 3. Lowe and Butt (2005) summarized information on cocoon production by *A. caliginosa* along with data on incubation times and viability. According to this review and other articles (e.g., Lofs-Holmin 1982; Spurgeon et al. 2000), an individual *A. caliginosa* can produce between 0.6 and 2.6 cocoons per week at 15 °C in a field soil. Cocoon production rate depends on the environmental, physical and chemical soil characteristics but also on the earthworm's age because individuals may suffer reproduction fatigue. In petri dishes on a moist filter paper, Holmstrup et al. (1991) recorded cocoon incubation periods of 36, 62, 199, and 234 days at 20, 15, 10, and 5 °C, respectively. Other authors reported incubation periods of 56-63 days (Jensen and Holmstrup 1997) at 15 °C in petri dishes or 70-84 days at 15 °C in soil (Boström and Lofs-Holmin 1996). The hatching rate (i.e., cocoon viability) was found to vary between 90 and 97 % at 15°C in petri dishes (Holmstrup 2001; Jensen and Holmstrup 1997). Similarly, Boström and Lofs-Holmin (1996) recorded a hatching rate of 91-95% at 15 °C in a field soil, but Booth et al. (2000a) reported a rate of 61 and 80% after 28 and 56 days respectively at 20 °C in petri dishes on moist filter paper. Booth et al. (2000a) found that 75 to 80% of

individuals became adult after 18 weeks at 15 °C. Lofs-Holmin (1983) found a shorter maturation time of 6.5 weeks at the same temperature in a mixture of farmyard manure and clay. From our own observations, under laboratory conditions, i.e. field loamy soil with horse dung as food, 15 °C, 60-70% of the soil Water Holding Capacity (WHC), this species needs an average of 13 weeks to mature from hatchling emergence. Cocoon biomass ranges from 10 to 20 mg and juveniles generally become adults at a biomass of 400 to 700 mg. To summarize the literature and our own observations, under laboratory conditions at 15 °C, the life cycle duration of *A. caliginosa* is between 4 and 6 months (Fig. 3). The life span is unfortunately unknown but is regularly more than two years under laboratory conditions (personal observation).

V. Sensitivity to pesticides

Twenty seven publications dealing with the impacts of pesticides on life history traits and behaviour of *A. caliginosa* are summarized in Table 1. In 75% of the studies, authors used commercial formulations of pesticides to allow better understanding and assessment of ecotoxicological effects under field conditions. However, 78% of the articles assessed the impacts of insecticides, while only 26%, 15% and 7% dealt with herbicides, fungicides and acaricides, respectively (some articles assessed different types of pesticides). Since herbicides and fungicides represent the main pesticides applied in agroecosystems (respectively 45% and 37% of the market in France in 2015 (UIPP 2016)), further research is needed to assess the ecotoxicological effects of these products on *A. caliginosa*.

Table 1. Effects of pesticides on life history traits and behaviour of *A. caliginosa* under laboratory conditions.

Reference	Studied parameters	Origin	Development stage	Pesticide used	Active substance	Action	Method of addition	Duration	Main Results
Alshawish et al. (2004)	Biomass and cocoon hatching	NA	NA	NA	Chlorpyrifos Cypermethrin Mancozeb	Insecticide Insecticide Fungicide	Mixed	12 weeks	Higher body mass increase in the control. Toxicity of chlorpyrifos (100 % hatching failure) > mancozeb (>73 % hatching failure) > cypermethrin (80 % survival and normal hatching).
Badawy et al. (2013)	Mortality and biomass	Collected	Adult	F	Buprofezin Triflumuron Lufenuron	Insecticide Insecticide Insecticide	NA	28 days	LC50 lufenuron: 1.87 mg/kg > LC50 buprofezin: 421mg/kg-> LC50 triflumuron : 477mg/kg. The reduction in biomass was dose-dependent in all treatments.
Bart et al. (2017)	Mortality, biomass and avoidance	Collected	Adult	F	Dimoxystrobin and epoxiconazole	Fungicide	Mixed	14 days for the acute test, 48H for the avoidance test	LC50: 6.3 times the RD. No avoidance from 1 to 10 times the RD. No biomass loss.
Booth et al. (2000a)	Growth, cocoon production, hatching success	NA	Juvenile	F	Chlorpyrifos Diazinon	Insecticide Insecticide	Mixed	4 weeks	For both insecticides : at the RD, no significant effect on growth, cocoon production and hatching success. At 5 times the RD for diazinon and 7 times for chlorpyrifos, there was a decrease in biomass (but reversible after 8 weeks without pesticide). A slow maturation and a reduction in the cocoon production was observed only for chlorpyrifos at 7 times the RD.
Booth et al. (2000b)	Mortality	Cultured	Juvenile	F	Chlorpyrifos Diazinon	Insecticide Insecticide	Mixed	14 days	LC50 chlorpyrifos : 69 mg/kg, LC50 diazinon : 100 mg/kg.
Booth and O'Halloran (2001)	Growth, maturation, cocoon production and viability	Cultured	Adult and juvenile	F	Chlorpyrifos Diazinon	Insecticide Insecticide	Mixed	12 weeks	At 7 times the RD of chlorpyrifos and from the RD of diazinon, negative effects on growth, maturation and cocoon production. The maturation was less sensitive than cocoon production. Growth and cocoon production in earthworms exposed as juveniles were more sensitive than earthworms exposed as adult.
Dalby et al. (1995)	Mortality and biomass	Collected	NA	NA	Dimethoate Glyphosate 2,4-DB	Insecticide and Acaricide Herbicide Herbicide	Sprayed	10 days or 3 weeks	No effects on survival or biomass, with or without plant cover.
Dittbrenner et al. (2010)	Biomass and cast production	Collected	Adult	M	Imidacloprid	Insecticide	Mixed	7 days	Significant biomass loss from the RD (0.66 mg/kg) to 4 mg/kg. Decrease in cast production at 0.66 and 2 mg/kg.
Dittbrenner et al. (2011a)	Burrowing behaviour	Collected	Adult	M	Imidacloprid	Insecticide	Mixed	1, 7 or 14 days, 24-96 hours for short-term effects and 6 weeks for long-term effects	Negative effects for the short-term experiment on the burrow depth from 0.2 to 4 mg/kg. Significant linear decrease in burrow volume with increasing imidacloprid concentration. RD = 0.66 mg/kg.
Dittbrenner et al. (2011b)	Biomass	Collected	Adult	M	Imidacloprid	Insecticide	Mixed	7 and 14 days	Significant biomass loss from 0.2 to 4 mg/kg after 7 days and from 0.66 to 4 mg/kg after 14 days. RD = 0.66 mg/kg.

Table 1. (Continued)

Reference	Studied parameters	Origin	Development stage	Pesticide used	Active substance	Action	Method of addition	Duration	Main Results
Dittbrenner et al. (2012)	Avoidance behaviour	Collected	Adult	M	Imidacloprid	Insecticide	Mixed	48 hours	No avoidance of imidacloprid-contaminated soil.
Falco and Momo (1991)	Mortality, biomass, cocoon production and fecal pellets production.	NA	Adult	NA	Heptachlor	Insecticide	NA	8 days	At 1.69 mg/kg, mortality increased with the average mass of earthworms. The decrease in mean biomass was maximal for individuals with higher average mass and minimal for individuals with an intermediate mass. No effect on cocoon production or fecal pellet production.
Gaupp-Berghausen al. (2015)	Cocoon hatching, surface casting activity	Collected	Adult/Sub-adult	F	Glyphosate	Herbicide	Sprayed	4 weeks for casting activity and 3 months for cocoon hatching	At 176.12 mL/m ⁻² of herbicide, which is 53% lower than the recommended plant-based application rate, reproduction was reduced by 56% within three months after herbicide application. Casting activity was not affected.
Hodge et al. (2000)	Avoidance behaviour	NA	Juvenile	F	Chlorpyrifos Diazinon	Insecticide Insecticide	Sprayed	1 or 4 days	No avoidance.
Kula and Kokta (1992)	Mortality	Collected	Adult	F	Parathion Propoxur	Insecticide Insecticide	Mixed	14 days	LC50 parathion: 126mg/kg and LC50 propoxur: 4.5 mg/kg.
Kula and Larink (1997)	Mortality	Collected	Adult and juvenile	NA	Dimethoate	Insecticide and Acaricide	Mixed	28 days	LC50 : 179 and 10 mg/kg for adults and juveniles respectively in OECD soil, and 47 and 32 mg/kg for adults and juveniles in LUF A soil, respectively.
Ma and Bodt (1993)	Mortality	Collected	Adult	M	Chlorpyrifos	Insecticide	NA	14 days	LC50 : 755 mg/kg, NOEC : 486 mg/kg.
Martin (1986)	Mortality, biomass and cocoon production	Collected and Cultured	Adult and juvenile	F	Carbofuran Aldicarb Phorate Aldoxycarb Methomyl Oxamyl Isazophos Fenamifos Fensulfothion Diazinon Citowett	Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide Herbicide		7 days for juvenile and 14 days for mature individuals	Concentration (mg/kg of dry soil) of pesticide causing zero growth (compared with the standard) : carbofuran (0.10), aldicarb (0.09), phorate (0.30), aldoxycarb (0.40), methomyl (0.54), oxamyl (0.59), isazophos (0.93), fenamifos (5.72), fensulfothion (7.35), diazinon (12.4), and citowett (525). For mature individuals, 0.5, 1 and 5 mg/kg of carbofuran caused 10, 80 and 100% of mortality respectively. These concentrations also reduced cocoon production.
Mosleh et al. (2003)	Mortality and biomass	Collected	Adult	F	Aldicarb Cypermethrin Profenofos Chlorfluazuron Atrazine Metalaxyl	Insecticide Insecticide Insecticide Insecticide Herbicide Fungicide	Mixed	7, 14, 21 and 28 days	LC50 after 28 days of exposure was 0.68, 72.96, 127.00, 139.90, 381.20 and 518.00 mg/kg for aldicarb, cypermethrin, profenofos, chlorfluazuron, atrazine and metalaxyl respectively. There was a decrease in biomass at the LC25 for all these pesticides.
Mosleh (2009)	Mortality	Collected	Adult	NA	Isoproturon	Herbicide	Mixed	60 days	No mortality. LC50 > 1200 mg/kg

Table 1. (Continued)

Olvera-Velona et al. (2008)	Mortality, biomass and burrowing behaviour	Collected	Adult	F	Ethyl-parathion	Insecticide	Mixed	7 and 14 days	Mortality occurred at lower concentrations after 14 days than 7 days. LC50 between 11 to 57 times RD, depending on the soil and time of exposure (7 or 14 days). Body mass change was significantly affected by pesticide concentration and in a lower extent by soil type (since 10 x RD). Reduction in burrow length and number of branches from 1 time the RD.
Rault et al. (2008)	Biomass	Collected	Adult	F	Ethyl-parathion	Insecticide	Mixed	14 days exposure and 56 days recovery	At 10 mg/kg, after 28 days, significant biomass loss. At 1 mg/kg, after 3 days, decrease in biomass but <i>A. caliginosa</i> showed rapid mass recovery.
Reinecke and Nash (1984)	Mortality, body observation	NA	Juvenile	NA	Dioxin	Herbicide	NA	85 days	No worms died or showing any other observable toxicological effects when exposed to concentrations up to 5 mg/kg. The lethal threshold concentration was between 5 and 10 mg/kg.
Reinecke and Reinecke (2007a)	Biomass	Collected	Adult	F	Chlorpyrifos	Insecticide	Sprayed	2 weeks	Significant decrease in biomass at the commercially recommended rate.
Reinecke and Reinecke (2007b)	Biomass, state of estivation	Collected	Adult	F	Chlorpyrifos	Insecticide	Sprayed	5 weeks	The highest biomass loss was observed with earthworms exposed to the highest pesticide concentration (8 µg/kg). Estivation was higher among earthworms exposed to higher exposure concentrations.
Springett and Gray (1992)	Growth, maturation and mortality	Cultured	Juvenile	F	Captan Glyphosate Azinphos-methyl	Fungicide Herbicide Insecticide	Sprayed	100 days	Azinphos-methyl and glyphosate reduced growth at all the concentration (realistic field rates). Captan had the lowest effect on growth and mortality. Worms took more time to reach maturity in all the other treatments except with Captan at the mid rate.
Stenersen (1979)	Mortality	Collected	Adult	M	Aldicarb Carbaryl Carbofuran Oxamyl Paraoxon Parathion Trichloronate gamma-HCH	Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide Insecticide	Mixed	14 days in soil and 30 minutes in pesticide solution	The range of LC50 (pesticide solution) were 3.1-6.3, >200, 3.1-6.3, 0.39-0.78 and 400-800 µg/mL for aldicarb, oxamyl, carbaryl, carbofuran and Gamma-HCH respectively. Carbaryl and carbofuran were lethal in soil at low concentration (4mg/kg).

For Origin: cultured (in laboratory) or collected (in the field). Pesticide used: F = Formulation and M = pure molecule. Method of addition: mixed (into the soil) and sprayed (at soil surface). RD = Recommended dose. LC50: Lethal concentration for 50% of exposed individuals. NOEC = No Observed Effect Concentration. NA= Not Available. ECX: Effective concentration.

Fifteen publications presented data on mortality to estimate LC50 values. This endpoint is commonly used in ecotoxicological studies and it is mandatory for pesticide registration. LC50 values have been also used to compare sensitivity between species and taxa (Pelosi et al. 2014; Bart et al. 2017). In most studies LC50 was estimated using adult individuals but a few studies have compared these values for adults and juveniles. Kula and Larink (1997) assessed the effects of the insecticide dimethoate on *A. caliginosa* in OECD artificial soil (10% sphagnum, 20% kaolin clay, 70% sand) and estimated LC50 at 179 mg kg⁻¹ for adults and 10 mg kg⁻¹ for juveniles. With the same pesticide in a LUFA 2.2 soil (Speyer, Germany) (standardized soil composed of 6.7% clay, 15.4 % silt and 78.1 % sand), a LC50 of 47 and 32 mg kg⁻¹ was recorded for adults and juveniles, respectively. This could be due to the higher availability of the contaminant in LUFA soil. For the insecticide chlorpyrifos, LC50 was estimated to be 755 mg kg⁻¹ for adult individuals (Ma and Bodt 1993) and 69 mg kg⁻¹ for juveniles i.e., 1-3 months old (Booth et al. 2000b). Although these two studies were not undertaken in the same conditions (e.g. soil, temperature), juveniles appeared to be more sensitive to pesticides than adults. Several authors have reported the same findings for other earthworm species (e.g. Spurgeon and Hopkin 1996). Therefore, acute toxicity of pesticides to juveniles should be more widely examined to better understand and to assess agroecotoxicological effects of pesticides on earthworm life cycles.

In addition to acute studies, different sublethal (chronic) end points have also been used. Firstly, fecundity (cocoon production and/or viability) was studied in 6 out of 27 articles. Booth et al. (2000a) found a reduction in cocoon production at 7 times the recommended dose (RD) of chlorpyrifos (28 mg kg⁻¹). Similarly, Gaupp-Berghausen et al. (2015) showed that glyphosate reduced hatching rate by 56% at half of the RD. In addition, Booth et al. (2001) found that juveniles exposed to realistic field applications of chlorpyrifos (4 mg kg⁻¹) and diazinon (12 mg kg⁻¹) produced subsequently fewer cocoons when they became adults than mature earthworms exposed to the same concentrations of these pesticides. These results suggested that (i) the reproduction of *A. caliginosa* individuals can be affected by pesticides at realistic concentrations and (ii) pesticides are more harmful to juveniles than adults. This may have consequences for life cycle and population dynamics of *A. caliginosa* in natural conditions.

Some authors used the term “growth” to describe biomass change of adult individuals (Dalby et al. 1995; Mosleh et al. 2003; Badawy et al. 2013). From our point of view, “growth” should be restricted to juveniles and we recommend use of the terms “biomass”, gain or loss, or “weight change” for adult individuals. Biomass monitoring of adult individuals was used in 13 out of 27 studies and provided useful information on earthworm health at low pesticide concentrations. For example, Reinecke et al. (2007a) and Dittbrenner et al. (2011b) showed a significant decrease in biomass at realistic concentrations of chlorpyrifos and imidacloprid respectively.

Data on the influence of pesticides on growth and maturation (defined by the presence of a fully developed clitellum) were only found in three publications. Booth et al. (2000a) showed that the growth of *A. caliginosa* was significantly reduced by chlorpyrifos at the RD and by diazinon at 5 times the RD. However, there was no effect during the recovery period (i.e. period in a control soil after exposure to a contaminated soil). In this study, juvenile individuals exposed to 7 times the RD of chlorpyrifos and 5 times the RD of diazinon matured more slowly than earthworms in control soil without pesticides. It has also been reported that the herbicide glyphosate and the insecticide azinphos-methyl repeatedly applied at 2-weekly intervals at lower concentrations than the commercial RD decreased growth rates and increased time to reach maturity of *A. caliginosa* (Springett and Gray 1992). However, these studies were performed with juveniles of unknown ages. In this study and more generally, the authors did not assess the impact of pesticides on earthworm growth from hatching to maturity. In all reviewed studies, only the biomass of juveniles was given, being at least 200 mg at the beginning of the experiment while just after hatching the biomass of *A. caliginosa* individuals is between 10 to 30 mg (Lofs-Holmin 1983; Bostrom et al. 1986; Bostrom 1988). It would therefore be relevant for the assessment of pesticide impacts to assess effects on recently hatched individuals. For that, cohorts of newly hatched juveniles can be produced by slowing growth of recently hatched earthworms by keeping individuals at 4 °C (see section 6.).

Finally, *A. caliginosa* behaviour in response to pesticide exposure can be assessed using avoidance, casting or burrowing activity. At 3 times the RD of imidacloprid (Dittbrenner et al. 2012), 16 times the RD chlorpyrifos (i.e. Lorsban 40EC, Hodge et al. 2000) and 10 times the RD of dimoxystrobin and epoxiconazole (i.e. Swing Gold®, Bart et al. 2017), no avoidance

was reported. Although these results give some indication of earthworm behaviour in response to pesticide application in field soils, they are not sufficient to generalize on the capability of *A. caliginosa* to avoid pesticides in soils and further studies are required. Moreover, the two chambers test is too simplistic and we thus advocate use of the six chambers method proposed in the ISO 17512-1 (2008) which would allow for a more realistic test. Alternatively avoidance behaviour may be assessed in a linear pollution gradient as proposed by in Lowe et al. (2016) and demonstrated in Bami et al. (2017b).

Five publications were found on casting and burrowing activity that can be related to ecological functions provided by *A. caliginosa* under pesticide exposure. Burrowing activity is involved in soil aeration and water infiltration (Ernst et al., 2009), and cast production can be related to organic matter degradation (Frouz et al. 2011). Dittbrenner et al. (2011a) showed a decrease in burrow volume at the RD and with increasing concentrations of imidacloprid. Similarly, Olvera-Velona et al. (2008) found that the insecticide ethyl-parathion applied at the RD decreased burrow length and the number of branches in a Calcosol. Dittbrenner et al. (2010) showed a decrease in cast production of between 45 and 97% due to imidacloprid at an application rate of 0.66 mg kg⁻¹ (1 times the RD).

To summarize, pesticides used at realistic field concentrations can induce sublethal negative effects on *A. caliginosa*. Chronic endpoints are useful to understand and predict pesticide impacts on the life cycle and population dynamics of this earthworm species. To go further, relationships between earthworm population dynamics and ecological functions should be assessed that would allow an understanding and quantification of the impacts of pesticide application on ecosystem services provided by *A. caliginosa*. To reach this goal, there is a need for more knowledge and data of the impacts of pesticides on different developmental stages and ecological functions. However, it is difficult to get cohorts of sufficient number of individuals for robust and reliable experimentations. The next section provides information on overcoming this technical issue.

VI. Laboratory culture and experimental design

As discussed in the previous sections, there is a need to produce data on the impacts of pesticides on *A. caliginosa* life history traits, behaviour and ecological function. A large

number of individuals is thus required for these experiments. Earthworms can be collected from the field but retrieving a large number of individuals (>200) is not always easy or feasible throughout the year. Furthermore, the age and exposure history of field-collected earthworms is unknown. Another solution is to culture *A. caliginosa* under controlled laboratory conditions. However, the procedure is poorly documented, except in Lowe and Butt (2005) who reviewed the optimal laboratory conditions to be used for the culture of *A. caliginosa*. Based on their work, other publications and our own experience, we have summarized and carefully described the optimal conditions and steps for the establishment of a culture and the implementation of laboratory experiments using *A. caliginosa*.

Table 2. Guidelines for sustained culture of *A. caliginosa* (adapted from Lowe and Butt, 2005).

Culture parameters	
Soil type	Natural Loamy/clay soil (pre-treated to remove macro- and meso-invertebrates)
Soil depth (cm)	> 3 cm
pH	6-7
Soil moisture (%)	25-30 % or 60-70% of the water holding capacity
Food	Dried and rewetted animal dung (cattle or horse)
Food amount for juveniles < 300 mg (ind ⁻¹ month ⁻¹)	2-3 g
Food amount for adults and juveniles > 300 mg (ind ⁻¹ month ⁻¹)	4-6 g
Food location	Mixed into the soil
Food particle size (mm)	< 1
Temperature (°C)	15 ± 1
Light	24h dark
Vessel type	Sealed, opaque, preferably plastic with ventilation holes in the lid

The culture parameters are summarized in Table 2 (adapted from Lowe and Butt, 2005). Thereby, for maintenance and development of *A. caliginosa*, a field loamy/clay soil (pH 6-7) is more appropriate than standardized soils such as OECD and LUFA 2.2 soil (Brami et al., 2017a). Kula and Larink (1997) reported no cocoon production in a LUFA 2.2 soil (a sandy soil) due to earthworm inactivity. More generally, standardized soils, such as OECD soil, have been shown to be unsuitable for soil dwelling species (Brami et al. 2017a). The soil needs to be pre-treated (air dried and sieved or crushed to 2-3 mm) to remove macro- and meso-invertebrates. The soil moisture must be adjusted to 25-30 % (or 60-70% of the WHC), and the temperature must be around 15 °C (use of a temperature controlled room or incubator is advisable). The supplied food should be animal dung (preferably from cattle or horse) previously dried, milled (<1mm), rewetted and mixed into the soil. For optimal growth, it is

suggested to provide 2-3 g and 4-6 g of dried food per individual per month for juveniles and adults respectively.

1. Development and maintenance of a laboratory culture

The different steps for the establishment of a culture of *A. caliginosa* in laboratory have been summarized in Fig. 4. The first step is to prepare soil and food stocks as previously described. Then collect mature specimens from the field using a digging and hand-sorting method consisting of removal (with a spade or a fork) and searching in the upper 20 centimeters of the soil profile.

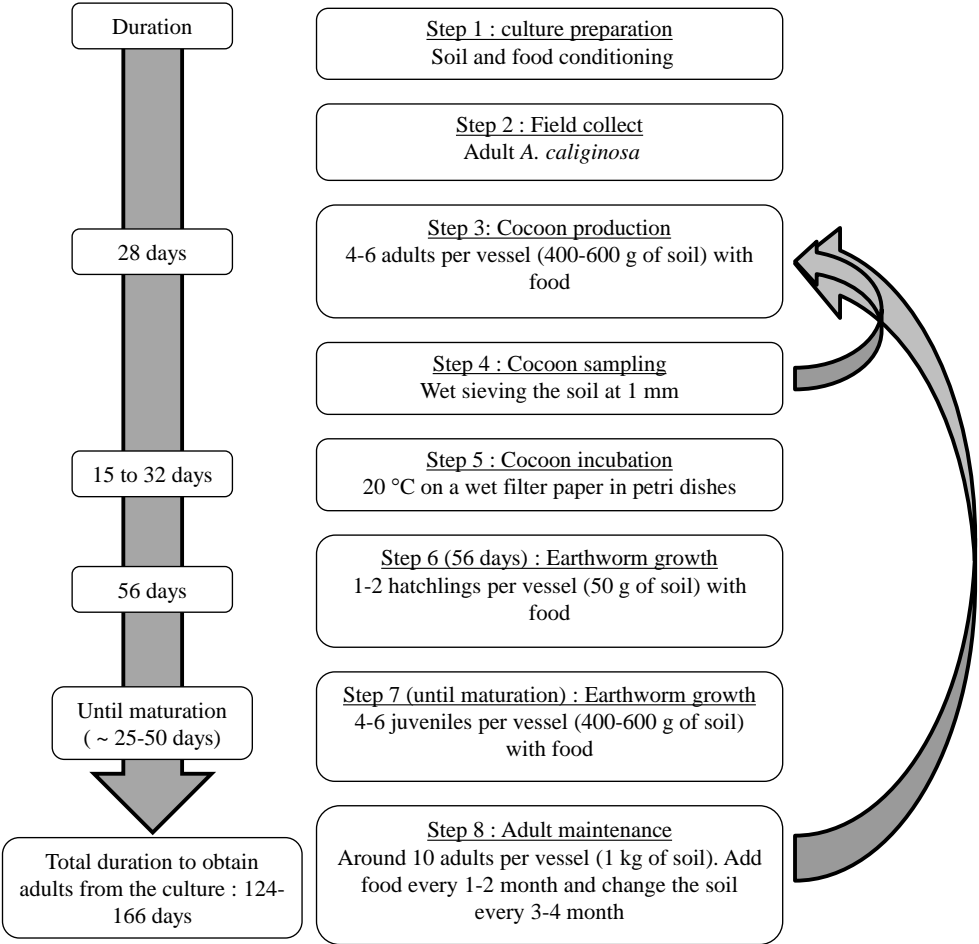


Figure. 4. Steps and their duration for the culture of *A. caliginosa* under laboratory conditions.

For cocoon production (i.e. step 3), 4-6 adults can be placed in a 1 L plastic vessel, with 400-600 g of soil (dry mass) and food supply. All vessels should be sealed with a perforated (using

a mounted needle) lid to allow gaseous exchange and prevent earthworms from escaping. After one month, individuals should be transferred into fresh substrate (food and soil). To collect cocoons, the soil should be wet sieved through a 1 mm mesh size in order to remove the soil but retain the cocoons in the sieve (Fig. 5). The collected cocoons can then be placed on wet filter paper (e.g. Whatman number 1) in petri dishes (Holmstrup et al., 1991) and incubated at 20 °C. This temperature allows a more rapid hatching than at 15°C (Holmstrup et al., 1991), and thus optimizes hatchling production. During this period (15-32 days after cocoon collection), we recommend regular monitoring to maintain moisture level and remove hatchlings (e.g. every 2-3 days). To synchronize individuals (i.e., to get a cohort of individuals at a similar level of development), we suggest that new hatchlings are maintained (maximum 40 individuals) in a small vessel (100-200 mL, with 50 g of moist soil) at 4 °C to minimize their development until the required number of cocoons have hatched (Lof-Holmin 1982). Then, in order to optimize growth of juveniles (step 6 in Fig. 4), it is recommended that earthworms are initially maintained individually in small vessels (e.g., 100-200 mL with 50 g of dry soil) for 56 days during which soil moisture has to be maintained. Juveniles need to be fed with 2-3 g of food per individual per month and the substrate needs to be prepared as previously described. The food should be added at the beginning (mixed with the soil). After 56 days, juveniles should be transferred to larger vessels (1 L with 400-600 g of soil), with 4 to 6 juveniles per vessel, until the development of the clitellum (at this stage individuals are sexually mature). These earthworms need to be fed with 4-6 g of food ind-1 month-1. The development of the clitellum can take between 25 to 50 days depending on the soil, food quality and individuals (i.e. intraspecific variability). For the feeding of individuals without soil changing, the vessel should be emptied, earthworms removed and food mixed with the soil and returned to the vessel prior to addition of the earthworms. Soil disturbance during this procedure does not disrupt *A. caliginosa* development (personal observation).



Figure 5. The wet sieving method for cocoon retrieving (1 mm mesh size).

Adult individuals can be maintained in culture for future experiments. For adult maintenance, we recommend reducing the food supply (1-2 g per individual per month is enough) to avoid excessive organic matter enrichment that could acidify the soil. Furthermore, the soil containing adults should be replaced every 3-4 months to avoid soil compaction and food accumulation which could lead to unsuitable conditions for earthworms.

To produce more individuals, cocoons or new hatchlings, it is also possible to go back to step 3 with adult stock. However, as suggested by Lowe and Butt (2007), we recommend avoiding the use of individuals from long-term isolated laboratory cultures. After several generations, there is a risk of inbreeding or adaptation to laboratory conditions, thus reducing the representativeness of cultured individuals compared to earthworms living in natural conditions. To avoid this situation, we recommend that laboratory cultures are renewed every 2-3 years with earthworms collected from the field.

2. Experimental design for the assessment of lethal and sublethal effects of pesticides

The ISO tests (i.e. using *E. fetida*) can be used as a basis for the assessment of pesticide effects on *A. caliginosa*. However, we recommend adoption of the advice presented at the beginning of section 6 for soil, food type and quantity, moisture and temperature parameters.

The ISO 11268-1 (2012) guideline can be adopted for the assessment of acute toxicity of pesticides and other contaminants. The recommended duration of the test is 14 days.

However, as shown by Khalil et al. (1996), LC50 decreased with increasing exposure duration. Therefore it may be relevant to use longer durations of exposure (e.g. 28 or 56 days). The recommended density of 10 individuals per container with 500-600g of soil (originally for *E. fetida*) is too high and 6 to 8 *A. caliginosa* individuals is proposed in this volume of soil. However, for longer exposures (i.e., more than the standard 14 days), we recommend reducing the density to 4-5 individuals per container to avoid a depletion of soil quality due to high earthworm activity. Furthermore, in order to avoid biomass loss during the experiment, the addition of food at a minimum rate of 3 g ind⁻¹ month⁻¹ is required at the beginning of the test (Bart et al. 2017). Acute toxicity tests are normally performed with adult individuals, but can be undertaken with juveniles that are potentially more sensitive to contaminants (see section 5.) and change in biomass can also be monitored by weighing earthworms at the beginning and at the end of the test.

It is suggested that reproduction tests with *A. caliginosa* can follow the ISO 11268-2 (2012) guideline with the following changes. First, a density of 4 to 5 individuals per container of 500 g of dry soil is more suitable than 10 individuals as suggested in the guideline (see above). Then, we suggest using cohorts of the same age as reproduction rates decrease with age. We also propose, as suggested by Lofs-Holmin (1982) that cocoon production instead of juvenile production is assessed. Indeed, at a temperature of 15 °C, the average hatching time of cocoons is 62 days (Jensen and Holmstrup 1997) which would result in a minimum test duration of 3 months (28 days for adult exposure, and then 60-70 days for hatching). For quicker tests, we thus propose directly assessing cocoon production, using the wet sieving method (see section 6.1.). This measurement should be complemented by monitoring cocoon viability and hatching rates.

No standardized test exists for the assessment of chemical effects on earthworm growth. For a better understanding of the impact of pesticides on earthworm development, we suggest assessing this endpoint at different stages of development, from hatchlings to older juveniles (2-3 months old). In addition, we recommend assessment of maturation time of juveniles (i.e. time from hatching until the emergence of a fully developed clitellum). For experiments with a cohort of hatchling earthworms see section 6.1. To assess growth, regular (e.g. every 14 days) monitoring of individual growth rates is recommended. For that, earthworms should be extracted from vessels while trying to minimize substrate

disturbance. The individuals extracted should be weighed and put back in vessels immediately. The experiment should stop when at least 80% of the earthworms have reached maturity (i.e. with a fully developed clitellum). We strongly support the development of a standardized test to assess the impact of chemicals on earthworm growth.

Finally, *A. caliginosa* behaviour in response to chemicals can be assessed by avoidance tests. From our own experience (Bart et al. 2017) and other publications (Hodge et al. 2000), the procedure described in the ISO 17512-1 (2008) is suitable for *A. caliginosa*, except for the soil (see section 6.). However, as discussed in section 5., we recommend use of the six chambers method or exposure to a linear pollution gradient (Lowe et al. 2016; Bami et al. 2017b). Assessment of the capability of earthworms to detect and to avoid pesticides is relevant in risk assessment as it may more accurately reflect what happens in the field. In this situation, avoidance behaviour would mean that earthworms could avoid sublethal effects of pesticides. Furthermore, if earthworms avoid pesticides and thus disperse from a contaminated area, they no longer provide soil ecological functions related to ecosystem services.

VII. Conclusion

A. caliginosa is one of the most dominant earthworm species in temperate agroecosystems. Its wide distribution and sensitivity to pesticides makes it a relevant soil dwelling species for a posteriori pesticide risk assessment and more generally in soil-based ecotoxicological tests. The use of this species, along with the guidelines presented in this article, can help for a better assessment of risks related to pesticide use. While *E. fetida*, remains a suitable test species for initial screening of chemical effects, the use of *A. caliginosa* is relevant in complementing knowledge on the effects of pesticides, especially for pesticides already widely used on crops.

Our review highlighted the need for further research on the impacts of pesticides on sublethal endpoints (i.e. growth and reproduction), with a focus on ecological functions such as burrowing or casting activities. Moreover, herbicides and fungicides, widely used in temperate areas, deserve more attention. This review also established that knowledge on *A. caliginosa* biology is sufficient to design laboratory experiments allowing assessment of

pesticide impacts on life history traits and behaviour. Data obtained from such tests could be used to understand and predict the effects of pesticides on population dynamics using modelling tools (e.g., Johnston et al. 2014).

Finally, while this review focused on *A. caliginosa* s.s, the information provided may also be appropriate for other species of the *A. caliginosa* complex species, i.e. *A. trapezoides*, *A. tuberculata* and *A. nocturna*.

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Principales conclusions de l'article

Cette revue bibliographique a permis de mettre en avant l'intérêt de l'utilisation de l'espèce *Aporrectodea caliginosa* comme espèce modèle pour l'évaluation du risque. En effet, cette espèce ubiquiste est très représentative des sols tempérés et en particulier des sols d'agroécosystèmes. De plus, la synthèse bibliographique sur les effets des pesticides a souligné la sensibilité de cette espèce aux pesticides. Elle a également permis de souligner le manque de données concernant les effets sur la reproduction et encore d'avantage sur la croissance. Cette dernière variable réponse est d'ailleurs apparue comme un point sensible du cycle de vie, alors qu'aucune norme pour son évaluation n'est disponible à l'heure actuelle. Enfin, il est apparu qu'un frein à l'utilisation de cette espèce est l'approvisionnement en individus aux différents stades (i.e., cocons, juvéniles, adultes). Cette revue a permis, au travers de la présentation des méthodes d'élevage, de démontrer que cet approvisionnement est possible pour tous les stades de développement, incluant la synchronisation de cohortes. Cela permet d'évaluer les effets des pesticides sur l'ensemble du cycle de vie, mais aussi d'acquérir des données sur la biologie de l'espèce et son fonctionnement bioénergétique.

Chapitre 2 – Compréhension du cycle de vie du modèle biologique, proposition d'un modèle bioénergétique

Ce chapitre correspond à un article intitulé « Towards a better understanding of the life cycle of the earthworm *Aporrectodea caliginosa*: new data and energy-based modelling », prochainement soumis dans la revue *Environmental Science and Technology*.

Dans le chapitre précédent, la manipulation en laboratoire de l'espèce de vers de terre *Aporrectodea caliginosa* a été présentée. L'objectif, avant d'évaluer les effets des pesticides sur cette espèce, est de comprendre le fonctionnement bioénergétique de son cycle de vie afin de proposer un modèle basé sur sa biologie. Ce modèle a pour vocation de servir ensuite à l'analyse des effets des pesticides sur le cycle de vie de cette espèce et servira, au-delà de la thèse, à nourrir des modèles de changement de niveau d'organisation « individu centré », c'est à dire s'appuyant sur une description fine du cycle de vie des individus. L'énergie d'un organisme provient de la nourriture absorbée qui est ensuite répartie dans les fonctions de maintenance, croissance et reproduction. Ainsi différentes expérimentations de croissance et de reproduction ont été réalisées en faisant varier la quantité de nourriture apportée pour calibrer le modèle proposé et estimer ses capacités prédictives.

Résumé: Les vers de terre jouent un rôle majeur dans les sols et sont utilisés comme indicateurs biologiques pour l'évaluation des risques écologiques. *Aporrectodea caliginosa* s.s. est une espèce dominante dans les agroécosystèmes et donc un bon candidat pour les tests écotoxicologiques. Afin d'améliorer les connaissances sur son cycle de vie, nous avons réalisé des expériences de croissance et de reproduction. Ces données ont été utilisées pour calibrer un modèle basé sur la gestion de l'énergie qui a fourni une description pertinente du cycle de vie d'*A. caliginosa*, lorsque les vers étaient nourris avec différentes quantités de nourriture (*ad libitum*, c'est-à-dire sans aucune restriction, en quantités limitées ou en absence de nourriture). Une comparaison entre les prédictions du modèle et de nouvelles données (qui n'ont pas été utilisées pour l'estimation des valeurs des paramètres) a permis

de valider notre bonne compréhension des mécanismes de gestion de l'énergie au cours du cycle de vie. Nous avons ainsi montré que les coûts de maintenance étaient négligeables, ce qui s'est traduit par une croissance linéaire en taille en condition *ad libitum*. Nous avons également mis en évidence que le passage du stade juvénile au stade adulte dépendait de la biomasse des individus et que la biomasse seuil n'était pas influencée par la quantité de nourriture disponible. De plus, nous avons montré qu'une fois adultes, s'ils ne sont pas isolés, les individus investissent toute leur énergie dans la production de cocons, qui ne dépendait que de la quantité de nourriture disponible, sans aucun effet de la densité des vers de terre. Enfin, nous mettons en avant l'intérêt de notre modèle, basé sur la gestion de l'énergie, pour la mise en place et l'analyse de tests écotoxicologiques.

Towards a better understanding of the life cycle of the earthworm *Aporrectodea caliginosa*: new data and energy-based modelling

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Abstract

Earthworms have a major role in soils and are used as biological indicators for ecological risk assessment. *Aporrectodea caliginosa* s.s. is one of the dominant earthworm species in agroecosystems and a good candidate for ecotoxicological testing. In order to improve knowledge on its life cycle, we performed growth and reproduction experiments. These data were used to calibrate an energy-based model that displayed good descriptions of *A. caliginosa* life cycle parameters under different feeding conditions (ad libitum, food limitations, or absence of food). A comparison between model predictions and other data (which were not used for the estimation of parameter values) permitted to validate our good understanding of the mechanisms behind the life cycle. Here we showed that the maintenance costs were neglectable, resulting in a linear growth in length when food was provided ad libitum (i.e. without any restriction). We also found that the switch from the juvenile to adult stage depended on the individual weight, and that the threshold weight was not influenced by the amount of provided food (horse dung). Moreover, we provided evidence that once adults, the individuals invested all the energy for cocoon production, which only depended on the available food amount, without any effect of the earthworm density. Finally, we discussed the usefulness of our energy-based model to support the set up and analysis of ecotoxicological tests and experiments.

Keywords: Lumbricidae, Soil ecotoxicology, Energy budget, Life history traits

I. Introduction

Earthworms represent the largest part of animal biomass in most temperate soils (Edwards, 2004; Lavelle and Spain, 2001; Paoletti, 1999). They are considered as typical soil ecosystem engineers (Jones et al., 1994) and provide ecological functions in agroecosystems such as organic matter degradation or soil structure improvement (Bertrand et al., 2015; Blouin et al., 2013; Palm et al., 2014). They are thus considered as indicators of soil biological activity and they are used as model organisms to assess pesticide effects on soil fauna (OECD 1984; Spurgeon et al., 2003).

Aporrectodea caliginosa is one of the dominant earthworm species in arable soils (Bouché, 1972; Decaëns et al., 2011). The interest for this species in the recently published scientific literature has been growing up as several authors proposed it as a reference species in ecological risk assessment (Bart et al., 2018; Klobucar et al., 2011; Pelosi et al., 2013; van Capelle et al., 2016;), instead of *Eisenia fetida* (Savigny). This latter species is recommended as a model species in standardized tests and is widely used in ecotoxicological studies because it is easy to breed and has a short generation time (OECD 1984), allowing for quick and cost effective tests. However, living mainly in organic matter, it is not representative of earthworm species present in the field (Lowe and Butt 2007).

The use of *A. caliginosa* for risk assessment requires sufficient knowledge and understanding of its life cycle and the drivers of its life history parameters, i.e. growth and reproduction. This would allow not only to propose relevant ecotoxicity tests and endpoints, but also to extrapolate the effects from individuals to populations using energy-based modelling. To date, the life cycle of the different earthworm species, and in particular of *A. caliginosa* is poorly known, so as the influence of environmental factors on life cycle parameters. Soil temperature and moisture are known to be key parameters driving earthworm life cycle (Eriksen-Hamel and Whalen, 2006; Holmstrup et al., 1991; Moreau-Valancogne et al. 2013). Furthermore, food quality and quantity are also recognized as major factors influencing life parameters of *A. caliginosa*. For instance, Boström and Lofs-Holmin (1986) showed that particle size had a strong effect on growth curve of *A. caliginosa* and cocoon production in laboratory experiments. Unfortunately, the relationship between food

availability and *A. caliginosa* growth and reproduction is still poorly documented and understood.

In ecotoxicological tests exceeding 14 days with *A. caliginosa*, food supply is necessary because earthworms may starve, stop growing or lose weight without food adding, especially in experiments with juveniles (Bart et al., 2018). This could increase the risk of false positives. In contrast, too much food could limit the contact of earthworms with the soil since they would not have to explore much of the soil to find food. Understanding and modelling the influence of food quantity and quality on ecotoxicological endpoints such as growth and reproduction is thus essential to use appropriate feeding conditions in the tests. Moreover, modelling the life cycle can permit the development of energy-based models, which can be the basis for models to analyse growth and reproduction toxicity data (Kooijman and Bedaux, 1996; Péry et al., 2002a) for effects data available at many time points. Such models permit to assess the toxicological mode of action of chemicals and permit to estimate toxicity parameters that do not depend on the exposure time, such as a no effect concentration (NEC) (Péry et al. 2003). Different authors (Klok, 2008; Jager et al., 2006, Jager and Klok, 2010; Johnston et al., 2014) proposed energy-based model for different earthworm species to analyse toxicity data or for population models. However, these models did not examine if the assumptions made are biologically correct for the considered species (e.g. relative to the energy costs of maintenance or energy allocation at adult stage) and often did not use dedicated experiments for model calibration, but data from previous study.

We here aimed at modelling the influence of food quantity and quality on *A. caliginosa* life cycle using an energy-based model with dedicated experiments. Data were provided by experiments on growth and reproduction under different food quantities, and quality, and the model was inspired by the DEB theory (Kooijman 1986, 2000, 2010) which separates the use of energy between growth, maintenance, and reproduction. We calibrated the model based on a first set data and challenged it with data obtained in other experimental conditions.

II. Material and methods

1. Animals, soil and food

Adults of *Aporrectodea caliginosa* s.s. (Sims and Gerard, 1998) were hand sorted from an agriculture field at INRA (Estrées-mons, 49°52' N 3°01'E) and cultured in a climate room at 15 ± 1 °C in a natural soil with food adding. The soil was a Luvisol (FAO soil classification), sampled from the top 0–20 cm in a permanent meadow in Versailles (48°48' N, 2°5' E). It was air-dried and milled (< 2 mm). Soil physical and chemical properties were as follows: pH 7.5, organic matter 32.6 g kg^{-1} , C/N 12.7, 29% sand, 48% silt, and 23% clay (see Bart et al., 2017 for more details). The food was horse dung which was frozen and defrosted twice and then milled (< 1 mm) as presented in Lowe and Butt (2005).

Cocoons were obtained through water sieving (1 mm) the culture soil (Bart et al., 2018), incubated at 20 °C in petri dish on wet filter papers (Holmstrup et al., 1991), and checked every two days. New hatchlings were collected and stored in soil at 4 °C to slow their development. This procedure allowed synchronizing cohorts of individuals of same age.

2. Description of the energy-based model

We first assumed that the length-to-width ratio remained constant during growth, and that there was a linear relationship between body length and cubic root of the body weight. This assumption, named isomorphism, is reasonable for earthworms. To validate it, length (mm) and weight (mg) of 40 individuals from the breeding culture and previous experiments (prior to this study) were monitored at different ages. For that, individuals were placed on a sheet of graph paper and at least 5 pictures were taken to catch an image of individuals in retracted position. Then, we used the ImageJ software for length measurement (Schneider et al., 2012). The same individuals were weighted using an analytical balance instrument (± 0.1 mg). The data set was used to check the linear correlation between the cubic root of the fresh body weight and the length (Figure1). In the model we then chose to use the cubic root of the fresh body weight. Indeed, body weight

was easier and more precise to obtain than length as individuals contract and relax all the time.

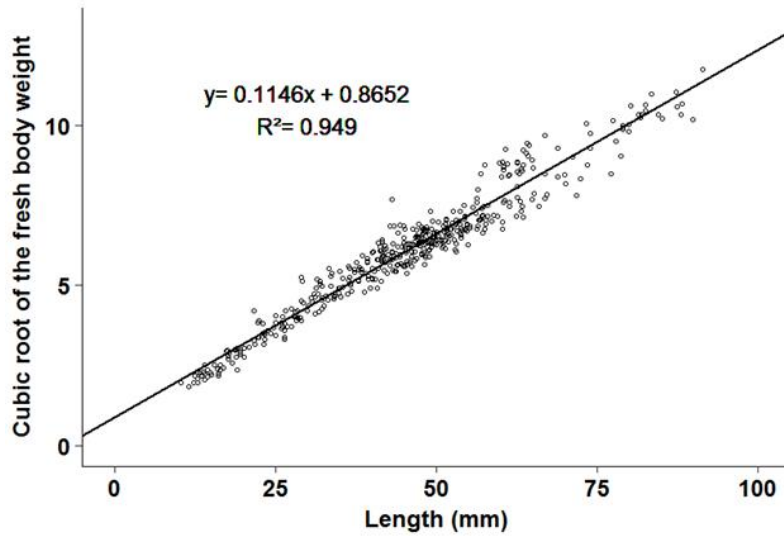


Figure 1. Relationship between the cubic root of the fresh body weight and the length of *Aporrectodea caliginosa*.

Secondly, we assumed that the energy costs of maintenance were neglectable compared to the energy costs of growth throughout the development of the individuals. This assumption was supported by preliminary results showing a linear growth in length even for the biggest individuals. In the context of the DEB theory, this suggests neglectable maintenance costs (Péry et al., 2002b). Finally, we assumed that there was a fixed weight threshold for juveniles to become adults (appearance of a fully developed clitellum).

Based on these assumptions and in the case of unlimited food (i.e. *ad libitum* conditions), the assumptions of isomorphic growth and neglected maintenance costs lead to the equation (1) (Péry et al., 2002b):

$$\frac{d}{dt} l = a \quad (\text{Eqn 1})$$

Where l is the cubic root of the fresh weight, and a is a constant depending on the ability of the organism to assimilate the food. This parameter can be modulated by food particle size as all food particles are not available for very small individuals, thus reducing their feeding

rate. The model account for this process by adding a correction factor to the a parameter for the small individuals (i.e. under a critical size). This leads to the following equation:

$$\text{If } l < C_s, \text{ then } \frac{d}{dt} l = a(1 - b) \quad (\text{Eqn 1})$$

$$\text{If } l > C_s, \text{ then } \frac{d}{dt} l = a \quad (\text{Eqn 1})$$

Where C_s is the Critical size, below which the individual cannot access all the food, and b is the correction factor which corresponds to 1 minus the fraction of the particles reachable for the individual (if $b = 0$, all the food is available since hatchling).

In the case of limited amount of food for earthworms, the daily weight increase was considered proportional to the amount of food that can be taken from the soil plus the added food (i.e. horse dung). This leads to the equation (2):

$$W_{n+1} - W_n = (p \times Q) + S \quad (\text{Eqn 2})$$

Where Q is the quantity of food added to the soil during the feeding event (in mg of horse dung), p is the fraction of this food which can be used to create weight, S is the weight created with the food initially available in the soil, and W_n is the individual weight at the feeding event n . The S parameter value depends on the soil used in the experiment, the time until the soil is renewed (i.e. 28 days in our experiments) and the weight of the individual. For S , we proposed the following equation:

$$S = I_{max} (1 - \exp^{-KW}) \quad (\text{Eqn 3})$$

Where I_{max} is the maximum weight increase between two feeding events, K is a constant and W is the weight of the individual.

Once C_s , a , b , Q , p and the parameters of Eqn 3 (I_{max} and K parameters) were determined, the growth pattern was calculated. For that, the weight increase was first calculated using Eqn 1, assuming *ad libitum* conditions. Then, we checked with Eqn 2 if there was enough food available to obtain the expected growth increase. If not, Eqn 2 was used to calculate the expected weight.

3. Experimental procedures

a. Growth experiment

In all the experiments, the soil was renewed every 28 days and the moisture was adjusted and maintained at 70% of the water holding capacity. The horse dung was also adjusted at 70% of the water holding capacity and mixed with the soil. Earthworms were individually placed in 1 L plastic vessels filled with 400 g dry soil. All the vessels had a removable perforated cover for gas exchanges and were stored in a climate room at 15 ± 1 °C. Individuals were carefully extracted from the vessels with hands every 14 days at the beginning of their growth to be weighted. When they reached approximately 500 mg, they were weighted every 7 days to monitor precisely their weight when a fully developed clitellum was observable.

Experiment 1 was used to (i) test the ability of the model to describe the growth curve of *A. caliginosa* in optimal food conditions (i.e. *ad libitum*), and (ii) estimate the parameters C_s , a , and b of the growth model (Eqn 1). Hatchling earthworms ($n=10$) were fed *ad libitum*, which corresponded to $3 \text{ g ind}^{-1} 14 \text{ days}^{-1}$ of horse dung. This food amount was previously determined and it was close to the amount of $5 \text{ g ind}^{-1} 14 \text{ days}^{-1}$ proposed by Lowe and Butt (2005) for the same species.

Experiment 2 was used to (i) test the capabilities of the model to predict the growth of *A. caliginosa* in *ad libitum* conditions with finer food particles (finer than in Experiment 1), and if not, to (ii) re-estimate the parameter b of the growth model within these new feeding conditions and compared the two descriptions. In this goal, hatchling earthworms ($n=7$) were fed *ad libitum* (i.e. $3 \text{ g ind}^{-1} 14 \text{ days}^{-1}$), but, contrarily to Experiment 1, the food particles were $< 0.5 \text{ mm}$. We expected small juvenile individuals to reach more food, thus decreasing the value of the parameter b (i.e. the correction factor corresponding to 1 less the fraction of the particles reachable for the individual). Thus, the parameters estimated in the Experiment 1 (C_s , a , and b) were first used to predict growth data of these 7 hatchlings. Then, a new value of the parameter b was estimated (keeping C_s and a value), called b_2 , to describe the growth pattern. We finally compared the two fitted growth patterns to the data

set with a Fisher test to determine if the re-estimation of the parameter b led to a significantly better fit of the data.

Experiment 3 was used to (i) estimate the parameters I_{max} and K of the S equation (Eqn 3). For that, we experimentally followed the growth pattern with only the food initially available in the soil) by monitoring the individual weight of new hatchlings ($n=5$), without any food addition. Moreover, because growth was expected to be very slow in that condition, we alternated growth in soil without added food and growth in soil with *ad libitum* food (same origin as in the experiment 2). We used these *ad libitum* periods to assess the ability of the model (Eqn1) to predict the growth pattern of new data. In this way, we first predicted the growth with Eqn 1 (i.e. with C_s , a and b_2 estimated in the experiments 1 and 2, as the food was the same as in the Experiment 2) and then compared to the produced data.

Experiment 4 was used to estimate the parameter p of Eqn 2. For that, the growth pattern of 10 individuals fed with $1 \text{ g ind}^{-1} 14 \text{ days}^{-1}$ (same origin as in the experiment 1) was followed by monitoring individual weight. We used the parameters C_s , a , b (estimated in the experiment 1) and the S equation (Eqn 3) with parameters estimated in the experiment 3, and we estimated the parameter p from the data.

Experiment 5 was used to test the ability of the model to predict a new data set (not used for the calibration of the parameter values) with a new limited food conditions. Five individuals were fed with a new amount of food corresponding to $1.5 \text{ g ind}^{-1} 14 \text{ days}^{-1}$. The predictions of the model using the parameters estimated in the first four experiments were compared with the data obtained in this experiment.

b. Switch to adult stage

The data obtained in the growth experiments with the three tested food amounts (i.e. 1, 1.5 and $3 \text{ g ind}^{-1} 14 \text{ days}^{-1}$) were used to determine the weight at which juveniles switched to adult stage (i.e. apparition of a fully developed clitellum). For each individual, we used the latest weight recorded as juvenile and the first weight recorded as adult. We first statistically tested if the food amount had an impact on the average of these two weights. Then, these two weights were used to provide the percent of individuals which reached

maturity in term of their weight. Finally, we fitted a linear regression between the smallest weight adult recorded and the biggest juvenile weight recorded of the data set.

c. Reproduction

Experiment 6 was used (i) to understand the energy allocation strategy between growth and reproduction at the adult stage, (ii) to estimate the impact of food amount on cocoon production, and (iii) to test the impact of earthworm density on cocoon production. For that, we used individuals of the experiment 1 and 4 (fed with 1 and 3 g ind⁻¹ 14 days⁻¹, respectively). When they became adults, they were separated into two groups. Four individuals were kept individually (same conditions as in the growth experiment) and the growth was monitored by weight measurements every 14 days. A second group of 6 individuals were pooled by 2 for 84 days, then by 3 for 84 days and finally by 6 for 84 days per vessel. The total food amount was adapted to suit the needed amount of food per individual per unit of time. Growth was monitored by weighting the earthworms individually every 14 days. Cocoon production was monitored when renewing the soil (i.e. every 28 days) by wet sieving it through a 1 mm mesh size (Bart et al 2018).

4. Model calibration and statistical analysis

The model equations were implemented in the R software R Core Team (2015) and the set of differential equations were solved using the DeSolve package. All the data for a given experiment were fitted simultaneously. Optimization was performed through a least square method. The 95% Confidence Interval (CI) of the parameters were estimated with the Bootstrap method (Efron, 1977). The impact of food on earthworm weight that determined the switch from juvenile to adult stage was tested using Kruskal-Wallis tests.

III. Results

1. Growth experiments

All the growth curves are presented in fresh weight for more realism in representing the earthworm growth. Based on the results of the Experiment 1, the model well described the growth data (Figure 2), with parameter values estimated at 3.93, 0.084, and 0.39 for C_s , a , and b respectively (Table 1). The growth rate in length (i.e. in weight cubic root of the fresh weight) of small earthworms (bellow C_s) was 39% lower than the rate of bigger individuals (above C_s).

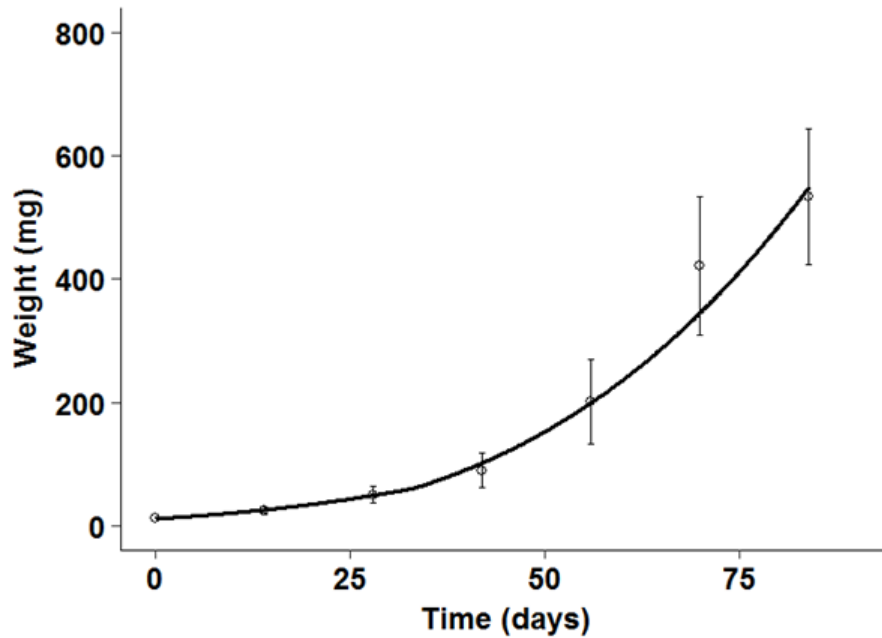


Figure 2. Growth pattern of *A. caliginosa* with food *ad libitum* as a function of time since hatchling ($n=10 \pm SD$). The line represents the description obtained with the growth model (Eqn. 1).

Table 1. Parameter values of the equation 1, 2 and 3, optimized with the different experiments and their confidence intervals (CI 95%)

Optimize with data set of	Symbol	Unit	Description	Value	CI 95%
Experiment 1	C_s	$\text{mg}^{1/3}$	Critical size allowing reachability to the all food	3.927	3.262 - 4.531
Experiment 1	a	$\text{mg}^{1/3} \text{ day}^{-1}$	Constant	0.084	0.074 - 0.093
Experiment 1	b	-	Correcting factor for growth constant for individual under the Critical size (C_s)	0.391	0.297 - 0.496
Experiment 2	b_2	-	Correcting factor for growth constant for individual under the Critical size (C_s)	0.256	0.202 - 0.309
Experiment 4	p	-	Proportion of food transformable into weight unit	0.048	0.044-0.052
Experiment 3	l_{max}	mg	Maximal weight increase between two feeding event (14 days)	80.97	-
Experiment 3	K	mg^{-1}	Constant	0.00159	-

From Experiment 2, we showed that the previous growth model (Eqn 1) (i.e. with the parameter values estimated with the data of experiment 1) underestimated the beginning of the growth pattern (full line, Figure 3). The re-estimation of the parameter b , called b_2 , allowed for a better description of the data (dash line, Figure 3) as there was a significant improvement of the fit (Fisher test, F-value < 0.01).

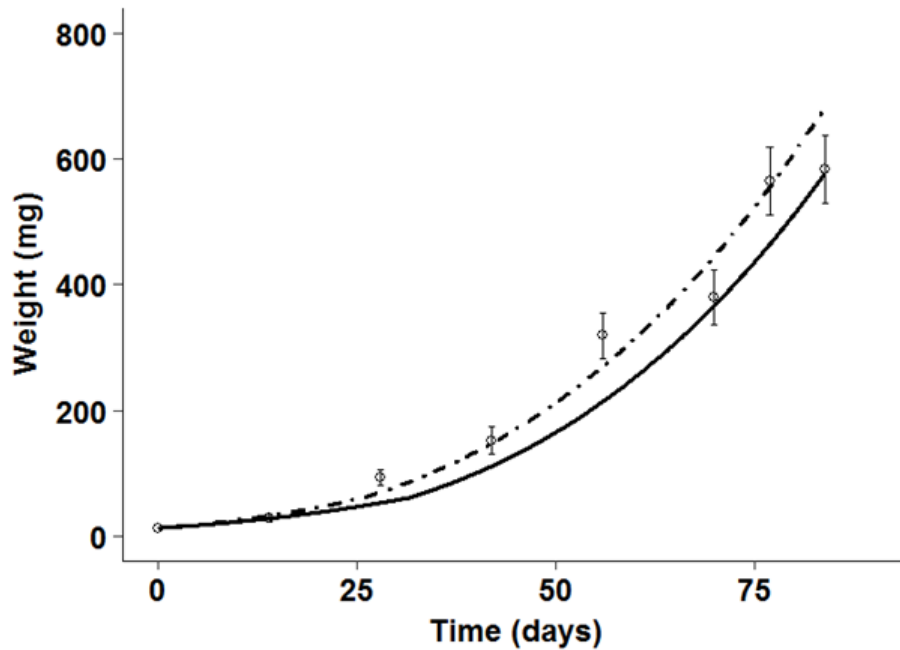


Figure 3. Growth pattern of *A. caliginosa* with food *ad libitum* as a function of time since hatching ($n=7 \pm SD$). The full line represents the predictive model with food *ad libitum* (Eqn. 1) with parameter values obtained in experiment 1. The dash line represents the model with food *ad libitum* (Eqn 1) with C_s and a parameter values from the experiment 1 and the b_2 parameter adjusted to the data.

Based on the data of the Experiment 3, we estimated parameters values for I_{max} and K of the S equation (Eqn 3) at 80.97 and 0.048 respectively (Figure 4, Table 1). The S equation (Eqn 3) provided a relevant description of the growth pattern when individuals were fed only with soil (Figure 5, full line). The growth model (Eqn 1) with parameter values previously estimated (in experiment 1 and 2) provided a relevant prediction of the growth pattern when individuals were fed with *ad libitum* food, with a maximum error of 11% between predicted values and data (Figure 5, dash line).

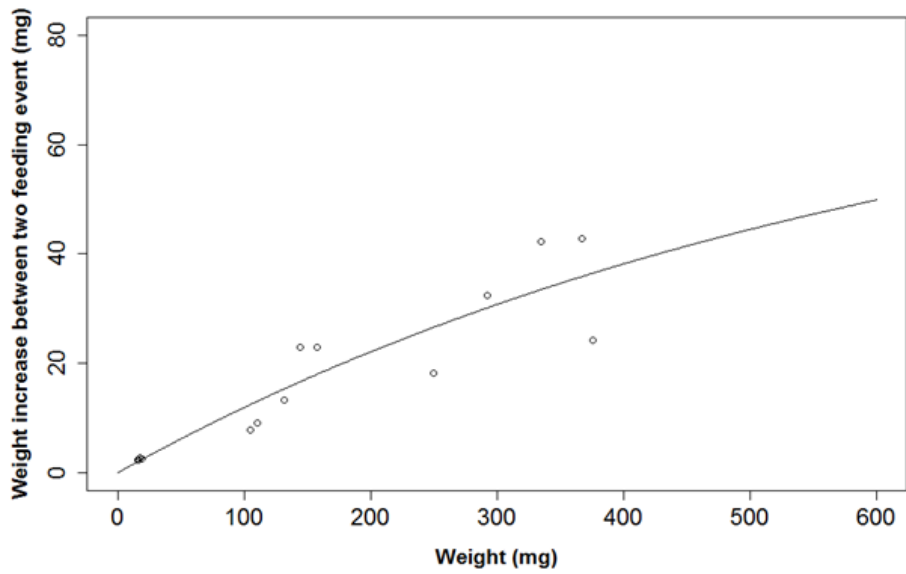


Figure 4. Individual weight increase in mg between two feeding events (i.e. every 14 days) with the food available in the soil in term of the individual weight ($n=5 \pm SD$). The line represents the description obtained with the S equation (Eqn 3).

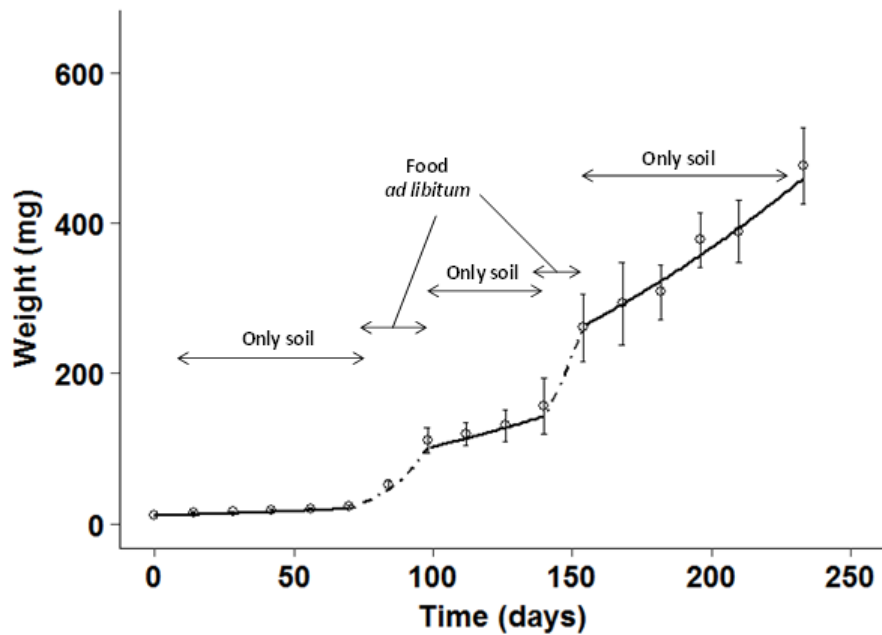


Figure 5. Growth pattern of *A. caliginosa* since hatching with only soil as food or with food *ad libitum* ($n=5 \pm SD$). The full line is the description of the data using the S equation (Eqn 3) and the dash line is the prediction of the model using Eqn 1 (with parameter values estimated in experiment 1 and 2, i.e. a , b_2 and C_s).

Eqn 1 predicted the beginning of the growth pattern of data of the experiment 4 and Eqn 2 described the rest of the growth curve (Figure 6) with the parameter p estimated at 0.048 (table1).

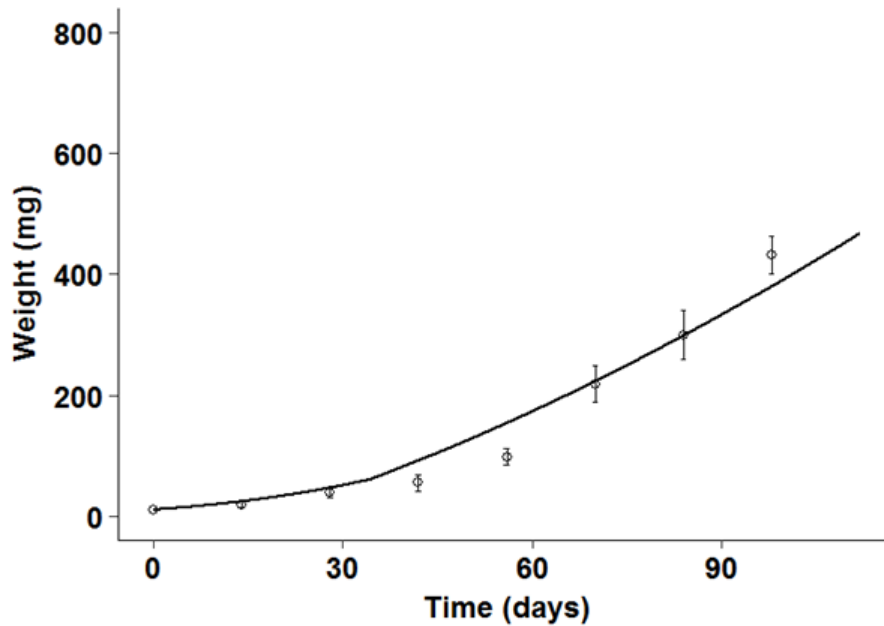


Figure 6. Growth pattern of *A. caliginosa* with $1 \text{ g ind}^{-1} 14 \text{ days}^{-1}$ of food as a function of time since hatching ($n=10 \pm \text{SD}$). The line represents the data described by the model using Eqn 1 until individuals were under limited food condition switching to the model with limited food with Eqn 2.

From the data obtained in Experiment 5, the growth of individuals fed with $1.5 \text{ g ind}^{-1} 14 \text{ days}^{-1}$ was predicted with our growth model using Eqn 1, Eqn 2 and parameter values optimized in experiments 1, 2, 3 and 4 (i.e. $a, b_2, C_s, p, K, I_{max}$). It was then compared to the data (Figure 7). The model provided predictions which never exceeded 18% of error between the predicted values and the data.

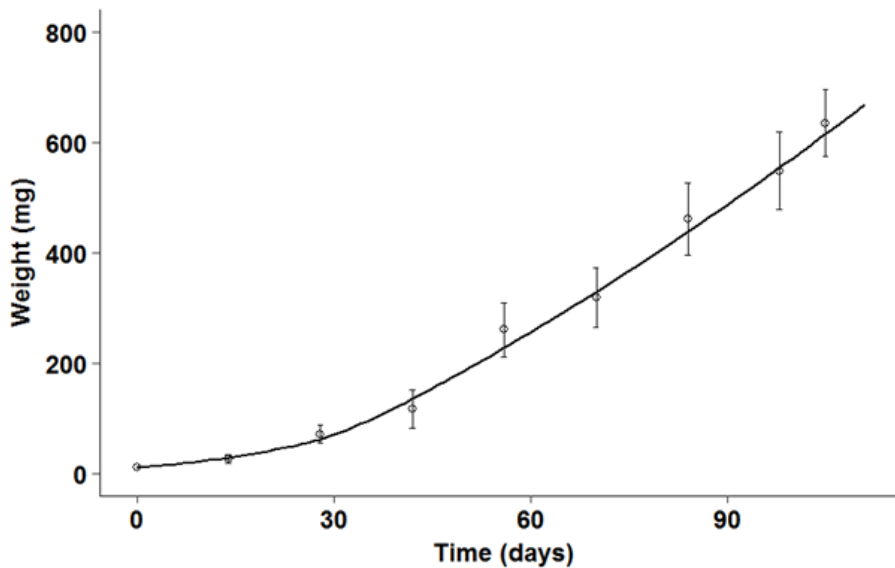


Figure 7. Growth pattern of *A. caliginosa* with $1.5 \text{ g ind}^{-1} 14 \text{ days}^{-1}$ of food as a function of time since the hatching ($n=5 \pm \text{SD}$). The line represents the prediction of the model with Eqn 1 and 2 with parameter values estimated in the experiment 1, 2, 3 and 4 (i.e. $a, b_2, C_s, p, k, I_{max}$).

2. Switch to adult stage

The statistical analyses revealed that the food amount did not impact the weight at which the individuals switched from juvenile to adult stage (Figure 8 A, p-value = 0.115). A linear regression was used to represent the percentage of adults according to the weight of the individuals. This equation correctly describes the data (Figure 8B) and can be associated to the growth model to predict the switch from juvenile to adult of an individual according to its weight.

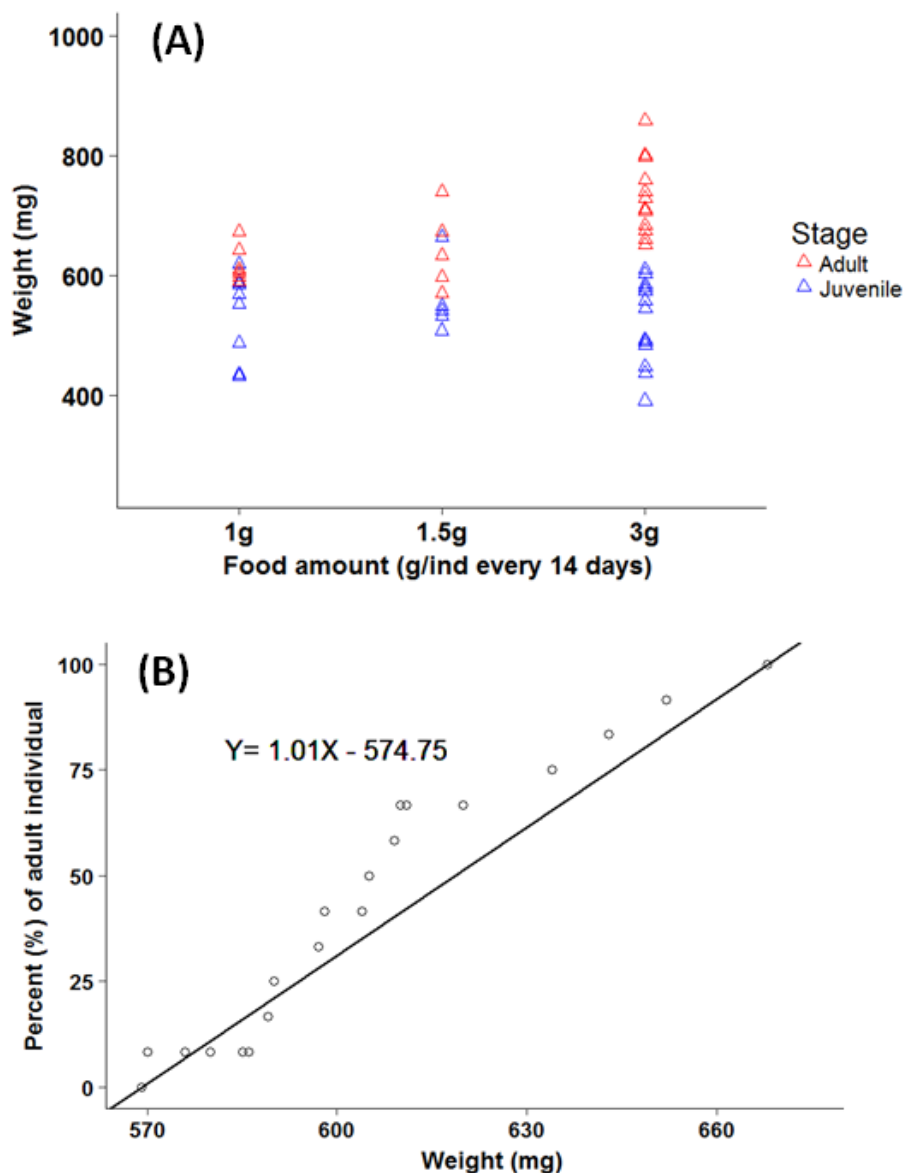


Figure 8. (A) First recorded weight as adult (in red) and last recorded weight as juvenile (in blue) of the individuals in growth experiments and (B) the percent (%) of adult individual in terms of individual weight, the line being the model to describe the data which are the points.

3. Reproduction experiment

Energy allocation strategy

The earthworms maintained individually in vessels at the adult stage continued to grow at both food treatments (red and dark blue lines, Figure 9). The individuals pooled in vessels for reproduction stopped their growth at both food treatments (orange and light blue lines, Figure 9).

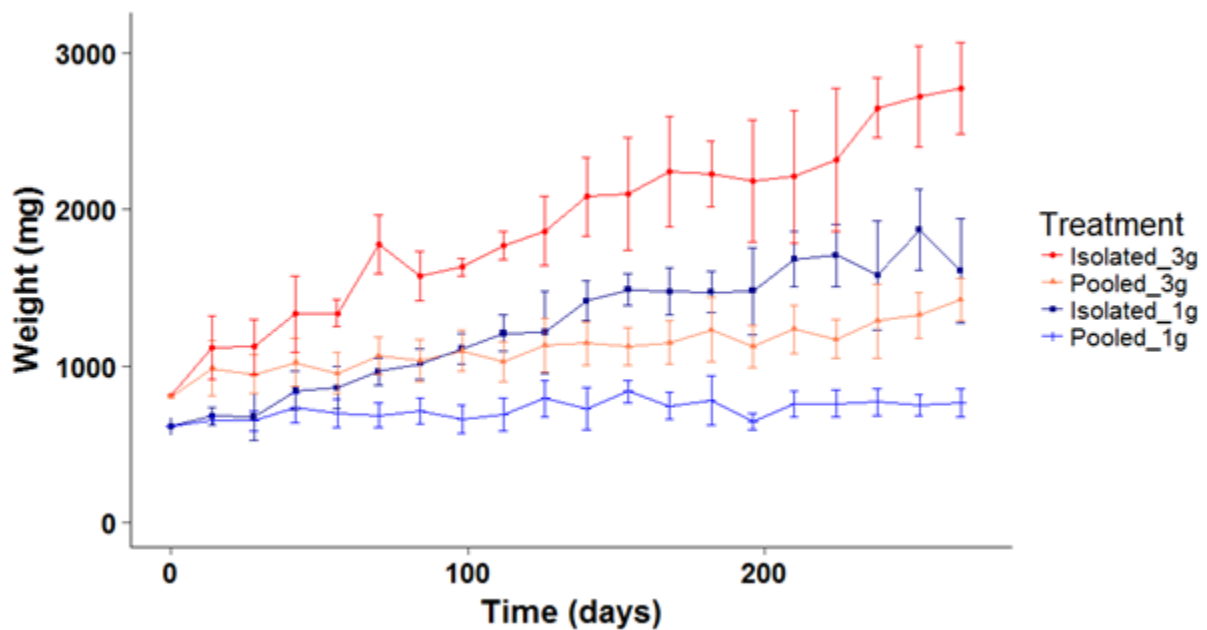


Figure 9. Growth pattern of adult individuals isolated ($n=4$) or pooled for reproduction ($n=6$). Individuals were fed with two different food amount (1 and $3 \text{ g ind}^{-1} 14 \text{ days}^{-1}$).

Cocoon production

The mean cocoon production was on average 1.6 and 2.4 cocoons $\text{ind}^{-1} \text{ week}^{-1}$ with 1 and $3 \text{ g food ind}^{-1} 14 \text{ days}^{-1}$ respectively. When the density was 6 individuals per vessel, the cocoons production decreased by 50% but only for the individuals fed with $1 \text{ g ind}^{-1} 14 \text{ days}^{-1}$ (Figure 10).

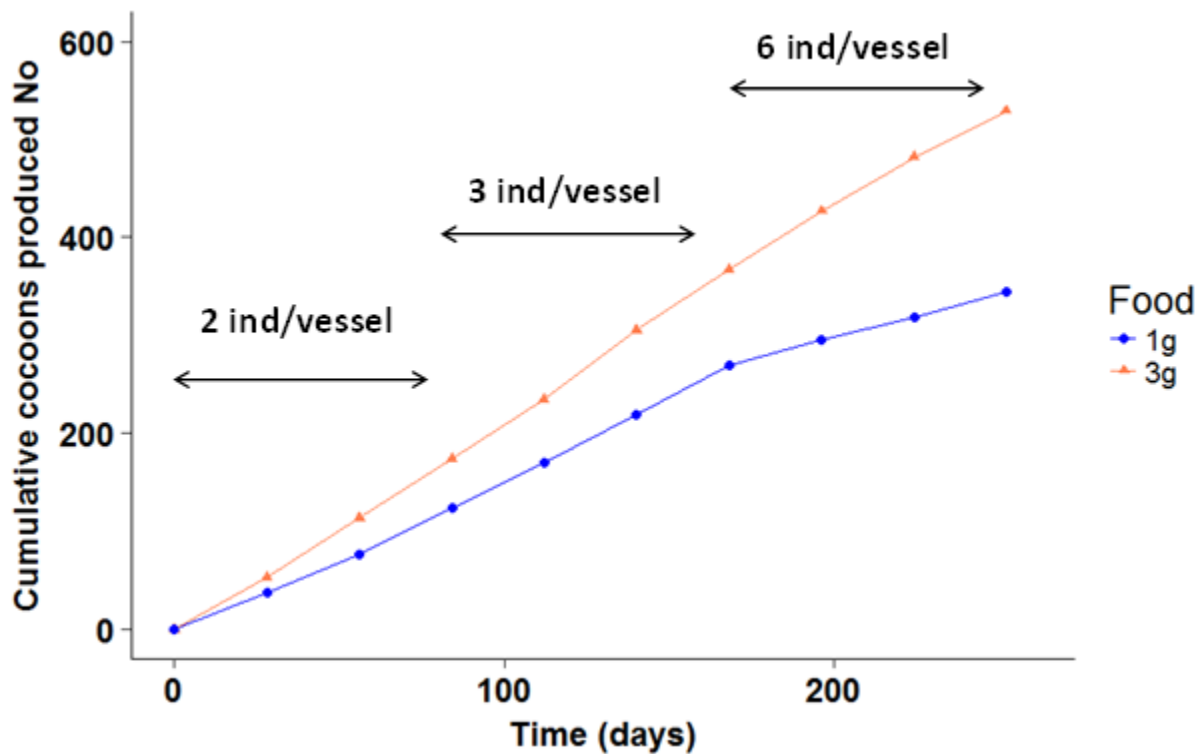


Figure 10. Cumulative cocoon production of 6 individuals, with two various food conditions (1 and 3 g ind⁻¹ 14 days⁻¹) pooled by 2 for 84 days, then by 3 for 84 days and finally by 6 the last 84 days.

IV. Discussion

While the interest towards the earthworm species *A. caliginosa* is growing up in ecology risk assessment (Bart et al., 2018), we here provided new data (growth and reproduction) and a modelling approach allowing to describe the life cycle of *A. caliginosa* under different food regimes.

The data allowed to assess the impact of food and density on reproduction and on the energy allocation between reproduction and growth at the adult stage. First, results showed that adult individuals produced on average 2.4 and 1.6 cocoons ind⁻¹ week⁻¹ for modality with respectively 3 g and 1 g food ind⁻¹ 14 days⁻¹. These results are in accordance with other authors (Lofs-Holmin, 1982; Lowe and Butt, 2005; Spurgeon et al., 2000) who reported that cocoon production for *A. caliginosa*, in field soils at 15 °C, is between 0.6 to 2.6 cocoons ind⁻¹ week⁻¹. According to our results, we here provided evidence that the cocoon production by *A. caliginosa* depends on the amount of available food. The reproduction

results also showed that at the highest density tested (i.e. 6 individuals per vessel) the cocoon production decreased for individuals fed with a limited food amount but not for individuals fed with *ad libitum* food. This result could be explained by the food available in soil, which was divided by the number of individuals per vessel. Indeed in limited food conditions, the influence of the food available in the soil can be high. There was twice less food from soil when 6 individuals were exposed than when they were 3. For individuals fed *ad libitum*, because the amount of food provided (i.e. horse dung) was sufficient, there was no decrease in cocoon production. The highest density tested would corresponded to 3200 ind⁻¹ m⁻² (if we considered that *A. caliginosa* is found in the first 20 cm of soil and a medium soil density of 1.2), which is very higher than densities found in the field ranging from 50 to 400 ind⁻¹ m⁻² (Amossé et al., 2018; Edwards and Bohlen, 1996). We thus conclude that the earthworm density do no affects the cocoon production as it was found by Klok et al. (2008) for *L. rubellus* species.

Finally, the results of the reproduction experiment highlighted that when in contact with each other, adult individuals pooled for reproduction invested all their energy for the production of cocoons and stopped growing. In ecology, this adaptive comportment corresponds to the r strategy which is related to a high growth rate, a production of many offsprings and a relatively low probability of surviving to adulthood (MacArthur and Wilson, 1967). This strategy has been already highlighted for other endogeic earthworm species (Satchell, 1980).

For the juvenile stage, the DEB theory (Kooijman, 2000) considered that the energy is invested to maintenance and growth from one part, and to maturity from the other part (this corresponds to the Kappa law). For the somatic part, the maintenance requirements are supposed to take precedence over growth and the food uptake is assumed proportional to surface area, whereas maintenance is proportional to volume. Given a faster increase of the volume than surface area with growth, it is expected an attenuating growth curve, which is captured by the Von bertalanffy growth curve. Different authors conserved these assumptions for different earthworm species: *Dendrobaena octaedra* (Jager and klok, 2010), *Eisenia fetida* (Jager et al., 2006), *Lumbricus rubellus* (Hobbelen and van Gestel, 2007; Klok, 2008) and *Aporrectodea caliginosa* (Johnston et al., 2014) but without testing the biological relevance of the assumptions of the model towards the considered species. In our study, we

tested a different modelling approach based on neglectable maintenance costs. This approach was successfully demonstrated as correct for several aquatic species (*Chironomus* species, Péry et al., 2005), and our results showed that this approach works for *A. caliginosa*. Our model provided a good description of the growth data measured in the different experiments, confirming that the maintenance costs are neglectable. Our model was able to account for food limitations (even absence of additional food), and was, in particular, able to predict the growth pattern from a new data set with a different food amount (i.e. limited quantity). When coupled with the equation determining the probability for an individual to switch to adult stage according to its weight, it became possible for any condition of feeding, and of soil, to predict the time for a juvenile individual to reach maturity.

The results indicated that the parameter corresponding to the growth rate should be optimized for any new food type (quality and quantity). This is particularly required for taking into account the part of the food available for small juveniles. This result is in accordance with Bostrom and Lofs-Holmin (1986) who showed that food particle size had a strong influence on the growth of *A. caliginosa*. Moreover, the equation for the soil (Eqn 3) should also be recalibrated for any new soil because of the organic matter content available (i.e. the food) is different for every soil type.

The model proposed in this study can be used to analyze ecotoxicological effects data throughout time. In ecotoxicology, growth and reproduction data are usually analyzed through statistically based models, with *ad hoc* curves to estimate EC_x or NOEC values, for a given duration of exposure, including earthworms (De Silva et al., 2009; Garcia et al., 2008; Rico et al., 2016). These parameters estimated are thus time dependent, and do not allow for any extrapolation of the effects at longer time steps. Energy-based models have been first proposed by Kooijman and Bedaux (1996) to analyze toxicity data from growth and reproduction tests at several time steps. Such models provided a relevant description of the data for different species including fish, nematodes, chironomids, and earthworms (Goussen et al., 2015; Hobbelen and van Geslel, 2007; Jager and Klok, 2010; Kooijman and Bedaux, 2010; Péry et al., 2003). In those models, the chemicals are supposed to affect one of the parameters of the energy-based model, as a function of their mode of action. For instance, an energy-based model was used by Péry et al. (2003) to assess if copper impacted the growth efficiency or the food assimilation of chironomids with models very similar to the

one proposed here. Moreover, these models allow the estimation of a No Effect Concentration (NEC) which does not depend on the duration of the test and make easier the extrapolation to other conditions (e.g. other times measurement) or other levels of biological organization (at population level).

To conclude, we provided new knowledge and understanding on the life cycle of *A. caliginosa* through experiments and the calibration of an energy based model. The model proposed should be of interest in ecological risk assessment and ecology for the analysis of toxicity data and, later, for population dynamics models.

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Principales conclusions de l'article

Cet article a permis de comprendre et de modéliser le fonctionnement bioénergétique du cycle de vie de l'espèce de vers de terre *A. caliginosa*. Les principales conclusions sont: (i) les couts énergétiques de maintenance sont négligeables; ainsi, la croissance en taille est linéaire en condition de nourriture *ad libitum*; (ii) le passage au stade adulte semble déterminé par la biomasse des individus; (iii) la biomasse seuil permettant la transition du stade juvénile au stade adulte ne dépend pas de la quantité de nourriture disponible dans le milieu; (iv) les individus adultes, s'ils ne sont pas isolés, allouent toute leur énergie à la reproduction (production de cocons), qui dépend de la quantité de nourriture disponible mais pas de la densité des individus.

Maintenant que le cycle de vie d'*A. caliginosa* a été décrypté, quels sont les effets des pesticides sur celui-ci ?

Partie 2 - Effets des pesticides sur le cycle de vie *d'A. caliginosa*, application du modèle bioénergétique

Chapitre 1 – Effet des pesticides sur la mortalité et le comportement

Ce chapitre correspond à un article intitulé “Differences in sensitivity between earthworms and enchytraeids exposed to two commercial fungicides”, publié dans la revue *Ecotoxicology and Environmental Safety*. <https://doi.org/10.1016/j.ecoenv.2017.02.052>

Pour évaluer les effets des pesticides sur le cycle de vie d'*A. caliginosa*, il a tout d'abord été nécessaire de déterminer la toxicité aiguë des pesticides choisis mais aussi d'évaluer la capacité d'*A. caliginosa* à éviter ces produits dans le sol. Cette étape est essentielle pour déterminer les concentrations sublétales que nous utiliserons ensuite. Ainsi, des expérimentations d'évaluation de la toxicité aiguë et d'évitement (Annexe 3) ont été réalisées avec deux fongicides. Le premier fongicide est une formulation commerciale de cuivre (50% Oxychlorure de cuivre) et le second est le Swing® Gold, dont les substances actives sont la dimoxystrobine (133g L⁻¹) et l'époxiconazole (50g L⁻¹) (Annexe 4). Dans ce chapitre, les tests ont été réalisés sur notre espèce modèle *A. caliginosa* mais aussi sur une espèce d'enchytréide, *Enchytraeus albidus* (Annexe 1). Les enchytréides sont des annélides oligochètes du sol, au même titre que les vers de terre. Ils participent à des fonctions écologiques clés du sol de la même manière que les vers de terre (dégradation de la matière organique, création de porosité) mais à une échelle inférieure à ces derniers puisqu'ils appartiennent à la mésofaune du sol. Les enchytréides ont été beaucoup moins étudiés que leurs homologues oligochètes du sol, les vers de terre. Pourtant, quelques études laissent penser que leur rôle serait complémentaire. Les uns, plus gros, fragmentent la matière organique peu décomposées de surface, ce qui profiterait aux autres, plus petits. Par ailleurs, les enchytréides seraient un peu moins sensibles aux facteurs environnementaux et aux pratiques agricoles (ex. utilisation de pesticides, labour) que les vers de terre. Ils pourraient ainsi assurer certaines fonctions (dégradation de la matière organique, création de porosité) quand les vers de terre ne le peuvent plus (ou moins), comme par exemple dans les systèmes cultivés de manière intensive.

L'article présenté ci-dessous est ainsi orienté vers une comparaison de sensibilité entre ces deux espèces, même si la partie qui intéresse directement ce travail de thèse concerne *A. caliginosa*.

Résumé: L'utilisation de pesticides dans les champs cultivés peut avoir des effets négatifs sur les annélides oligochètes du sol, c'est-à-dire les vers de terre et les enchytréides, et affecter ainsi la qualité du sol. Cette étude avait pour objectif d'évaluer les effets de deux formulations commerciales de fongicides sur l'espèce de vers de terre *Aporrectodea caliginosa* et l'espèce d'enchytréide *Enchytraeus albidus* dans un sol naturel. Les fongicides étaient le Cuprafor micro® (oxychlorure de cuivre), couramment utilisé en agriculture biologique, et le Swing® Gold (époxyconazole et dimoxystrobine), un fongicide synthétique largement utilisé dans l'agriculture conventionnelle pour protéger les cultures céréalières. Des expériences en laboratoire ont été réalisées pour évaluer les effets sur la survie, la perte de biomasse et le comportement d'évitement. Aucun effet létal n'a été observé après une exposition au fongicide cuivré pendant 14 jours, même pour des concentrations de 5 000 mg kg⁻¹, c'est-à-dire 650 fois la dose recommandée (DR). Cependant, une diminution significative de la biomasse a été observée à partir de 50 mg kg⁻¹ de cuivre (6.5 fois la RD) pour *A. caliginosa* et à 5 000 mg kg⁻¹ de cuivre (650 fois le RD) pour *E. albidus*. Ces effets sublétaux suggèrent qu'une plus longue période d'exposition aurait probablement conduit à des effets létaux. L'EC50 évitement pour le fongicide cuivré a été estimé à 51,2 mg kg⁻¹ de cuivre (6.7 fois le DR) pour *A. caliginosa* et à 393 mg kg⁻¹ de cuivre (51 fois le DR) pour *E. albidus*. Concernant le Swing Gold®, la CL50 estimée était de 7.0 10⁻³ mL kg⁻¹ (6.3 fois le DR) pour *A. caliginosa* et de 12,7 10⁻³ mL kg⁻¹ (11.0 fois la DR) pour *E. albidus*. Aucun effet sur la biomasse ou l'évitement n'a été observé aux concentrations sublétales. Il a été conclu que les enchytréides étaient moins sensibles que les vers de terre aux deux fongicides commerciaux en termes de mortalité, de perte de biomasse et de comportement d'évitement. Par conséquent, nous discutons quant aux différentes stratégies utilisées par ces deux espèces pour lutter contre la présence de ces pesticides, ainsi que les conséquences potentielles sur le fonctionnement du sol.

Differences in sensitivity between earthworms and enchytraeids exposed to two commercial fungicides

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Abstract: The use of pesticides in crop fields may have negative effects on soil Oligochaeta Annelida, i.e., earthworms and enchytraeids, and thus affect soil quality. The aim of this study was to assess the effects of two commercial fungicide formulations on the earthworm *Aporrectodea caliginosa* and the enchytraeid *Enchytraeus albidus* in a natural soil. The fungicides were Cuprafor micro[®] (copper oxychloride), commonly used in organic farming, and Swing[®] Gold (epoxiconazole and dimoxystrobin), a synthetic fungicide widely used in conventional farming to protect cereal crops. Laboratory experiments were used to assess the survival, biomass loss and avoidance behaviour. No lethal effect was observed following exposure to the copper fungicide for 14 days, even at 5,000 mg kg⁻¹ of copper, i.e. 650 times the recommended dose (RD). However, a significant decrease in biomass was observed from 50 mg kg⁻¹ of copper (6.5 times the RD) for *A. caliginosa* and at 5,000 mg kg⁻¹ of copper (650 times the RD) for *E. albidus*. These sublethal effects suggest that a longer period of exposure would probably have led to lethal effects. The EC50 avoidance for the copper fungicide was estimated to be 51.2 mg kg⁻¹ of copper (6.7 times the RD) for *A. caliginosa*, and 393 mg kg⁻¹ of copper (51 times the RD) for *E. albidus*. For the Swing[®] Gold fungicide, the estimated LC50 was 7.0 10⁻³ mL kg⁻¹ (6.3 times the RD) for *A. caliginosa* and 12.7 10⁻³ mL kg⁻¹ (11.0 times the RD) for *E. albidus*. No effect on biomass or avoidance was observed at sublethal concentrations of this synthetic fungicide. It was concluded that enchytraeids were less sensitive than earthworms to the two commercial fungicides in terms of mortality, biomass loss and avoidance behaviour. Therefore we discuss the different strategies possibly used by the two Oligochaeta species to cope with the presence of the pesticides were discussed, along with the potential consequences on the soil functions.

Keywords: Potworms, ecotoxicology, pesticides, risk assessment, mortality.

I. Introduction

Pesticide applications in agricultural systems decrease biodiversity and biological activity in cultivated soils (Bengtsson et al., 2005; Hole et al., 2005; Pelosi et al., 2014). The non-intentional effects of pesticides on soil organisms have to be understood and quantified in order to assess their possible consequences on soil functioning (Lavelle et al., 2006; Blouin et al., 2013; Bertrand et al., 2015). Earthworms are often used as soil biological indicators (Spurgeon et al., 2003). They represent the largest living biomass in terrestrial ecosystems (Edwards, 2004) and have positive impacts on soil structure and porosity, nutrient cycling, and organic matter fragmentation (Lavelle et al., 2004; Seeber et al., 2008; Bottinelli et al., 2010). Although enchytraeids are less studied, they have also been recognized as indicators of soil quality (Graefe and Beylich, 2002; Jänsch et al., 2005), chemical stress (Didden and Römbke, 2001) and agricultural management practices (Pelosi and Römbke, 2016). Like earthworms, enchytraeids play key functional roles in the soil but at a smaller scale (van Vliet et al., 1993; Marinissen and Didden, 1997). They are involved in the evolution of the soil structure (Didden, 1990; van Vliet et al., 1995). Moreover, they influence organic matter decomposition and nutrient cycling (i) directly by consuming large amounts of organic matter (Briones et al., 1998; Cole et al., 2000; Maraldo et al., 2011), and (ii) indirectly by creating favourable microhabitats for microbial activity (Kasprzak 1982; Bardgett 2005).

Interactions between earthworms and enchytraeids can increase organic matter consumption and decomposition rates as compared to the same organisms taken separately (Sandor and Schrader 2012). In a cultivated soil under tillage pressure, enchytraeid activity increased when earthworm activity decreased (Topoliantz et al. 2000). The authors concluded that enchytraeids played a key role for soil structure and porosity when earthworm density was low. Thus, the use of enchytraeids in addition to earthworms, for testing chemicals, may provide relevant and complementary information. This is unfortunately rarely documented.

In 2009, Marques et al. highlighted “a need for a careful assessment of the impacts of formulated products”. Indeed, most of the studies assessing the impact of pesticides on Oligochaeta Annelida had used only active substances or pesticides no longer authorized in Europe (Kobeticova et al., 2009; Pelosi et al., 2014). The effects of active substances can

substantially differ from those of commercial formulations because formulations can contain more than one active substance as well as adjuvants also likely to have adverse ecotoxicological effects (De Silva et al., 2010; Lajmanovich et al., 2011; Piola et al., 2013).

The aim of the present study was to assess the impact of two commercial pesticide formulations on the survival, biomass and avoidance behaviour of two species of terrestrial oligochaetes: an earthworm species, *Aporrectodea caliginosa* and an enchytraeid species, *Enchytraeus albidus*. Experimental studies were conducted under laboratory conditions using a natural soil and different pesticide concentrations. Commercial pesticides formulations were given preference over pure molecules to be as close as possible to the field conditions. The two pesticides are widely used in cultivated fields in Europe. The first one was Cuprafor micro[®], a copper fungicide composed of 50% copper oxychloride, used in organic farming to prevent spore germination. The second one was Swing[®] Gold, a synthetic fungicide used to protect cereal crops in conventional farming. It is composed of two active substances, dimoxystrobin (DMX) and epoxiconazole (EPX) recognized as dangerous for the environment as well as carcinogenic, mutagenic and reprotoxic (PPDB, 2016). *Eisenia* sp. is currently recommended as the model earthworm species in standardized tests (OECD 1984). However, it is not found in arable lands and is less sensitive to pesticides and metabolites than species found in cultivated fields (Pelosi et al., 2013). That is why we chose *Aporrectodea caliginosa*, a dominant earthworm species in cultivated fields in temperate areas (Bouché 1972; Perez-Losada et al., 2009; Decaëns et al., 2011), for this study. As knowledge about enchytraeids is relatively poor and species found in fields are difficult to breed, we used *Enchytraeus albidus*, the species recommended in standardized tests (OECD 220, 2004; Römbke 2003).

II. Material and methods

1. Soil, pesticides, animals

The same soil was used for all experiments. We chose a natural soil rather than an artificial soil to be closer to field conditions. It was a Luvisol (FAO soil classification), sampled from a fallow in Versailles (48°48' N, 2°5' E) where no chemicals had been applied for more

than 20 years. It was collected from the top 0-20 cm, air-dried and passed through a 2-mm sieve. Its main physical and chemical properties were as follows: pH 7.5, organic matter 32.6 g kg⁻¹, C/N 12.7, 29 % sand, 48 % silt, and 23% clay (Table 1). The dry soil was stored at 20°C for a maximum of 50 days before the experiments.

Table 1. Main characteristics of the studied soil

Characteristics	Soil
Clay (< 2 µm, g kg ⁻¹)	226.0
Fine silt (2-20 µm, g kg ⁻¹)	174.1
Coarse silt (20-50 µm, g kg ⁻¹)	298.9
Fine sand (50-200 µm, g kg ⁻¹)	239.1
Coarse sand (200-2000 µm, g kg ⁻¹)	47.9
CaCO ₃ total (g kg ⁻¹)	23.3
Organic matter (g kg ⁻¹)	32.6
P ₂ O ₅ (g kg ⁻¹)	0.1
Organic carbon (g kg ⁻¹)	18.9
Total nitrogen (N) (g kg ⁻¹)	1.5
C/N	12.7
pH	7.5
Total Cu (mg kg ⁻¹)	25.2

The French RD for Cuprafor micro[®] (Quimicas del Valles, 50% copper oxychloride) is 10 kg ha⁻¹ on potato and in vineyards (E-phy, 2016a). The French RD for Swing[®] Gold (Bayer Agro SAS, dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹) is 1.5 L ha⁻¹ on wheat (E-phy, 2016b). Considering that the active compounds of this fungicide are almost always found in the top 10 cm of soil (McDonald et al., 2013; Chabauty et al., 2016) the RD for Swing[®] Gold was calculated as 1.16 10⁻³ mL kg⁻¹ of dry soil, with a soil density of 1.29. Considering that copper is mainly found in the top 5 cm of soil, the Cuprafor micro[®] RD was calculated as 15.5 mg kg⁻¹ of dry soil. Different concentrations of the two fungicides were tested separately. The natural dry soil was spiked with aqueous solutions of the fungicides, and the soil water holding capacity was adjusted concomitantly.

Mature individuals of *Aporrectodea caliginosa* s.s (Savigny, 1826) were collected by hand-sorting from an agricultural field in Estrées-Mons (49°52' N 3°01' E). They were maintained in the soil used for the experiments at 15 ± 1°C, 24h darkness for at least 7 days

before the experiments. Mature individuals of *Enchytraeus albidus* (Henle, 1837) were from a laboratory culture (INRA Versailles-Grignon, France) where they were kept at $15 \pm 1^\circ\text{C}$, 24h darkness in the soil used for the experiments. They were fed once a week with rolled oats.

2. Ecotoxicological tests

d. Acute toxicity tests

The active compound of the Cuprafor micro[®] formulation is copper oxychloride, whose LC50 for *E.fetida* was found to be $> 489.6 \text{ mg kg}^{-1}$ for 14 days of exposure (PPDB, 2016). This data was used to define a range of concentrations as follows: 5, 50, 500, and 5,000 mg kg^{-1} of copper corresponding to 0.65, 6.5, 65 and 650 times the RD. In the Swing[®] Gold commercial formulation, DMX is the main active compound (see section 2.1). It also has more potential harmful effects on the studied animals than EPX (For *E. fetida*: the DMX LC50 is 23.65 mg kg^{-1} and the EPX LC50 is $>500 \text{ mg kg}^{-1}$). Moreover, the DMX LC50 for *E. fetida* in a formulation such as Swing[®] Gold (i.e dimoxystrobin 133 g L^{-1} , epoxiconazole 50 g L^{-1}) was found to be 3.35 mg kg^{-1} (Agritox 2016). This data was used to define a range of concentrations based on the DMX concentration in Swing[®] Gold, as follows: 0.01, 0.1, 1, 10 and 50 mg kg^{-1} of DMX, corresponding to 0.065, 0.65, 6.5, 65, and 324 times the RD of Swing[®] Gold. These concentrations are detailed in Table 2, with simplified abbreviations for the different concentrations used in the acute toxicity test, i.e. C1, C2, C3 and C4 for the copper fungicide, and SGA1, SGA2, SGA3, SGA4 and SGA5 for Swing[®] Gold.

Acute toxicity tests on earthworms were adapted from the ISO 11268-1 (2012). Three replicates were used for each concentration of the two fungicides. For each replicate, 500 g of dry soil and 10 g of horse dung were mixed and placed in a 1 L plastic vessel with a removable perforated cover for gas exchanges. Horse dung used as a food source was frozen and defrosted twice and then milled ($<1 \text{ mm}$) before use (Lowe and Butt 2005). Previous tests had revealed the need to supply food to *A. caliginosa* individuals at that density to avoid weight loss in the control treatment after 14 days (personal observation). The soil moisture was adjusted to 65% of the soil water holding capacity using tap water. Five

individuals were introduced into each plastic vessel that were stored in a climate room ($15 \pm 1^\circ\text{C}$, 24h darkness) for 14 days. To evaluate earthworm biomass loss during the experiment, individuals were first weighed before being introduced into the vessels, and then after 14 days, at the end of the experiment. Before being weighed, earthworms were individually placed in petri dishes on damp filter paper for 48 hours in the dark at $15 \pm 1^\circ\text{C}$ to void gut contents (Hartenstein et al., 1981). After 14 days, dead earthworms were counted, and the LC50 was calculated (see section 2.3). The health and activity status (active, dead, in quiescence) of each earthworm was visually evaluated at the end of the experiment. Quiescence is a state of reduced metabolic activity during which earthworms remain coiled up in a chamber (Satchell 1967) (see the picture on Fig. 2 and Fig. 3).

Acute toxicity tests on enchytraeids were adapted from the ISO 16387 (2014). Based on the results obtained with Swing[®] Gold (see section 3.1.2), a second test was performed with lower concentrations (10, 25, and 50 times the RD) to precisely estimate the LC50. Four replicates were used for each treatment of the two fungicides. For each replicate, 30 g of dry soil, and 40 mg of rolled oats as a food source, were mixed and placed in a 75 mL plastic vessel with a removable perforated cover for gas exchanges. Ten individuals were introduced into each plastic vessel. The soil moisture was also adjusted to 65% of the soil water holding capacity using tap water. The vessels were stored in a climate room ($15 \pm 1^\circ\text{C}$, 24h darkness) for 14 days. After 14 days, dead individuals were counted using a binocular microscope and the LC50 was calculated (see section 2.3). To evaluate enchytraeid biomass before pesticide application and after 14 days of exposure, each individual was photographed under a binocular microscope. The volume was then estimated by measuring the length and diameter of each individual using ImageJ software (Schneider et al., 2012). Diameters were measured at two different positions (anterior and posterior sections) and averaged for each individual. Enchytraeids were considered as cylinders to estimate their volumes, and fresh biomass values were indirectly estimated from their volume multiplied by a factor of 1.051 (Phillipson et al., 1979).

Table 2. Concentrations of fungicides tested as commercial forms and active compounds, for a) acute and b) behavioural tests. RD: Recommended Dose.

a)

Swing® Gold					Cuprafor micro®			
Abbreviation	Times RD	Commercial form (mL kg ⁻¹)	Dimoxystrobin (mg kg ⁻¹)	Epoxiconazole (mg kg ⁻¹)	Abbreviation	Times RD	Commercial form (mg kg ⁻¹)	Copper (mg kg ⁻¹)
SGA1	0.065	7.52 10 ⁻⁵	1 10 ⁻²	3.76 10 ⁻³	C1	0.65	10	5
SGA2	0.65	7.52 10 ⁻⁴	1 10 ⁻¹	3.76 10 ⁻²	C2	6.5	100	50
SGA3	6.5	7.52 10 ⁻³	1	3.76 10 ⁻¹	C3	65	1000	500
SGA4	65	7.52 10 ⁻²	10	3.76	C4	650	10000	5000
SGA5	324	3.76 10 ⁻¹	50	18.80	--	--	--	--

b)

Swing Gold®					Cuprafor micro®			
Abbreviation	Times RD	Commercial form (mL kg ⁻¹)	Dimoxystrobin (mg kg ⁻¹)	Epoxiconazole (mg kg ⁻¹)	Abbreviation	Times RD	Commercial form (mg kg ⁻¹)	Copper (mg kg ⁻¹)
SGB1	1	1.16 10 ⁻³	1.55 10 ⁻¹	5.81 10 ⁻²	C1	0.65	10	5
SGB2	2	2.32 10 ⁻³	3.10 10 ⁻¹	1.16 10 ⁻¹	C2	6.5	100	50
SGB3	5	5.81 10 ⁻³	7.75 10 ⁻¹	2.91 10 ⁻¹	C3	65	1000	500
SGB4	10	1.16 10 ⁻²	1.55	5.81 10 ⁻¹	C4	650	10000	5000

e. Avoidance behaviour tests

The range of concentrations of each fungicide was determined according to the results of the acute toxicity tests so as to apply sublethal concentrations. The Cuprafor micro® concentrations were the same as those used for the acute toxicity test because no mortality was observed in this test; consequently, abbreviations remained the same (Table 2: C1, C2, C3 and C4). For the Swing® Gold, concentrations were adjusted at 1, 2, 5, and 10 times the RD; abbreviations were SGB1, SGB2, SGB3 and SGB4 (Table 2).

The avoidance behaviour test on earthworms was adapted from the ISO 17512 (2009), using the test procedure with two-section vessels. Five replicates were performed for each concentration of each fungicide and 700 g of dry soil were placed in each plastic vessels (the same vessels as for acute toxicity tests, see section 2.2.1). A removable piece of plastic-coated cardboard was placed in each vessel to divide it into two equal sections. Then, 350 g of the control soil were placed in one-half of the vessel and 350 g of the spiked (i.e. contaminated) soil were inserted into the other half. The plastic divider was removed, and then ten earthworms were placed in the centre of the vessel on the two neighbouring soil types. Soil moisture was adjusted to 65% of the soil water holding capacity. The vessels were stored in a climate room (15 ± 1°C, 24h darkness) for 48 hours. At the end of the test, the

divider was placed again to separate the control soil from the spiked soil, and the number of individuals on each side was counted.

The test procedure for *E. albidus* was the same as for earthworms. The only difference was the amount of soil in the vessels. We used the same vessels as those used for the enchytraeid acute toxicity tests (see section 2.2.1). In each replicate, we inserted 50 g of dry soil, 25 g of the control soil on one side and 25 g of the spiked soil on the other side.

3. Statistical analysis

The Regtox model was used to calculate (i) the concentration that was lethal for 50 % of the individuals (LC50), and (ii) the effective concentration that induce 50 % of avoidance (EC50 avoidance) (Vindimian, 2016).

All the other data were analysed with the statistical software R Core Team (2015). For all the statistical tests, differences were considered significant if $p < 0.05$. When normality and homoscedasticity conditions were satisfied, ANOVA and Fisher's least significant difference (LSD) post hoc tests were performed to analyse biomass losses and the ratios of active, quiescent, or dead earthworms between the different tested concentrations of both substances. When normality and homoscedasticity conditions were not satisfied, non-parametric Wilcoxon-Mann-Whitney tests were used.

In the avoidance test, correlations were tested using Spearman's method to determine potential dose-response relationships. Moreover, Kruskal-Wallis tests and post hoc multiple comparison tests were performed to analyse the avoidance response between concentrations tested. The results were graphed in terms of average net-response (NR) expressed as percentage and calculated as follows (Amorim et al., 2005; ISO 17512, 2009): $NR = \{(C-T)/N\} * 100$, where C is the number of earthworms or enchytraeids observed in the control soil; T is the number of earthworms or enchytraeids observed in the treated soil; N is the total number of earthworms or enchytraeids per replicate.

According to the ISO 17512 (2009), the habitat function of the soil was considered as limiting if on average more than 80% of the individuals were found in the control soil. When

the avoidance rate was below 25%, the results were considered as a non-response to the polluted soil (Kobeticova et al., 2009).

III. Results

1. Acute toxicity tests

a. *Cuprafor micro*[®]

No mortality was observed for enchytraeids and earthworms in the control treatment or following exposure to any of the different concentrations of *Cuprafor micro*[®]. Therefore it was not possible to determine an LC50 value for *Cuprafor micro*[®] after 14 days of exposure. For earthworms, significant biomass losses of 13.3%, 15.2% and 34.1% were calculated at 50, 500, and 5,000 mg kg⁻¹ of copper (6.5, 65, and 650 times the RD, respectively) compared to the control (Fig. 1). For enchytraeids, a significant biomass loss of 59.3% was recorded at 500 mg kg⁻¹ of copper (650 times the RD) but no significant difference was observed at lower concentrations (Fig. 1).

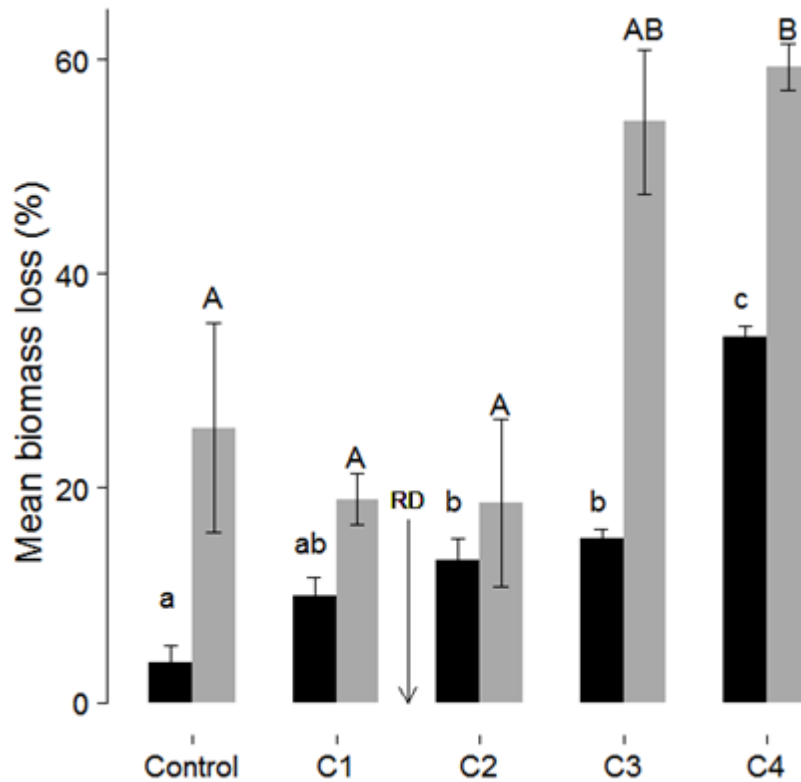


Fig.1. Mean percentage of biomass loss for *A. caliginosa* (black bars, n = 3, \pm SE) and *E. albidus* (grey bars, n = 4, \pm SE) exposed to a control soil (without pesticides) and the same soil spiked with different Cuprafor micro[®] concentrations (0.65, 6.5, 65 and 650 times the RD or 5, 50, 500, and 5,000 mg kg⁻¹ of copper for C1, C2, C3 and C4 respectively). Different letters indicate significant differences between the tested concentrations. One statistical analysis per species (ANOVA: p < 0.05). RD: Recommended Dose.

At the end of the experiment, the number of earthworms in quiescence was significantly higher at 500 and 5,000 mg kg⁻¹ of copper (65 and 650 times the RD, the two highest concentrations) than in the other three treatments (Fig. 2). At these concentrations, 86 % and 66% of the earthworms were found to be in quiescence after 14 days of exposure, respectively. After gut voiding, 20% of the earthworms from the C4 treatment (5,000 mg kg⁻¹ of copper or 650 times the RD) displayed autotomy i.e., separation of a body part (data not shown).

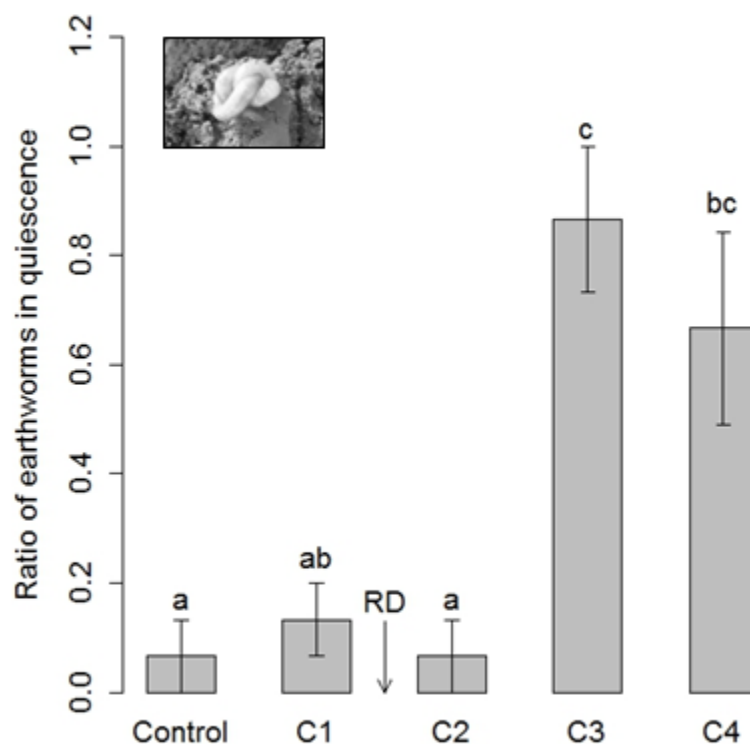


Fig.2. Ratio of quiescent *A. caliginosa* individuals at the end of the experiment (14 days) exposed to soils spiked with different Cuprafor micro[®] concentrations (0.65, 6.5, 65 and 650 times the RD or 5, 50, 500, and 5,000 mg kg⁻¹ of copper for C1, C2, C3 and C4 respectively) (n = 3, ± SE). Different letters indicate significant differences between concentrations according to Kruskal-Wallis tests (p < 0.05). RD: Recommended Dose. Photo: *A. caliginosa* in quiescence.

b. Swing[®] Gold

No mortality was observed for earthworms in the control, SGA1, and SGA2 treatments. A mortality rate of 53% of the individuals was recorded in the SGA3 treatment, corresponding to 6.5 times the RD. 100% of the individuals died in the SGA4 and SGA5 treatments, corresponding to 65 and 324 times the RD. The LC50 (Table 3) for *A. caliginosa* exposed to Swing[®] Gold for 14 days was estimated to be $7.0 \cdot 10^{-3}$ mL kg⁻¹ dry soil (confidence interval = $[5.8 \cdot 10^{-3} - 9.6 \cdot 10^{-3}]$) of the commercial formulation or 6.3 times the RD (confidence interval = $[4.9 - 8.5]$), corresponding to 0.97 mg kg⁻¹ dry soil of DMX (confidence interval = $[0.80 - 1.29]$) and 0.37 mg kg⁻¹ dry soil of EPX (confidence interval = $[0.29 - 0.47]$). For the surviving earthworms, no significant difference in biomass loss was observed between the

different treatments (7.8%, 11.3%, 9.5%, and 10% of biomass loss for the control, 0.065, 0.65, and 6.5 times the RD respectively). At the end of the experiment, no significant difference in the ratio of quiescence of the surviving earthworms was observed between the treatments (Fig. 3).

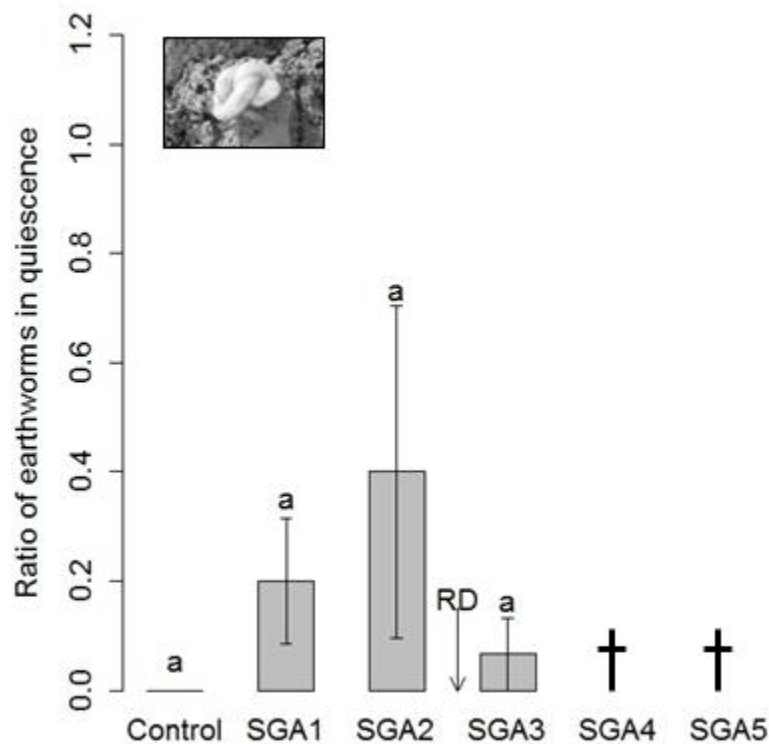


Fig.3. Ratio of quiescent *A. caliginosa* individuals at the end of the experiment (14 days) exposed to soils spiked with different Swing® Gold concentrations (0.065, 0.65, 6.5, 65 and 324 times the RD or 1×10^{-2} , 1×10^{-1} , 1, 10, and 50 mg k^{-1} of DMX or 3.76×10^{-3} , 3.76×10^{-2} , 3.76×10^{-1} , 3.76, and 18.8 of EPX for SGA1, SGA2, SGA3, SGA4 and SGA5 respectively) ($n = 3$, \pm SE). Different letters indicate significant differences between concentrations according to Kruskal-Wallis tests ($p < 0.05$). Black crosses indicate 100% mortality. RD: Recommended Dose.

Photo: *A. caliginosa* in quiescence.

No mortality was observed for enchytraeids in the control, SGA1, SGA2 and SGA3 treatments. However, 100% mortality occurred in the SGA4 and SGA5 treatments, corresponding to 65 and 324 times the RD. Due to this marked threshold effect, it was not possible to accurately estimate an LC50 value from this first experiment. In the second experiment, 30% of mortality occurred at 10 times the RD and 100% mortality occurred at 25 and 50 times the RD. According to these results, the LC50 (Table 3) for *E. albidus* exposed to

Swing Gold® for 14 days was estimated to be $12.7 \times 10^{-3} \text{ mL kg}^{-1}$ (confidence interval = $[12.0 \times 10^{-3} - 14.2 \times 10^{-3}]$) of the commercial formulation or 11.0 times the RD (confidence interval = $[10.3 - 12.2]$), corresponding to 1.69 mg kg^{-1} of DMX (confidence interval = $[1.598 - 1.893]$) and 0.63 mg kg^{-1} of EPX (confidence interval = $[0.287 - 0.471]$). For the surviving enchytraeids (in the control and in 0.065, 0.65 and 6.5 times the RD), no significant differences in biomass loss were observed between the different treatments (2.5%, 9.5% and 10% of biomass gain for the control, 0.065 and 0.65 times the RD, respectively, and 12% of biomass loss for 6.5 times the RD).

2. Avoidance behaviour tests

A significant positive correlation was found between the Cuprafor micro® concentration and earthworm avoidance responses (Fig. 4 A). Based on the 80% effect criterion (ISO 17512, 2009), avoidance was significant in the C3 and C4 treatments (500 and 5,000 mg kg^{-1} of copper, or 65 and 650 times the RD, respectively). The EC50 avoidance value for *A. caliginosa* (Table 3) was estimated to be 51.2 mg kg^{-1} of copper (confidence interval = $[46.0 - 77.5]$) corresponding to 6.7 times the RD (confidence interval = $[6.0 - 10.1]$) and 102 mg kg^{-1} of Cuprafor micro® (confidence interval = $[92 - 155]$).

Table 3. LC50 and EC50 avoidance of a) *A. caliginosa* and b) *E. albidus* exposed to Swing® Gold and Cuprafor micro® fungicides. RD: Recommended Dose.

a)

	Swing® Gold				Cuprafor micro®		
	Times RD	Commercial form (mL kg^{-1})	Dimoxystrobin (mg kg^{-1})	Epoxiconazole (mg kg^{-1})	Times RD	Commercial form (mg kg^{-1})	Copper (mg kg^{-1})
LC50	6.3	7.0×10^{-3}	0.97	0.37			
95% confidence interval	4.9 - 8.5	$5.8 \times 10^{-3} - 9.6 \times 10^{-3}$	0.80 - 1.29	0.29 - 0.47		No mortality	
AC50 Avoidance		No avoidance			6.7	102	51.2
95% confidence interval					6.0 - 10.1	92-155	46.0 - 77.5

b)

	Swing® Gold				Cuprafor micro®		
	Times RD	Commercial form (mL kg ⁻¹)	Dimoxystrobin (mg kg ⁻¹)	Epoxiconazole (mg kg ⁻¹)	Times RD	Commercial form (mg kg ⁻¹)	Copper (mg kg ⁻¹)
LC50	11.0	12.7 x 10 ⁻³	1.69	0.63			
95% confidence interval	10.3 – 12.2	12.0 10 ⁻³ - 14.2 10 ⁻³	1.60 - 1.89	0.29 - 0.47	No mortality		
EC50 Avoidance		No avoidance			51	786	393
95% confidence interval					27 - 72	412 - 1114	206 - 557

A significant positive correlation was found between the Cuprafor micro® concentration and enchytraeid avoidance responses (Fig. 4 B). Based on the 80% effect criterion (ISO 17512, 2009), avoidance was significant in the C4 treatments (5,000 mg kg⁻¹ of copper or 650 times the RD). The EC50 avoidance value for *E. albidus* (Table 3) was estimated to be 393 mg kg⁻¹ of copper (confidence interval = [206 – 557]) corresponding to 51 times the RD (confidence interval = [27 – 72]) and 786 mg kg⁻¹ of Cuprafor micro® (confidence interval = [412 - 1114]).

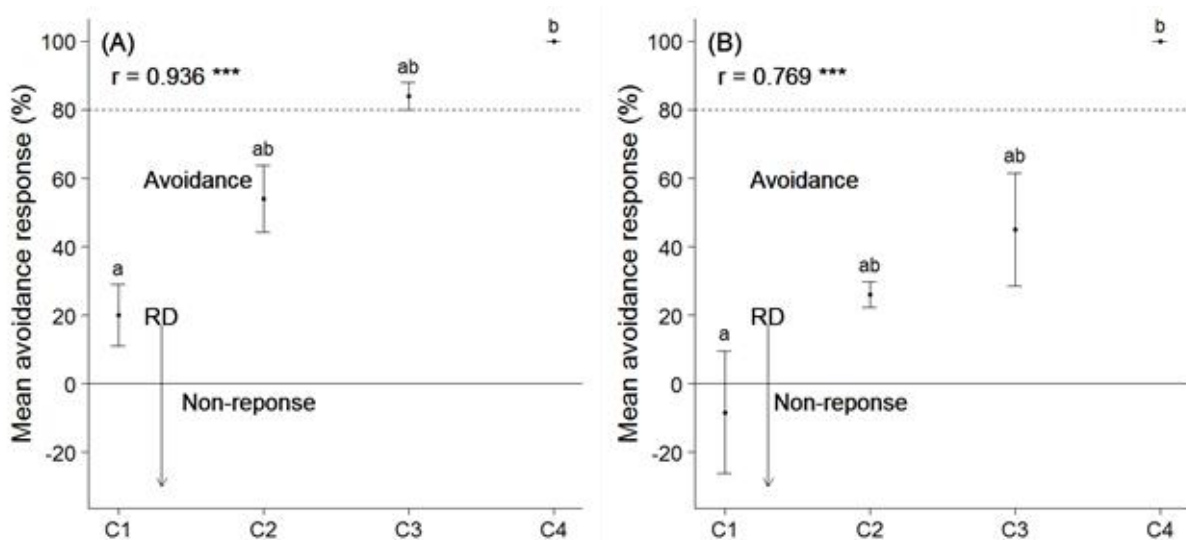


Fig.4. Mean avoidance net-response (n = 5, ± SE) of (A) *A. caliginosa* and (B) *E. albidus* exposed to soils spiked with different Cuprafor micro® concentrations (0.65, 6.5, 65 and 650 times the RD or 5, 50, 500, and 5,000 mg kg⁻¹ of copper for C1, C2, C3 and C4 respectively). The dashed line indicates the trigger value of 80% of the individuals. Different letters indicate significant differences in avoidance net-response between the concentrations (Kruskal-Wallis tests, $p < 0.05$). r indicates the rho correlation coefficient (Spearman correlation tests, * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$). RD: Recommended Dose.

For the Swing® Gold formulation, the threshold value of 80% was not reached at any of the tested concentrations (1, 2, 5, and 10 times the RD) and no significant dose-response

relationship was observed for earthworms or enchytraeids (Fig. 5). Therefore, it was not possible to determine an EC50 avoidance value for Swing® Gold.

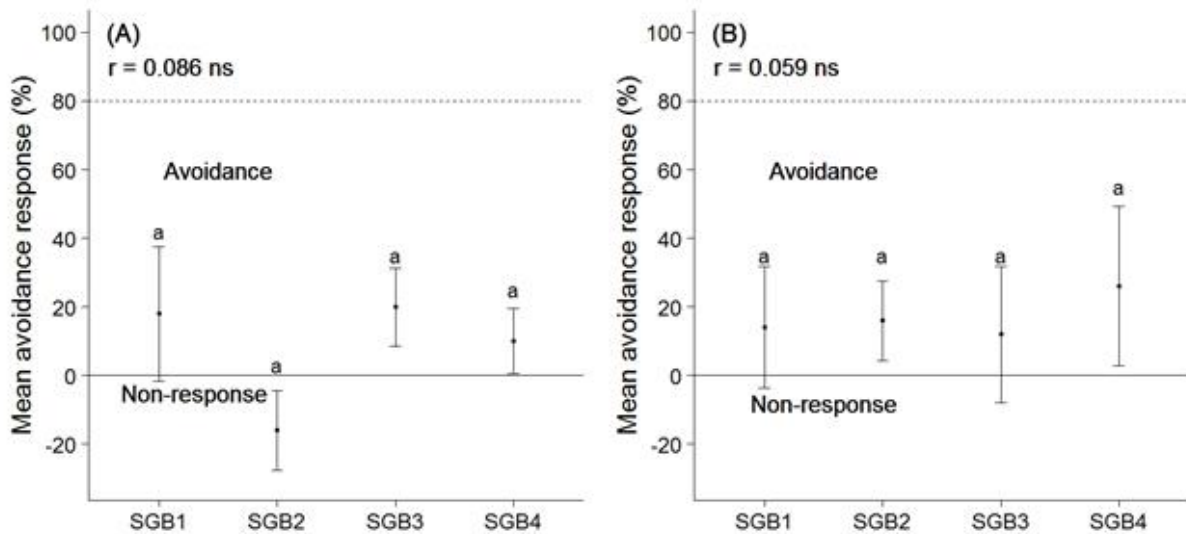


Fig.5. Mean avoidance net-response ($n = 5$, \pm SE) of (A) *A. caliginosa* and (B) *E. albidus* exposed to soils spiked with different Swing® Gold (1, 2, 5 and 10 times the RD or 1.55×10^{-1} , 3.10×10^{-1} , 7.75×10^{-1} , and 1.55 mg k^{-1} of DMX or 5.81×10^{-2} , 1.16×10^{-1} , 2.91×10^{-1} , and 5.81×10^{-1} of EPX for SGB1, SGB2, SGB3 and SGB4 respectively).

The dashed line indicates the trigger value of 80% of the individuals. Different letters indicate significant differences in avoidance net-response between the concentrations (Kruskal-Wallis tests, $p < 0.05$). r indicates the rho correlation coefficient (Spearman correlation tests, ns = no significant). RD: Recommended Dose.

IV. Discussion

1. The copper fungicide

We found no lethal effect of the copper fungicide on both species of terrestrial *Oligochaeta*. The high soil organic matter content (i.e. meadow soil, see Table 1) and the addition of horse manure may have decreased copper bioavailability (Ma and Rao, 1997) for earthworms and thus alleviate its ecotoxicological effects. However, the high rate of quiescence of earthworms, probably allowing them to avoid lethal direct effects by soil ingestion, and the biomass loss at the end of the experiment evidenced the adverse effects of the copper fungicide. Longer exposure would have probably led to lethal effects. Indeed, Khalil et al. (1996a) found LC50 values for *A. caliginosa* of 1 425 and 640 mg kg^{-1} copper sulfate after 14 and 56 days of exposure respectively in a natural soil. Copper could thus have harmful long-term effects that could lead to death, depending on the concentrations and the length of exposure. In accordance with our results, Paoletti et al. (1998) showed that

A. caliginosa biomass and abundance were negatively correlated to copper concentration in a vineyard soil. Furthermore, Eijsackers et al. (2005) showed that “vineyard soil conditions resulted in lower growth rates than in grassland soils”. This lower growth rate could delay earthworm maturation and thereby negatively affect reproduction rates. These authors concluded that copper oxychloride may hinder the population development and dispersal. Similarly, several authors highlighted the negative effects of copper fungicides on earthworm growth (Khalil et al., 1996b; Reinecke et al., 2002) and cocoon production (Helling et al., 2000) from 100 mg kg⁻¹ of copper. These concentrations, as those tested in our study, could be reached in agricultural systems because copper accumulates in soils (Brun et al., 1998).

For enchytraeids, as for earthworms, we found sublethal effects (i.e. biomass loss at the highest concentration) that could have led to lethal effects over a longer period of exposure. Copper was also found to induce other sublethal effects. For instance, Konečný et al. (2014) found that soils from a copper smelter (containing concentrations ranging between 37 and 8,980 mg kg⁻¹ of copper) had negative effects on *Enchytraeus crypticus* reproduction with an estimated EC50 of 351 mg kg⁻¹ of copper in the soil. In our study, the biomass loss of enchytraeids in the control treatment may have been due to an insufficient food supply for 14 days of experiment, although the supply was based on the recommendation given in the ISO 16387 (2014).

Moreover, our results highlighted a great ability of the tested animals to avoid the soil contaminated with the copper fungicide (with EC50 avoidance values of 51.2 mg kg⁻¹ and 393 mg kg⁻¹ of copper for earthworms and enchytraeids, respectively). For earthworm, our results are in accordance with other studies showing avoidance behaviour of *A. caliginosa* and other earthworm species when exposed to copper and other trace elements in soils (Eijsackers et al., 2005; Lukkari and Haimi, 2005). In our study, when earthworms could not escape from the contaminated zones, earthworms displayed another resistance mechanism by entering into quiescence. Quiescence is a period during which development is suspended, as a result of adverse environmental conditions (Diaz Cosin et al., 2006), in our case by contamination by a copper fungicide. For the two highest copper concentrations (500 and 5000 mg kg⁻¹ of copper), we observed 86% and 66% of earthworms in quiescence, respectively. This can probably explain the biomass loss. The autotomy observed among

earthworms at the highest copper concentration could be a detoxification process aimed at eliminating the copper from their body. Maleri et al. (2008) showed that *A. caliginosa* could concentrate trace elements (i.e. Cr, Mn and Ni) in their posterior region. The authors explained that they probably did so to get rid of pollutants by autotomy. These two processes, i.e. quiescence and autotomy, can be considered as survival strategies of *A. caliginosa* exposed to high copper concentrations, and probably to other trace elements in soils. For enchytraeids, Amorim et al. (2008) found 80% of avoidance response in a standard sandy loam LUFA 2.2 soil (Speyer, Germany) contaminated with 320 mg kg⁻¹ CuCl₂. We found an EC50 avoidance value of 393 mg kg⁻¹ of copper, corresponding to 51 times the RD of Cuprafor micro®. This difference could be explained by the soil used in both experiments since the bioavailability of some pollutants including trace elements has been found to be higher in standardized soil than in natural soil (e.g., Smit and Van Gestel 1996). Amorim et al. (2012), in a natural soil, found an EC50 avoidance value of 475 mg kg⁻¹ of CuCl₂ salt for *E. albidus*, which is close to our result (i.e. 393 mg kg⁻¹). Thus, as already demonstrated by some authors, the choice of the soil in ecotoxicological tests is crucial (Amorim et al., 2005).

2. The Swing® Gold fungicide

The responses of the tested annelids to the synthetic fungicide Swing® Gold, used in conventional farming, were qualitatively very different from those obtained with the copper fungicide. First of all, neither species was able to avoid the fungicide, even at 10 times the RD. These results suggest that both species could undergo potential lethal and sublethal (growth, reproduction) effects in the field. Moreover, mortality rates allowed us to estimate LC50 values, i.e., 6.3 and 11.0 times the RD for earthworms and enchytraeids, respectively. No data were available about enchytraeid sensitivity to the two active compounds of the Swing® Gold formulation, i.e., DMX and EPX. However, the earthworm species *E. fetida* presented LC50 values > 500 mg kg⁻¹ dry soil for EPX (PPDB, 2016) and 23.65 mg kg⁻¹ dry soil for DMX (PPDB, 2016). We did not find any LC50 value for EPX in a formulation such as Swing® Gold (i.e., 133 g L⁻¹ of dimoxystrobin and 50 g L⁻¹ of epoxiconazole). However, for DMX in such a commercial formulation, the LC50 value of *E. fetida* was 3.35 mg kg⁻¹ dry soil (Agritox, 2016), more than three times higher than the LC50 value found in this study for *A. caliginosa* (0.97 mg kg⁻¹ dry soil for DMX). Although the conditions of the tests were not the

same, it appeared that *A. caliginosa* was more sensitive than *E. fetida* to the tested pesticide. This is in accordance with previous studies showing lower sensitivity of *E. fetida* to pesticides as compared to species found in mineral soils (Pelosi et al., 2013). Moreover, according to regulatory test data (Agritox, 2016), DMX is seven times more harmful to *E. fetida* when tested in a formulation with an LC50 value of 3.35 mg kg⁻¹ dry soil, than when it is used as a pure molecule (LC50 of 23.65 mg kg⁻¹ dry soil). This substantial difference is probably due to the mixture of the two active substances as well as the adjuvants whose composition is unknown. Pelosi et al. (2016) investigated the effects of a commercial formulation of EPX on *Aporrectodea icterica* in a natural loamy soil. The authors found an LC50 of 45.5 mg kg⁻¹ dry soil, which is more than 10 times lower than the LC50 of *E. fetida* in standard condition tests with pure EPX. They suggested that this difference could be due to the use of a commercial formulation instead of a pure molecule, and/or to the lower sensitivity of *E. fetida* as compared to *A. icterica*. Thereby, along with other authors (Da Silva et al., 2010; Lajmanovich et al. 2011), Pelosi et al. (2016) underlined the importance of (i) comparing the toxicity of commercial formulations with that of pure molecules, and (ii) working on species that can be found in arable lands in addition to *E. fetida*.

3. Difference in sensitivity between earthworms and enchytraeids, and potential consequences on the services annelids can provide in crop fields.

This study showed that the LC50 value of earthworms exposed to for Swing® Gold was almost half as much as that of enchytraeids (6.3 and 11.0 times the RD for earthworms and enchytraeids, respectively). When exposed to the copper fungicide, earthworms lost biomass and avoided the polluted soil at a lower concentration than enchytraeids. Considering these results, enchytraeids were less sensitive than earthworms to the tested fungicides. These results could depend on the studied *Oligochaeta* species. Nevertheless, our findings are in accordance with other studies that suggest a difference in sensitivity to chemicals between earthworms and enchytraeids (Bezchlebova et al., 2007). For instance, Leitão et al. (2014) assessed the impact of azoxystrobin, chlorothalonil and ethoprophos on the reproduction of an earthworm (*Eisenia fetida*), a collembolan (*Folsomia candida*) and an enchytraeid species (*Enchytraeus crypticus*): enchytraeids were the least sensitive species.

Similarly, Kobetičová et al. (2010) found that *Eisenia fetida* presented a greater ability to avoid a contaminated soil than *Enchytraeus crypticus*. Under field conditions, McColl (1984) found no effect of a nematicide on enchytraeids while earthworm numbers depressed. The difference in sensitivity between these two *Oligochaeta Annelida* families could have important consequences on the soil functioning. Actually, as enchytraeids provide key roles in soils, close to those of earthworms, so that they could perform functions related to the evolution of the soil structure and organic matter that earthworms can no longer perform under pesticide pressure (Topoliantz et al., 2000). We support the need to further investigate this difference in sensitivity to assess the ecotoxicity of pesticide applications in the field and understand the impact on soil functioning and ecosystem services.

V. Conclusion

Acute toxicity and avoidance tests provided complementary information that allowed us to compare the sensitivity of earthworms and enchytraeids to two commonly used pesticides. The acute toxicity test assessed lethal (i.e., acute) effects of pesticides and a potential effect on biomass. As underlined by Römbke (2003), the avoidance test is relevant for a first evaluation of pesticide toxicity over a short period of time i.e. 48 hours. It can provide useful information not only on differences between annelid behavioural patterns in the field but also on their ability to avoid potential lethal and sublethal effects. This could provide clues to understand why some species are not found in the pesticide-treated fields and determine the potential consequences for soil functioning (e.g. Pelosi et al., 2014).

Our study also highlighted that considering the two groups of terrestrial *Oligochaeta*, i.e., earthworms and enchytraeids, is of prime importance when assessing the effects of pesticides. While earthworms might escape from or die in pesticide-treated areas, enchytraeids could provide functions such as organic matter decomposition that earthworms can no longer provide. We also underlined once again that it is quite relevant to study commercial pesticide formulations to assess the potential negative effects of adjuvants and substance mixtures. In the same way, and to address the challenge of putting more ecology into ecotoxicology (Beketov and Liess, 2012; Chapman, 2002), it is recommended to work on species that live in mineral soils where pesticides are applied.

Moreover, not only do we have to better investigate the sublethal effects (e.g., on reproduction and growth) of pesticides to understand their impacts at the population level, but we also need to study the effects of low concentrations such as field recommended doses on the behaviour of soil organisms. This could pave the way for a better assessment of the consequences of pesticide use on the “things that matter” in protecting the environment, i.e., ecosystem services (Forbes and Callow, 2012).

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Principales conclusions de l'article :

Cet article avait pour objectif de déterminer les effets de deux fongicides (i.e le Swing® Gold et le Cuprafor micro) sur la mortalité et la capacité d'évitement d'*A. caliginosa* et une espèce d'enchytréide *E. albidus* et de comparer la sensibilité de ces deux espèces. Dans le cadre de cette thèse, l'intérêt de cet article se focalise sur l'espèce *A. caliginosa*. Les données expérimentales produites ont permis d'estimer pour le Swing® Gold une CL50 correspondant à 6.3 fois la dose agronomique recommandée (DR) et de mettre en évidence que les individus ne sont en pas en mesure d'éviter ce fongicide dans le sol, même à 10 fois la DR. Concernant le fongicide cuivré, la CL50 est supérieur à 5000 mg kg⁻¹ de cuivre. Cependant une perte de biomasse a été observée à partir de 50 mg kg⁻¹ et les individus ont été capables d'éviter le cuivre dans le sol avec une CE50 évitement estimée à 51.2 mg kg⁻¹ de cuivre. Ces résultats ont permis de déterminer une gamme de concentrations sublétales pour évaluer les effets de ces pesticides sur la reproduction, la croissance et les fonctions écologiques. Ces aspects sont abordés dans les chapitres suivants.

Chapitre 2 - Effet des pesticides sur la reproduction

Ce chapitre correspond à un article intitulé "Assessing pesticide effects on earthworm reproduction by exposing adults and cocoons", prochainement soumis dans la revue *Ecotoxicology*.

Les valeurs de CL50 et CE50 évitement estimées précédemment, nous ont permis de définir les gammes de concentrations sublétales qui ont été utilisées dans ce chapitre pour évaluer les effets des pesticides sur la reproduction. Celle-ci a été évaluée en s'intéressant à plusieurs critères d'effet. En effet, lors de l'application d'un pesticide, la reproduction des vers de terre peut être affectée de différentes manières. L'exposition peut amener à une baisse de fécondité des adultes, conduisant à une réduction de la production de cocons, ou à la production de cocons défectueux (e.g. un plus faible taux d'éclosion). Cependant, il existe aussi au champ des cocons « naïfs » dans le sol, qui ont été déposés dans des zones non contaminées, par des individus adultes non exposés. Ces cocons peuvent être affectés par l'apport d'un pesticide dans le sol. Les conséquences de l'exposition sur des cocons « naïfs » ont donc également été évaluées (Annexe 3).

Résumé: L'utilisation de pesticides dans les agroécosystèmes peut avoir des effets négatifs sur les vers de terre, qui jouent un rôle clé dans le fonctionnement du sol, comme la décomposition de la matière organique. L'objectif de cette étude était d'évaluer les effets de deux fongicides (le Cuprafor micro[®], composé d'oxychlorure de cuivre et le Swing[®] Gold, composé d'époxiconazole et de dimoxystrobine) sur la reproduction des vers de terre en réalisant différentes mesures. En premier lieu, des individus adultes ont été exposés pendant 28 jours et différentes mesures ont été réalisées (production de cocons, masse des cocons produits, succès d'éclosion et temps d'éclosion des cocons produits). D'autre part, des cocons "naïfs" produits par des vers de terre non exposés (provenant d'un élevage) ont été déposés dans des sols contaminés par les mêmes fongicides, aux mêmes concentrations. Sur ces cocons, le succès et le temps d'éclosion ont été suivis. Dans la première expérience, la production de cocons a été réduite de moitié à la concentration de cuivre la plus élevée (232,5 mg Cu kg⁻¹) par rapport au témoin. Les cocons ont mis en moyenne 5 jours de plus

pour éclore et le succès d'éclosion a diminué de 35% par rapport au contrôle. Dans les traitements au Swing® Gold, la production de cocons a été divisée par 2,6 à 3 fois la dose recommandée (RD) et le succès d'éclosion a diminué significativement de 16% à la DR. Dans la seconde expérience, seul le succès d'éclosion des cocons a été affecté par le Swing® Gold à 3 RD (30% de moins d'éclosion). Nous en avons conclu que le critère d'évaluation le plus sensible était le succès d'éclosion des cocons produits par les adultes exposés aux pesticides.

Assessing pesticide effects on earthworm reproduction by exposing adults and cocoons

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Abstract

The use of pesticides in agroecosystems may have negative effects on earthworms, which play key roles in the soil functioning such as organic matter decomposition. The aim of this study was to assess the effects of two fungicides (Cuprafor micro[®], composed of copper oxychloride and Swing[®] Gold, composed of epoxiconazole and dimoxystrobin) on earthworm reproduction using innovative methods and endpoints. First, adult individuals were exposed for 28 days and cocoon variables (production, weight, hatching success, and hatching time) were monitored. On another hand, “naïve” cocoons produced by unexposed earthworms were deposited in soils contaminated with the same pesticides. We assessed the hatching success and hatching time. In the first experiment, the cocoon production was halved at the highest copper concentration (232.5 mg Cu kg⁻¹) compared with the control. Cocoons took 5 more days to hatch and the hatching success decreased by 35% compared with the control. In the Swing[®] Gold treatments, the cocoon production was divided by 2.6 at 3 times the recommended dose (RD) and the hatching success significantly decreased by 16% at the RD. In the second experiment, only the hatching success of cocoons was impacted by Swing[®] Gold at 3 RD (30% less hatching). We conclude that the most sensitive endpoint was the hatching success of cocoons produced by adults that had been exposed to pesticides.

Keywords: ecotoxicology; risk assessment; soil invertebrates; fungicide; copper

I. Introduction

During the last decades, the increasing demand in food products led to changes in land use and management. Along with an increase in cultivated areas, the enhancement of yields was promoted by the use of chemical inputs such as pesticides (Tilman et al., 2002). In temperate soils, earthworms represent a large proportion of living biomass (Gobat et al., 2004). These primary consumers of organic matter are involved in soil structure, thus allowing to maintaining soil health (Blouin et al., 2013; Bertrand et al., 2015). While in permanent grasslands, earthworm densities generally range from 200 to 400 individuals m⁻² (Edwards and Bohlen, 1996; Beylich and Graefe, 2012; Amossé et al., 2018), they can be lower than 100 individuals m⁻² in conventional agricultural fields (Scullion et al., 2002). It has been showed that mechanical tillage such as ploughing has a direct negative impact on earthworm populations (Mele and Carter, 1999; Chan, 2001). However, the impact of pesticides on earthworm populations, under field conditions, is much harder to assess and rarely documented. Using a concentration-response based field experiment, Schnug et al. (2015) showed that the mixture of three biocides (i.e., the insecticide esfenvalerate, the fungicide picoxystrobin and the bactericide triclosan) affected earthworm abundance along with juvenile proportions. They also highlighted that pesticide concentrations equaling EC50s (reproduction) for *E. fetida* (Schnug et al., 2013; Schnug et al., 2014) provoked effects on the earthworms in the field exceeding 50%, indicating an intensification of the effects from the laboratory to the field. This suggested that some endpoints such as reproduction or growth) were not sufficiently covered in laboratory experiments. To assess and understand the impact of pesticides on earthworm populations in the field, there is a need to perform more relevant and realistic laboratory experiments investigating the effects of pesticides on earthworm life history parameters. Currently, the reproduction test proposed in the ISO guideline (ISO 11268-2) allows assessing the number of juveniles produced by adults exposed to chemicals. However, it does not allow assessing specifically the impacts of chemicals on cocoon production and viability. Actually, unfavorable soil environmental conditions (e.g., drought, freeze and chemical stresses) can directly affect adult and juvenile individuals. The population recovery and survival can arise from the hatching of cocoons, when better environmental conditions occur. It is thus crucial to assess whether pesticides have any effect on cocoons. Consequently, it is relevant not only to assess the impacts of

pesticides on cocoon production and hatching success by exposing adult earthworms but also to study the viability of cocoons by exposing them directly to chemicals. Finally, the tests proposed by the ISO guidelines are performed with *Eisenia fetida*, which is not a species representative of agricultural fields and was shown to be less sensitive than other soil dwelling species to pesticides (Pelosi et al., 2013). The relevance of using other species than *E. fetida* in pesticide risk assessment is also increasingly argued in scientific literature (Lowe and Butt, 2007; Spurgeon et al., 2003). The species *Aporrectodea caliginosa* is one of the species which is increasingly advocated in ecotoxicity tests. This species is highly representative of temperate agricultural fields (Perez-Losada et al. 2009; Decaëns et al. 2011) and according to Pelosi et al. (2013), this species is three times more sensitive to pesticides compared with *E. fetida*.

In this context, our study aimed to assess the impacts of two commercial formulations of fungicides (Cuprafor micro[®], composed of copper oxychloride and Swing[®] Gold, composed of epoxiconazole and dimoxystrobin) on the reproduction of *A. caliginosa*. First, we exposed adult earthworms in contaminated soils and we monitored the impacts on cocoon production and hatching success. Secondly, we exposed “naïve” cocoons, produced in a breeding culture, in soils contaminated with the same fungicides in order to monitor the hatching success and the hatching time. We made the assumption that the hatching success and time of cocoons produced by exposed adults could be more affected by pesticides than “naïve” cocoons because they are able to resist to unfavorable environmental conditions. However, if this ability is affected by exposure to pesticides, the impacts on reproduction and on the renewing of the population in the field could be more important than expected.

II. Material and methods

1. Soil, animals, and pesticides

A loamy soil (FAO soil classification) was used in all experiment. The soil was collected in a permanent meadow in Versailles (48°48' N, 2°5' E) where no chemicals had been applied for more than 20 years. The soil was collected from the top 0-20 cm, air-dried and crushed at

2 mm. Its main physico-chemical characteristics were: pH 7.5, organic matter 32.6 g kg⁻¹, C/N 12.7, 29% sand, 48% silt, 23% clay and total Cu 25.2 mg kg⁻¹ (see Bart et al., 2017 for more details).

A. caliginosa individuals came from an agricultural field in Estrées-Mons, France (49°52' N 3°01'E). They were mature with a fully developed clitellum and their weight ranged from 600 to 1000 mg. These individuals were bred in the above mentioned soil at 15°C for 14 days.

We studied two formulations, Swing[®] Gold and Cuprafor micro[®]. Swing[®] Gold (BASF Agro SAS, dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹) is used to protect cereal crops in conventional farming. The French Recommended Dose (RD) for this product is 1.5 L ha⁻¹ on wheat (ANSES, 2017a). The RD in laboratory was calculated as 1.16 10⁻³ mL kg⁻¹ of dry soil with a soil density of 1.29 and considering that the active compounds of this fungicide are mainly found in the top 10 cm of soil (McDonald et al., 2013; Chabauty et al., 2016). For all experiments, we used the following concentrations: 0.33, 1 and 3 times the RD, abbreviated as SG0.33, SG1 and SG3, respectively (Table 1). These concentrations are expected to be sub-lethal considering that the 14 days LC50 was estimated at 6.3 times the RD for *A. caliginosa* (Bart et al., 2017). Cuprafor micro[®] is used to prevent spore germination in organic farming. The French RD for this product is 10 kg ha⁻¹ on potato and in vineyards (ANSES, 2017b). The RD in laboratory was calculated as 15.5 mg kg⁻¹ of dry soil (corresponding to 7.75 mg kg⁻¹ of copper) with a soil density of 1.29 and considering that copper is mainly found in the top 5 cm of soil (Couto et al., 2015). For all experiments, we used the following concentrations: 3.33, 10 and 30 times the RD abbreviated as Cu3.33, Cu10 and Cu30, which correspond to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper, respectively (Table 1). These concentrations were expected to be sub-lethal considering that for *Eisenia fetida*, the LC50 has been estimated > 486.6 mg kg⁻¹ of copper (which correspond at 63 times the RD) (PPDB, 2017). In all experiments, the dry soil was spiked with aqueous solutions of fungicides, and the soil water holding capacity was adjusted concomitantly at 70% of the Water Holding Capacity (WHC).

Table 1. Concentrations of fungicides tested as commercial forms and active compounds

Swing® Gold				
Abbreviation	Times RD	Commercial form (mL kg ⁻¹)	Dimoxystrobin (mg kg ⁻¹)	Epoxiconazole (mg kg ⁻¹)
SG0.33	0.33	0.39 x 10 ⁻³	5.2 x 10 ⁻²	1.94 x 10 ⁻²
SG1	1	1.16 x 10 ⁻³	1.55 x 10 ⁻¹	5.81 x 10 ⁻²
SG3	3	3.48 x 10 ⁻³	4.62 x 10 ⁻¹	1.74 x 10 ⁻¹

Cuprafor micro®			
Abbreviation	Times RD	Commercial form (mg kg ⁻¹)	Copper (mg kg ⁻¹)
Cu3.33	3.33	52	25.8
Cu10	10	155	77.5
Cu30	30	465	232.5

2. Cocoon production, hatching success and time to hatch through adult exposure

Five replicates were performed for each fungicide and concentration tested. For each replicate, 500 g of soil (dry mass) and 24 g of horse dung were mixed and placed in a 1 L plastic vessel with a removable perforated cover for gas exchanges. Horse dung used as a food source was frozen and defrosted twice and then milled (< 1 mm) before use (Lowe and Butt, 2005). The food amount corresponded to 6 g ind⁻¹ month⁻¹ which is enough to maintain the weight of *A. caliginosa* species. This food amount was previously determined and close to that proposed by Lowe and Butt, 2005. Four adult individuals were weighed and introduced in each vessel and stored in a climate room (15±1 °C, 24 h darkness) for 28 days. The soil moisture was weekly checked following the mass vessels and adjusted if necessary with tap water. At the end of the exposure period, individuals were removed, counted and weighed. The difference between the individual weight at the beginning and at the end of the experiment allowed calculating the percentage of adult weight change. The wet sieving method was used to retrieve cocoons produced and results were expressed as number of cocoon per vessel. The cocoons were then weighed and incubated at 20° ± 0.5 C in petri dish in aqueous tap water solution (i.e., semi-immersed). The hatched cocoons were monitored

every two days over a 60 days period. After this period we considered that the cocoons would never have hatched. Mean hatching time and hatching success were calculated for each replicate.

3. Cocoon hatching success and time to hatch through cocoon exposure

More than 120 cocoons were produced over a period of 7 days in the breeding culture of *A. caliginosa*. The 5% bigger and smaller cocoons were discarded to get a homogenous cocoon cohort. The sinks of 24-well microtitre plates were filled with 2 g of soil (dry mass). All plates had a removable perforated cover for gas exchanges. Five replicates were set up for each fungicide and concentrations tested. We introduced 3 cocoons, randomly selected among the cocoon cohort in each sink. Cocoons were gently immersed in order to be buried in the soil. The plates were incubated at 20 ± 0.5 °C and the soil moisture was checked weekly and adjusted if necessary with tap water. The hatching cocoons were checked every 2 days and the experiment was stopped after 60 days of incubation, considering that behind this delay, the remaining cocoons would never hatch. The mean hatching time and hatching success per replicates for each modality were calculated.

4. Statistical analyses

All data were analysed with the statistical software R Core Team (2015). For all the statistical tests, differences were considered significant if $p\text{-value} < 0.05$. When the homogeneity of variance (Bartlett-test, Snedecor and Cochran, 1989) and the normality of residuals (Shapiro test) were respected, ANOVA and Fisher's least significant difference (LSD) post hoc tests were performed to analyse difference between treatments. When normality and homoscedasticity conditions were not satisfied, the non-parametric Kruskal-Wallis test was used.

III. Results

1. Cocoon production, hatching success and time to hatch through adult exposure

Cuprafor micro[®]. At the end of the experiment, a mortality rate of 5% was recorded in the Cu3.33 and in the control treatments. No mortality occurred in the Cu10 and Cu30 treatments. No significant difference in adult weight change was recorded between the control and *Cuprafor micro*[®] treatments. However, in the Cu30 treatment, adult weight change (-7.3%) was significantly lower than in the Cu10 treatment (+9.6 %, Figure 1 A). Earthworms produced on average 15.9 cocoons (mean cocoon number per vessel of 4 individuals) in the control treatment. Cocoon production was significantly two times lower in the Cu30 treatment (corresponded to 30 times the RD or 232.5 mg kg⁻¹ of copper) than in the control (Figure 1 B). We did not find any significant effect of the copper fungicide on the mean weight of the produced cocoons (Figure 1 C).

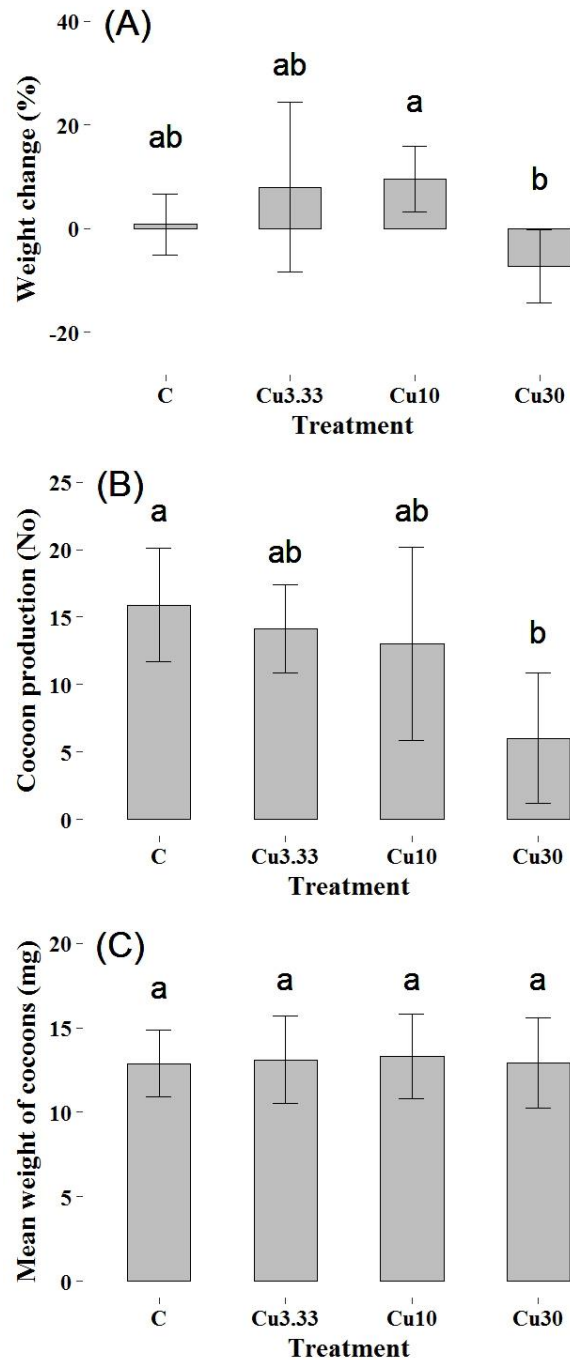


Figure 1. (A) Weight change (% of difference between the beginning and the end of the experiment, 28 days), (B) cocoon production (mean cocoon number per vessel of 4 individuals), and (C) mean weight of cocoons produced by earthworms exposed in control soil and soils spiked with different concentrations of Cuprafor micro® (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper, abbreviated Cu3.3, Cu10 and Cu30 respectively). Different letters indicate significant differences between concentrations (p<0.05).

The hatching dynamics of cocoons (including all replicates) in the Cu10 and Cu30 treatments were different than in the control treatment (Figure 2). More specifically, the mean hatching time of cocoons produced in the Cu30 treatment was significantly higher (5

more days) than in the control treatment (Table 2). Moreover, the hatching success was significantly two times lower in the Cu10 treatment than in the control (Table 2). Although the mean hatching success was 61% and 94% in the Cu30 and the control treatments respectively, the difference was not significant due to high variability in the Cu30 treatment. Indeed, only one cocoon was produced in one replicate (N=5) and did not hatch (hatching success of 0%) while 2 cocoons were produced in another replicate and hatched (hatching success of 100%).

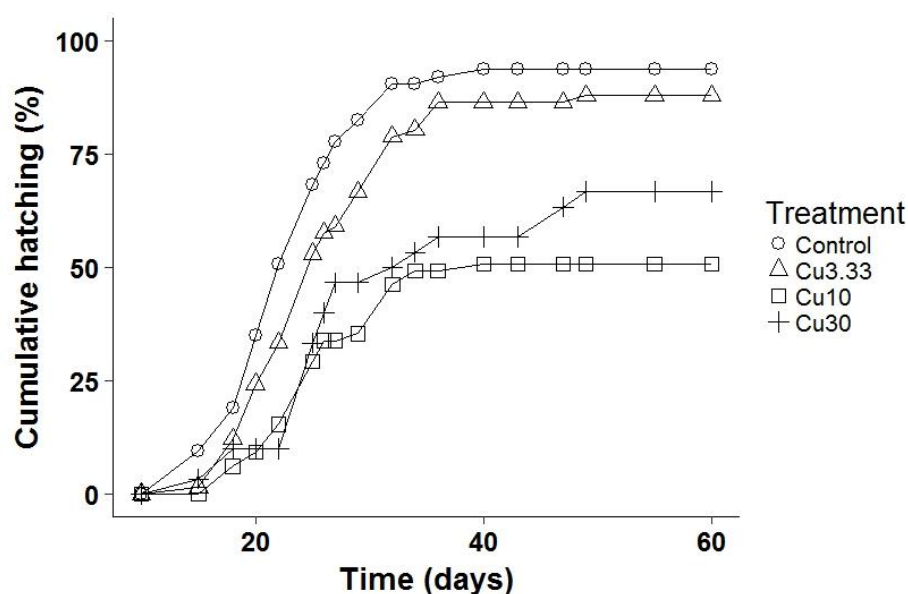


Figure 2. Hatching dynamics of cocoons produced for a 28 days period in control soil and in soils spiked with different Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper abbreviated Cu3.3, Cu10 and Cu30 respectively).

Table 2. Mean hatching time (days) and mean hatching success (%) of cocoons produced by adult exposed to Cuprafor micro[®]^a

	Treatment ^b			
	C	Cu3.33	Cu10	Cu30
Hatching time (days)	23.5 ± 1.7 A	25.6 ± 1.8 AB	26.8 ± 3.5 AB	28.5 ± 2.4 B
Hatching success (%)	94.4 ± 5.3 A	88.5 ± 13.7 A	45.9 ± 18.9 B	61.0 ± 38.5 AB

^a Cocoons were produced for a 28 days period in a control soil C and in soils spiked with different Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper abbreviated Cu3.3, Cu10 and Cu30 respectively). C = Control treatment.

^b Different letters indicate significant differences between concentrations (p<0.05).

Swing® Gold. At the end of the experiment, no mortality was recorded in the SG0.33 treatment. A mortality rate of 5% was found in the SG1 and control treatments, and 20% in the SG3 treatment. No significant difference in adult weight change was recorded between the control and *Swing® Gold* treatments. However, in the SG3 treatment, adult weight change (-13.4%) was significantly lower than in the SG0.33 treatment (+7.9%, Figure 3 A). Earthworms produced significantly three times less cocoons at 3 times the recommended dose (SG3) than in the control soil (Figure 3 B). As for the copper fungicide, the different *Swing® Gold* concentrations did not influence the weight of the produced cocoons (Figure 1 C).

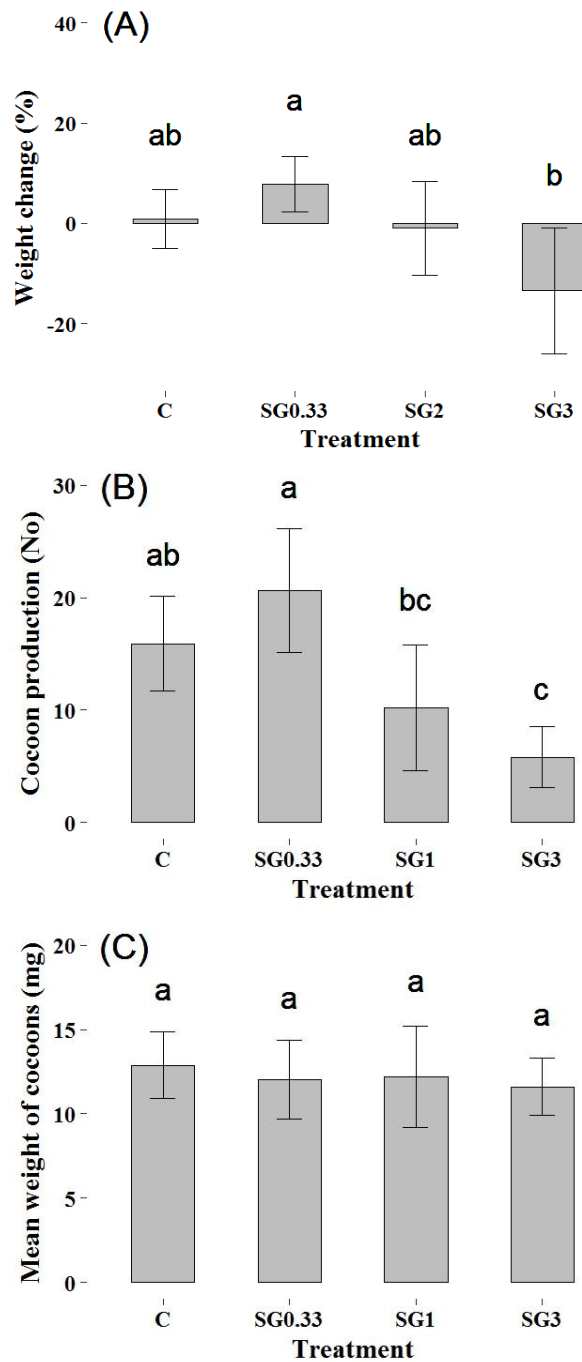


Figure 3. (A) Weight change (% of difference between the beginning and the end of the experiment, 28 days), (B) cocoon production (mean cocoon number per vessel of 4 individuals), and (C) mean weight of cocoons produced by earthworms exposed in control soil and soils spiked with different Swing® Gold concentrations (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively). Different letters indicate significant differences between concentrations ($p < 0.05$).

The hatching dynamics of cocoons (including all replicates) in the SG1 and SG3 treatments were visually different than in the control treatment (Figure 4). More specifically, the Swing® Gold fungicide negatively affected the hatching time of cocoons as they took approximately 5 more days to hatch in the SG3 treatment compared to the other treatments

(Table 3). The hatching success was 16% and 36% lower for the cocoons produced by individuals exposed in the SG1 and SG3 treatments, respectively, than in the control soil (Table 3).

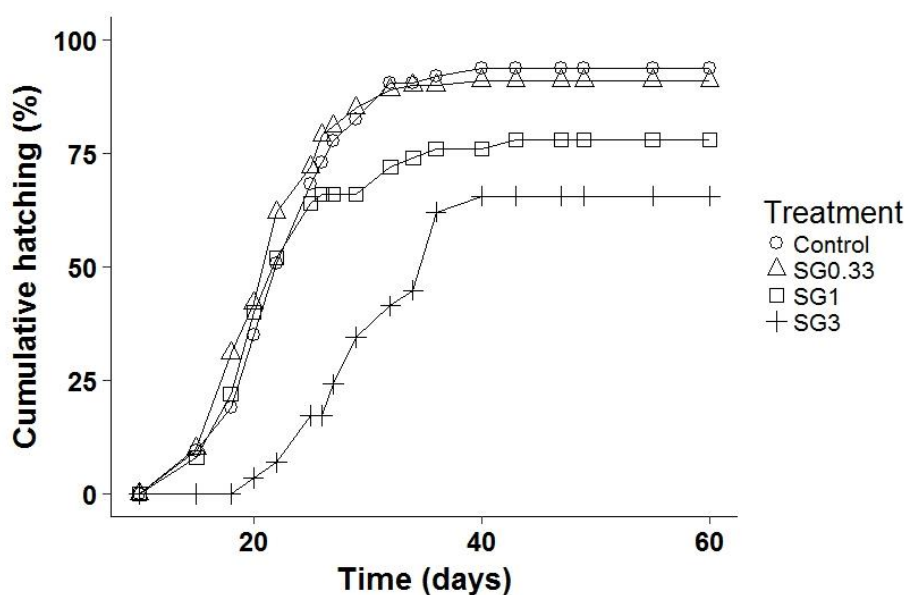


Figure 4. Hatching dynamics of cocoons produced for a 28 days period in control soil and in soils spiked with different Swing® Gold concentration (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively).

Table 3. Mean hatching time (days) and mean hatching success (%) of cocoons produced by adult exposed to Swing® Gold^a

	Treatment ^b			
	C	SG0.33	SG1	SG3
Hatching time (days)	23.5 ± 1.7 A	22.2 ± 1.4 A	22.9 ± 1.7 A	28.8 ± 3.4 B
Hatching success (%)	94.4 ± 5.3 A	91.1 ± 15.6 AB	78.9 ± 6.8 B	60.3 ± 14.4 C

^a Cocoons were produced for a 28 days period in a control soil C and in soils spiked with different Swing® Gold concentration (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively).

^b Different letters indicate significant differences between concentrations ($p < 0.05$).

2. Cocoon hatching success and time to hatch through cocoon exposure

Cuprafor micro[®]. The hatching dynamics of cocoons (including all replicates) produced in a non-contaminated and exposed to Cu3.33 and Cu10 treatment or in a control soil were

close (Figure 5). The dynamics in the Cu30 treatment was visually slower and stopped around 80% of hatching success while it was 100% in the control treatment. However, there was no significant effect on the hatching time and success, whatever the tested concentration (Table 4).

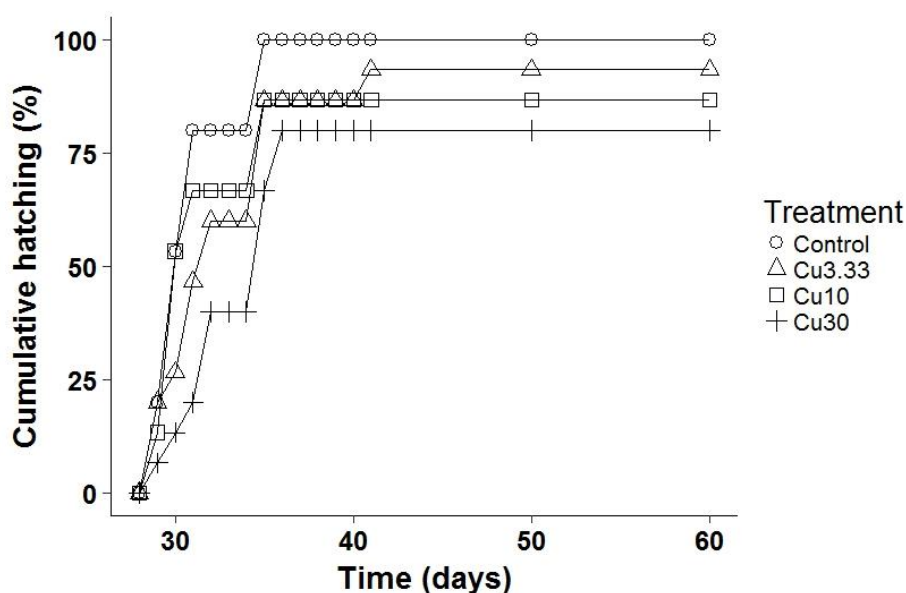


Figure 5. Hatching dynamics of cocoons produced for a 7 days period in the breeding culture and incubated at 20°C in control soil or in soils spiked with different Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper abbreviated Cu3.3, Cu10 and Cu30 respectively).

Table 4. Mean hatching time (days) and mean hatching success (%) of “naïve” cocoons exposed to Cuprafor micro^{®a}

	Treatment ^b			
	C	Cu3.33	Cu10	Cu30
Hatching time (days)	31.1 ± 1.1 A	32.4 ± 1.0 A	32.7 ± 3.6 A	33.1 ± 1.5 A
Hatching succes (%)	100 ± 0.0 A	93.3 ± 14.9 A	86.7 ± 18.3 A	80 ± 18.25 A

^a Cocoons were produced for a 7 days period in the breeding culture and incubated at 20°C in control soil C or in soils spiked with different Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper abbreviated Cu3.3, Cu10 and Cu30 respectively).

^b Different letters indicate significant differences between concentrations (p<0.05).

Swing[®] Gold. The hatching dynamics (including all replicates) of “naïve” cocoons produced in a non-contaminated soil and exposed to SG0.33 and SG1 treatment were close to the control (Figure 6). We found no significant effect of the different treatments on the

hatching time and success (Table 5). The hatching dynamics of cocoons exposed to the SG3 treatment was slower than in the control treatment (Figure 6) and the hatching success was significantly affected with only 67% of hatching success (Table 5).

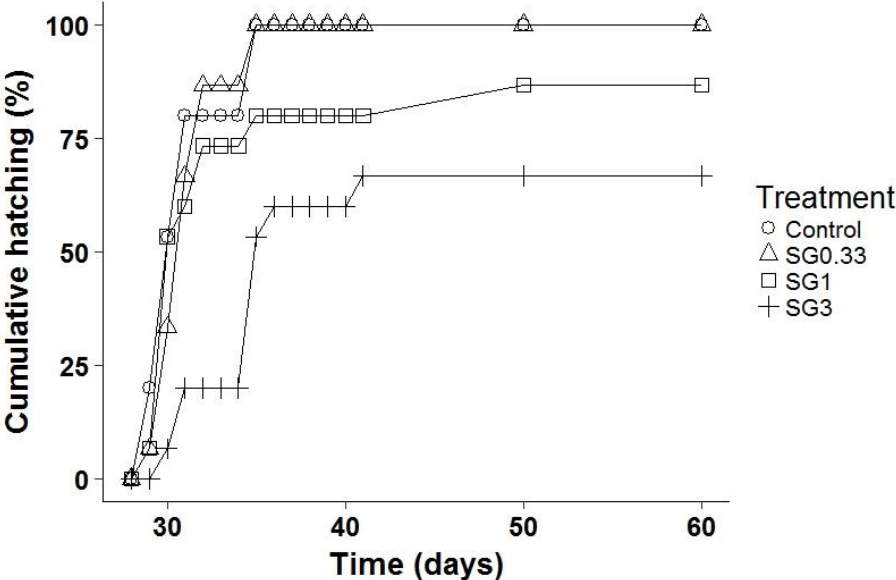


Figure. 6. Hatching dynamics of cocoons produced for a 7 days period in the breeding culture and incubated at 20°C in control soil or in soils spiked with different Swing® Gold concentrations (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively).

Table 5. Mean hatching time (days) and mean hatching success (%) of “naïve” cocoons exposed to Swing® Gold^a

	Treatment ^b			
	C	SG0.33	SG1	SG3
Hatching time (days)	31.1 ± 1.1 A	31.3 ± 1.4 A	32.6 ± 3.0 A	34.4 ± 2.6 A
Hatching success (%)	100 ± 0.0 A	100 ± 0.0 A	86.7 ± 29.8 A	66.7 ± 0.0 B

^a Cocoons were produced for a 7 days period in the breeding culture and incubated at 20°C in control soil C or in soils spiked with different Swing® Gold concentration (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively).

^b Different letters indicate significant differences between concentrations (p<0.05).

IV. Discussion

In agroecosystems, earthworms can be exposed to pesticides at different development stages, from cocoons to adults (producing cocoons). The impacts of pesticides are difficult to assess under natural conditions due to several factors operating at the same

time. Using an ecologically relevant species of earthworm, a natural soil, and commercial formulations of pesticides, this laboratory study revealed negative effects of two currently used fungicides on *A. caliginosa* reproduction at realistic concentrations. Among the tested ecotoxicological endpoints, the most sensitive was the hatching success of cocoons produced by exposed individuals of earthworms.

The tested concentrations of pesticides were sublethal for *A. caliginosa* in our experimental conditions (i.e. soil characteristic, moisture and temperature), except in the Swing® Gold treatment at three times the recommended dose where 20% of earthworms died. This result is in accordance with Bart et al., 2017 who estimated a LC50 at 6.3 times the RD for *A. caliginosa*. Furthermore, a recent field experiment showed that this fungicide applied at 10 times the recommended dose in the field, dramatically affected earthworm density and especially the *A. caliginosa* species (Amossé et al., 2018). In our experimental conditions, the weight change of adults exposed to the two tested fungicides was not significant compared to the control treatment, even if we observed, for both fungicides, a trend suggesting a weight loss with increasing concentrations. In the control treatment, the individuals did not lose weight, indicating that the amount of provided food was sufficient.

Adults of *A. caliginosa* produced one cocoon per individual per week in the control treatment. This is in accordance with other studies (Lofs-Holmin 1982; Spurgeon et al. 2000) which found between 0.6 to 2.6 cocoons per individual per week at 15 °C in natural soils for *A. caliginosa*. The cocoon production was strongly reduced in the two highest concentrations of both fungicides, although partially explained by the mortality rate in the SG3 treatment. Other studies with the same species reported EC50 values for cocoon production between 68 and 186 mg Cu kg⁻¹ (Ma 1988; Khalil et al. 1996b). Similarly, Martin (1986) estimated a NOEC of 50 mg Cu kg⁻¹ and for *Aporrectodea tuberculata*, which belong to the *A. caliginosa* complex of species (Sims and Gerard 1999). Mirmonsef et al. (2017) found an EC50 value of 220 mg kg⁻¹. In our experiment, the copper toxicity is a little lower than previously reported and could be due to the addition of horse manure that may have decreased copper bioavailability (Ma and Rao, 1997) for earthworms and thus alleviate its ecotoxicological effects. The copper toxicity highly depends on the copper form and the soil (EU 2008).

The weight of cocoons produced by adults exposed to fungicide or chemicals as an ecotoxicological endpoint is not documented in the scientific literature. However, it may

reveal key information on *A. caliginosa* reproductive strategy. Actually, the studied fungicides did not impact the weight of cocoons produced by adult exposed, even at the highest concentrations. When a cocoon is produced, the vitellus contained in the cocoon allows the development of the embryo (Bouché, 1972). When exposed to fungicides, the strategy of *A. caliginosa* would be to produce less cocoons but with the required vitellus amount to ensure the embryo development. However, cocoons produced by adults exposed displayed a longer time to hatch and a lower hatching success, even at the recommended dose of Swing® Gold. Previous studies showed that the sperm quality and quantity of earthworms, including *A. caliginosa*, can be affected by pesticides (Venter and Reinecke 1985; Mosleh et al. 2003; Espinoza-Navarro and Bustos-Obregon, 2004; Espinoza-Navarro and Bustos-Obregon 2005) and might explain our results.

In the second experiment, where “naive” cocoons were exposed to the two tested fungicides, we found a mean incubation period of 31 days in the control treatment (this number is underestimated because cocoons were produced over a 7 days period at 15°C before the beginning of the incubation) which is in accordance with Holmstrup et al., (1991) who found a cocoon incubation period in petri dishes on a moist filter paper of 36 days at 20 °C for cocoons of *A. caliginosa*. Several authors recorded a hatching success of more than 90% in a natural soil (Boström and Lofs-Holmin, 1996) or in petri dishes (Holmstrup, 2001; Jensen and Holmstrup, 1997) at 15°C. This is in accordance with our results in the control treatment where 100% of cocoons hatched. To our knowledge, the monitoring of “naive” cocoons incubated in a contaminated soil has never been documented in scientific literature. Nevertheless, even if this situation may less occur in the field than the previous one (cocoons produced by exposed earthworms), cocoons from “naive” earthworms can be exposed to pesticides after being produced. Here we found that these “naive” cocoons appeared less affected by the tested fungicides than the cocoons produced by the exposed adults. Indeed, differences in hatching time and success between the control and exposed cocoons were not significant while cocoons produced by exposed adults were negatively affected by pesticides. These results mean that the ecotoxicological effects are mainly related to the quality of cocoons produced and not to the environment in which they are deposited. Nevertheless, we observed that the hatching time tended to increase and the hatching success tended to increase with increasing fungicide concentrations. This result was

significant in the SG3 treatment with the hatching success. The reproductive success of earthworms would thus be determined by both the exposure of adults and cocoons. Under field conditions, cocoons produced by adults exposed to pesticides are generally deposited in the same contaminated soil. Thus, the impacts on reproduction could be more important than our observations.

V. Conclusion

Considering different ecotoxicological endpoints, our results showed that fungicides may negatively impact earthworm reproduction even at the recommended dose for the Swing® Gold fungicide. According to the EFSA Scientific Report 46, 1-82 (2005), the dimoxystrobin NOEC reproduction in a formulation with the same properties as Swing® Gold (i.e., 133 g L⁻¹ of dimoxystrobin and 50 g L⁻¹ of epoxiconazole) was estimated at <0.0887 mg kg⁻¹ of dimoxystrobin which is equivalent to 0.5 times the RD in our experimental conditions (i.e. 10 cm depth and 1.29 soil density). In addition, a NOEC reproduction for *E. fetida* and the epoxiconazole was estimated at 0.084 mg kg⁻¹ (PPDB, 2017). Our results with the endogeic species *A. caliginosa*, which inhabits agroecosystems and will consequently come in contact with this pesticide, strongly support the conclusion that the Swing® Gold formulation is harmful for earthworm reproduction, even at the RD.

In risk assessment procedures, the reproduction test with *Eisenia fetida* directly assesses the impact of chemicals on the number of produced juveniles. This does not allow understanding the mechanisms by which the pesticides affect earthworm reproduction. For a deeper understanding, some authors assessed the impacts on cocoon production. In this study, we went further by taking account the cocoon stage (i.e. weight, hatching success and time) after the cocoon production by exposed adults and separately by exposing “naïve” cocoons in contaminated soils allowing a better understanding of pesticide impact on earthworm reproduction.

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Principales conclusions de l'article

Cet article avait pour objectif d'évaluer les effets des deux fongicides (i.e. le Swing® Gold et le Cuprafor micro) sur la reproduction d'*A. caliginosa*. Il est apparu que les effets observés sur les cocons « naïfs » exposés aux fongicides étaient plus faible que les effets sur (i) la production de cocons par des adultes exposés et (ii) sur le succès et le taux d'éclosion de ces derniers. Nous avons ainsi mis en évidence un effet négatif sur le succès d'éclosion des cocons produits par des adultes exposés à 1 fois la dose recommandée de Swing® Gold, ce qui en fait le paramètre le plus sensible. Après la reproduction, la croissance des vers est un point important du cycle de vie. La prochaine étape est ainsi d'évaluer les effets sur celle-ci, depuis la sortie du cocon jusqu'au stade adulte.

Chapitre 3 - Effet des pesticides sur la croissance, application du modèle bioénergétique

Ce chapitre correspond à un article intitulé “ An energy-based model to analyse growth data of earthworms exposed to two fungicides.”, prochainement soumis dans la revue *Environmental Science and Technology*.

La croissance est un point sensible du cycle de vie des vers de terre. Au champ, des individus juvéniles de tout âge sont présents, de la sortie du cocon jusqu'à un stade précédant l'apparition des caractères sexuels (i.e. apparition du clitellum). Tous ces individus juvéniles peuvent être affectés dans leur croissance lors de l'application d'un pesticide, mais sans doute de manières différentes. J'ai donc pris en compte cet aspect dans ce chapitre pour évaluer les effets de concentrations sublétales de pesticides sur la croissance d'*A. caliginosa*. Ainsi, des juvéniles de 3 âges différents ont été exposés aux pesticides (Annexe 3) jusqu'à la maturation (c'est-à-dire l'apparition du clitellum). Le modèle bioénergétique, calibré dans la première partie de la thèse, et couplé à un modèle toxicocinétique (TK) tenant compte de la concentration mesurée dans le milieu d'exposition, a été utilisé pour analyser simultanément l'ensemble des données de croissance pour un pesticide donné. De plus, des mesures de pesticides ont été réalisées dans les vers à la fin de l'expérimentation afin d'évaluer l'accumulation des pesticides dans les tissus au cours de la croissance, et mettre ces résultats en relation avec l'analyse faite à l'aide du modèle.

Résumé:

L'évaluation des risques liés à l'utilisation de pesticides pour les vers de terre est actuellement réalisée à l'aide de tests standardisés, avec l'espèce modèle *Eisenia fetida*, et l'analyse des données est réalisée à l'aide d'outils statistiques de type *ad hoc*. La survie et la reproduction sont les paramètres couramment utilisés dans ces procédures et plus généralement dans les tests écotoxicologiques. La croissance est un paramètre sensible du cycle de vie du ver de terre, mais elle est encore mal renseignée. Dans cette étude, nous avons évalué les effets de deux formulations commerciales de fongicides (Cuprafor micro® composé oxychlorure de cuivre et le Swing® Gold composé d'époxiconazole et de

dimoxystrobine) sur la croissance de l'espèce *Aporrectodea caliginosa*, qui est très représentative des champs cultivés. Des individus de trois âges différents (de l'éclosion jusqu'à l'âge de 56 jours) ont été exposés à trois concentrations différentes des deux fongicides. Les données ont été analysées avec un modèle toxicodynamique basé sur la gestion de l'énergie, couplé à un modèle toxicocinétique. Le fongicide cuivré a provoqué une inhibition de la croissance lorsque la concentration sans effet (CSE), estimée à 65 mg kg^{-1} de cuivre, a été dépassée dans le modèle toxicocinétique. Le Swing® Gold a affecté négativement la croissance à 3 fois la dose recommandée. Nous avons pris en compte, dans notre analyse des données de croissance, la dégradation des composés actifs, qui ont été mesurés au cours du temps dans le sol. Les valeurs des CSE ont été estimées à $0,387 \text{ mg kg}^{-1}$ et $0,128 \text{ mg kg}^{-1}$ pour la dimoxystrobine et l'époxiconazole dans cette formulation de fongicide, respectivement. Les effets au cours du temps sur les individus ont été entièrement pris en compte par le modèle, quel que soit leur âge d'exposition. De plus, des analyses de données de toxicité, étayées par des mesures de concentrations des fongicides dans les vers de terre à la fin de l'expérience, ont permis de mieux comprendre les mécanismes d'action sur la croissance des vers de terre.

An energy-based model to analyse growth data of earthworms exposed to two fungicides.

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Abstract

The risk assessment linked to the use of pesticides for earthworms is currently performed using standardized tests, the model species *Eisenia fetida*, and the analyses of the data obtained are performed with *ad hoc* statistical tools. Survival and reproduction are commonly used endpoints in these procedures and more generally in ecotoxicological tests. Growth is a sensitive endpoint of earthworm life cycle but it is still poorly documented. We here assessed the impact of two commercial formulations of fungicides (Cuprafor micro[®] with copper oxychloride, and Swing[®] Gold with epoxiconazole and dimoxystrobin) on the entire growth pattern of the earthworm species *Aporrectodea caliginosa*, which is highly representative of agricultural fields. Individuals of three different ages (from hatching to 56 days old) were exposed to three different concentrations of both fungicides. Data were analyzed with an energy-based toxicodynamic model coupled with a toxicokinetic model. The fungicide made of copper oxychloride caused a drastic growth inhibition once the No Effect Concentration (NEC), estimated at 65 mg kg⁻¹ of copper, was exceeded. The Swing[®] Gold negatively affected the growth at 3 times the recommended dose. We accounted, in our analysis of the growth data, for the degradation of the active compounds, which were measured throughout time in the soil. The NEC values were estimated at 0.387 mg kg⁻¹ and 0.128 mg kg⁻¹ for the dimoxystrobin and the epoxiconazole in this fungicide formulation, respectively. The time-profile of the effects on *A. caliginosa* individuals was fully accounted for by the model, whatever their age of exposure. Furthermore, toxicity data analyses, supported by measurements of fungicide concentrations in earthworm at the end of the experiment, allowed better understanding of the mechanisms of action of the fungicides towards earthworm growth.

Keywords: Lumbricidae, bioaccumulation, ecotoxicology, pesticide, modelling, DEB.

I. Introduction

Pesticides used in agroecosystems can harm biodiversity and biological activity (Bengtsson et al., 2005; Hole et al., 2005). Among non-target soil organisms that can be impacted by pesticides, earthworms are commonly used as biological indicators of chemical stress (OECD, 1984) because they are key soil organisms, involved in nutrient cycling, soil water regulation and aeration (Blouin et al., 2013; Bertrand et al., 2015). During the last decades, different ecotoxicological laboratory tests have been developed such as the acute toxicity test (ISO 11268-1, 2012) or the reproduction tests (ISO 11268-2, 2012; OECD, 2004). In these ISO and OECD tests, the recommended species is *Eisenia fetida fetida* or *Eisenia fetida andrei* and they are often used to assess the impacts of pesticides or other chemicals on earthworms. Contrarily, growth tests with earthworms are poorly documented while some authors reported growth to be a very sensitive endpoint (Springett and Gray, 1992; Booth et al., 2000; Booth and O'Halloran, 2001). Spurgeon et al. (2004) highlighted the need to develop a test system for measuring key demographic traits in juvenile earthworms, especially growth. To move towards a more realistic and relevant assessment of the environmental risks of pesticide use, some issues have to be overcome.

First, the model species (i.e. *E. fetida fetida* or *andrei*) do not generally inhabit mineral soils (Lowe and Butt 2007) and are therefore rarely found in cultivated fields where pesticides are applied. To complement the use of *E. fetida* in pesticide risk assessment procedures, the earthworm species *Aporrectodea caliginosa* s.s. was recently proposed as a relevant species to be used in soil ecotoxicology tests (Klobucar et al., 2011; Bart et al., 2018). This species is one of the dominant species in agroecosystems in temperate areas (Boström and Lofs-Holmin 1996; Boag et al. 1997; Curry et al. 2008) and is found to be more sensitive to pesticides and metabolites than *E. fetida* (Pelosi et al, 2013). The second issue in currently used risk assessment procedures is data analysis. Ad hoc statistics are used to test differences between effects measured in polluted and unpolluted soils or to calculate no observed effect concentrations (NOEC), lowest observed effect concentrations (LOEC), or effective concentrations (ECx). However, these parameters cannot easily be extrapolated for other exposure durations than the one used for the test, and do not account for the kinetics of the toxicant in soil. To go towards a better understanding of the mechanisms of toxicants

on life cycle parameters, Kooijman and Bedaux (1996) proposed energy-based models to analyse toxicity data. These models are based on the dynamic energy budget (DEB) theory (Kooijman 1986, 2000, 2010) which separates the use of energy between growth, maintenance, and reproduction. Effects models, called DEBtox models, assume that the use of energy by an organism described in the DEB model can be disturbed by a toxicant. The effect is described as an impact on one of the parameters of the energy-based model and the magnitude of the effects is assumed to be related to the internal concentration of the toxicant of the organism. The exposure concentration and internal concentration of the organisms are related throughout time by a toxicokinetic model. The DEBtox models have proved their reliability in the analysis of data from growth and reproduction tests (Péry et al., 2002; Jager et al., 2004; Goussen et al., 2013). These models also allow estimating a no effect concentration (NEC) which is a threshold for toxicity that does not depend on the time of exposure. The NEC can be used to compare ecotoxicity of toxicants avoiding time dependency issues of classical parameters such as LOEC, NOEC or ECx (Baas et al., 2010; Jager, 2011; Heckmann et al., 2010).

We here tested the impact of two commercial formulations of fungicides on the growth of the earthworm *A. caliginosa*. The exposure was performed at three different ages to reveal potential differences in earthworm sensitivity over their development. Data were analysed with an energy-based model, calibrated for this species (Bart et al., 201X, Partie 1, Chapitre 2), in order to understand the mechanisms of action and the time-dependence of the two different fungicides on *A. caliginosa* growth. To support the understanding of the toxicity mechanisms provided by the model, we performed concentration measurements in earthworms at the end of the growth experiment.

II. Material and methods

1. Soil, animals, pesticides

All experiments were performed using a loamy soil (FAO soil classification) which was sampled from a permanent grassland in Versailles (48°48' N, 2°5' E) where no pesticides

have been applied for more than 20 years. The soil was collected from the top 0-20 cm, air-dried and crushed at 2 mm. Its main physico-chemical characteristics were: pH 7.5, organic matter 32.6 g kg⁻¹, C/N 12.7, 29% sand, 48% silt, 23% clay, and 25.2 ± 3.8 mg kg⁻¹ of initial copper content (see Bart et al., 2017 for more details).

The earthworm *Aporrectodea caliginosa* was used in all experiments and individuals were taken from our personal laboratory breeding. The individuals of this breeding were initially sampled from an agricultural field, 1 year before this study, in Estrées-Mons, France (49°52' N 3°01'E). The breeding soil was the same as presented before. To get cohorts of hatchlings, cocoons were collected in the breeding culture by wet sieving the soil through a 1-mm mesh size (Bart et al., 2018), and incubated at 20 °C in petri dishes on wet filter papers (Holmstrup et al., 1991). Cocoons were checked every two days and new hatchlings were collected and stored in the breeding soil at 4 °C to slow their development. This procedure allowed synchronizing cohorts of individuals of the same level of development (Bart et al., 2018).

The first studied fungicide was Swing[®] Gold (BASF Agro SAS, dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹), used to protect cereal crops in conventional farming. The French Recommended Dose (RD) for this product is 1.5 L ha⁻¹ on wheat (E-phy, 2017a). The RD in laboratory was calculated as 1.16 10⁻³ mL kg⁻¹ of dry soil (corresponding to 150 µg kg⁻¹ of dimoxystrobin and to 60 µg kg⁻¹ of epoxiconazole) with a soil density of 1.29 and considering that the active compounds of this fungicide are mainly found in the top 10 cm of soil (McDonald et al., 2013; Chabauty et al., 2016). We tested the following concentrations: 0.33, 1, and 3 times the RD, abbreviated SG0.33, SG1, and SG3, respectively. These concentrations were assumed to be sub-lethal considering the LC50 estimated at 6.3 times the RD for *A. caliginosa* (Bart et al., 2017).

The second studied fungicide was Cuprafor micro[®], used to prevent spore germination in organic farming mainly. The French RD for this product is 10 kg ha⁻¹ on potato and in vineyards (E-phy, 2017b). The RD in laboratory was calculated as 15.5 mg kg⁻¹ (corresponding to 7.75 mg kg⁻¹ of copper) of dry soil with a soil density of 1.29 and considering that copper is mainly found in the top 5 cm of soil (Couto et al., 2015). We tested the following concentrations: 3.33, 10, and 30 times the RD abbreviated Cu3.33, Cu10 and Cu30, corresponding respectively to 25.8, 77.5, and 232.5 mg kg⁻¹ of copper. These

concentrations were assumed to be sublethal (Ma 1984; Spurgeon et al., 2004; Bart et al., 2017; PPDB, 2017). In all experiments, the dry soil was spiked with aqueous solutions of the fungicides, and the soil water holding capacity was adjusted concomitantly at 70% of the Water Holding Capacity (WHC).

2. Growth experiment

In order to monitor the growth, the weight of individuals was measured as it was more precisely obtain than a length measurement as explained in Bart et al. (201X, Partie 1, Chapitre 2). The individuals were weighted using an analytical balance instrument (± 0.1 mg).

The experimental design of the growth experiment is summarized in Figure 1. The impact of fungicides was tested at three different ages as follows:

- **Age 1 (A1):** Individuals were exposed to the contaminated soil just after hatching. At this age, they weighed 12 ± 3 mg.
- **Age 2 (A2):** Individuals were exposed to the contaminated soil after 28 days of growth in a control soil. At this age, they weighed 90 ± 15 mg.
- **Age 3 (A3):** Individuals were exposed to the contaminated soil after 56 days of growth in a control soil. At this age, they weighed 300 ± 40 mg.

Earthworms were placed individually in 1 L vessels with 400 g of soil (dry mass). Seven replicates were used per age of exposure and fungicide concentration, including a control without fungicide. All the vessels were stored in a climate room at 15 ± 1 °C. Individuals were fed with horse dung in *ad libitum* conditions, as presented in Bart et al., (201X, Partie 1, Chapitre 2). Individuals were weighted at least every 14 days and the experiment was stopped when individuals had reached maturity, characterized by the apparition of a fully developed clitellum. If the growth of individuals stopped over time without maturation, we stopped the experiment, after around 35, 70 and 98 days without growth for individual exposed at Age 3, 2 and 1 respectively. At the end of the experiment, all individuals were placed in petri dishes on damp filter paper for 48 h in the dark at 15 ± 1 °C to void gut contents (Hartenstein et al., 1981). Then, they were weighted and frozen at -80 °C for fungicide analysis.

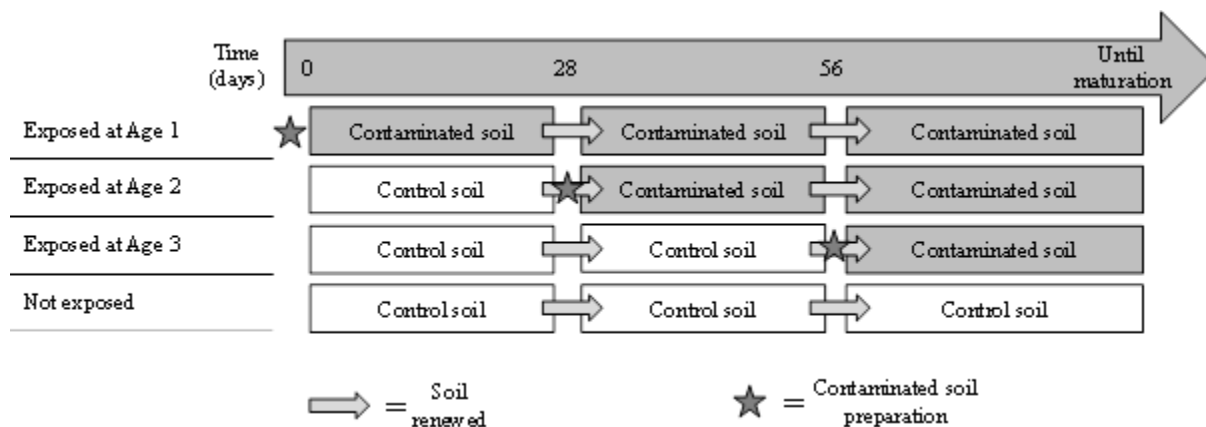


Figure1. Experimental design of the growth experiment.

For each treatment, the soil was renewed every 28 days to avoid unsuitable conditions for earthworm growth (e.g. soil compaction). All the soils for a given fungicide treatment were prepared at the same time (see Figure 1), to ensure a comparable evolution of pesticide concentrations and bioavailability. Swing® Gold fungicide concentration in the soil with earthworms and horse dung and in the new soil was monitored at each soil renewal. Moreover, in order to characterize the exposure concentration of the two active substances (dimoxystrobin and epoxiconazole), the total and bioavailable concentrations of the active substances were monitored over the time of the experiment, every 28 days.

3. Fungicides analyses

a. Soil

The copper fungicide

The soil was sampled just after the preparation of contaminated soils to verify the contamination level. The soil samples were air-dried and then, an aliquot was sieved at 200 µm for total Cu analysis. For this, 0.5 g of soil were weighed in Teflon containers and digested by HF/HNO₃ (1:3, v:v) and microwave heating (CEM MarsXpress, Matthews, NC, USA). After complete digestion, the excess of acid was evaporated and the samples were diluted in 50 mL with 1% HNO₃. All the reagents used were of analytical grade and deionized water of high purity (Water resistivity = 18 MΩ) was prepared by Milli-Q water system (Millipore). Total Cu content in solution was determined by flame atomic absorption

spectrophotometry (FAAS, Varian SpectrAA 220, quantification limit 0.04 mg Cu L⁻¹) following quality control assured by triplicate samples, running blanks, and using certified reference materials (TMDA-70.2, Environment Canada).

The Swing® Gold fungicide

The soil was sampled just after each preparation of contaminated soil to check that the contamination was correctly performed. Moreover, the two active substances, i.e., dimoxystrobin (DMX) and epoxiconazole (EPX) were measured over time (0, 28, 56 and 84 days after the contamination) in 4 of the 7 replicates. An aliquot of each sampled soil was immediately analysed to evaluate available fraction and the complement was stored at -40°C to evaluate the total content of DMX and EPX. For the bioavailable concentration determination, the extraction was performed according to Nélieu et al. (2016). Briefly, 9 mL of a 50 mM hydroxypropyl-β-cyclodextrin aqueous solution was added to 4 g of wet soil (in triplicate), agitated overnight, centrifuged and the supernatant was analysed. To evaluate the total content of DMX and EPX, freeze-dried soil was manually ground with a mortar. Triplicate soil subsamples of 5 g were extracted by 10 mL of methanol through agitation on orbital shaker (10 min, 300 rpm) and sonication for 20 min, before being centrifuged (10 min, 1300 g). The supernatant was retained and the soil re-extracted as before. Combined supernatants were analysed without further preparation for lower doses (SG0.33 and SG1) but diluted by a factor 10 for SG3. The absence of DMX and EPX in initial soil and controls was checked after a further purification-concentration by solid phase extraction. Analyses were performed on an ultra-high performance liquid chromatograph (Acquity UPLC, Waters) coupled through an electrospray interface to a triple quadrupole mass spectrometer (TQD, Waters). The analytical conditions were as described by Nélieu et al. (2016), except the UPLC gradient (95/5 to 45/55 of water/acetonitrile, each containing 0.1% acetic acid) and the introduction of conditions to detect DMX (cone voltage 23 V, MRM transitions 327>205 at 10 eV for quantitation and 327>116 at 17 eV for confirmation). The matrix effect was systematically evaluated and compensated, even if it remained within the 74-121% range.

b. Earthworms

The copper fungicide

The total copper (Cu) content in earthworms was quantified after individual lyophilisation and dry weight recording. Digestion of earthworms was done in a concentrated HNO₃ solution (normapur; VWR-Prolabo). Depending on the weight of the earthworms, this mineralisation was done either only in ependorf microtubes in the presence of 2mL HNO₃ for earthworms with dry weights under 40 mg, or in a microwave system (CEM, MarsXpress, Matthews, NC, USA) with 3mL of HNO₃ for earthworms with dry weights higher than 40 mg. In the first case, the digests were recovered in a final volume of 10 mL, and in the second case 20 mL with ultrapure water (MilliQ water, Millipore, 18 MΩ). Blanks were conducted to insure the absence of contamination during the mineralisation procedure. Total earthworms copper was performed by FAAS (Varian SpectrAA 220) following quality assurance procedures as for the soil.

The Swing[®] Gold fungicide

The dimoxystrobin (DMX) and epoxiconazole (EPX) concentration in earthworms were measured on individuals, using a method based on that described by Nélieu et al. (2016). The main differences with this publication, necessary for optimal extraction of both DMX and EPX, were the addition of a larger acetonitrile volume (4 mL instead of 2 mL) and higher (doubled) weights of salts to perform the QuEChERS purification. Briefly, the earthworms were homogenized in water and extracted in a water/acetonitrile mixture. After centrifugation, the supernatant was purified by QuEChERS and then by solid phase extraction on florisil. The final extract was analysed by UHPLC-MS/MS as described for soils.

4. The energy-based model

We used a growth model we previously built and which is fully presented in Bart et al. (201X, Partie 1, Chapitre 2). This model is based on the DEB (Dynamic Energy Budgets) theory (Kooijman 2000). Under *ad libitum* conditions and according to the assumptions of isomorphism and neglected energy costs of maintenance, the growth is expressed with the following equation:

$$\text{If } l < C_s, \text{ then } \frac{d}{dt} l = a(1 - b)$$

$$\text{If } l > C_s, \text{ then } \frac{d}{dt} l = a$$

Where l is the cubic root of the wet weight of the organisms, a is a constant and b is a correcting factor to take into account that when individuals are too small, they cannot access the whole food quantity. As explained in Bart et al. (201X, Partie 1, Chapitre 2) C_s , a , and b have to be calibrated for each new experiment to take into account the new experimental conditions, because these parameters depend on the food quality (particle size repartition) and soil characteristics. In our experimental conditions, the parameter values were optimized with the control treatment as follows $C_s = 3.99$, $a = 0.075$ and $b = 0.13$.

5. The effect model

The effect model is based on the energy-based model mentioned above. We assumed that the exposure to the toxicant increases the energy cost of growth. As in the DEBtox model (Kooijman and Bedaux, 1996), we assumed that there is a threshold for effect, a no effect concentration (NEC), and that the effects are proportional to the difference between the internal concentration and the NEC value. The toxicokinetic of the internal concentration (C_i) was deduced from the exposure concentration with a one compartment model:

$$\frac{dC_i}{dt} = K_u \times c_e(t) - K_e \times C_i(t)$$

where K_u and K_e are the uptake and the elimination rate, c_e and C_i are the external and internal concentrations of the toxicant, respectively. However, because we do not have access to the internal concentration, it was scaled by the bio-concentration factor (here K_u/K_e) as explained in Péry et al. (2001), leading to the following equation:

$$\frac{dci(t)}{dt} = K_e (c_e(t) - ci(t))$$

Where ci is proportional to the concentration in the tissue, but has the dimension of an external concentration. Moreover, the individuals had a significant growth during the

experiment which was taken into account with the dilution by growth in the toxicokinetic model (Kooijman and Bedaux, 2010), leading to the following equation:

$$\frac{dci(t)}{dt} = \frac{Ke (ce(t) - ci(t))}{l} - \frac{3a ci}{l}$$

In the case of an increase in the growth costs, as hypothesized here, we assumed that the costs of building a cell are multiplied by a factor $1 + e (ci(t) - NEC)$, e being a constant and accounting for the level of toxicity as soon as the NEC is exceeded by the scaled internal concentration, leading to the following equation:

$$\text{If } l < Cs, \text{ then } \frac{d}{dt} l = \frac{a(1-b)}{1 + e (ci - NEC)}$$

$$\text{If } l > Cs, \text{ then } \frac{d}{dt} l = \frac{a}{1 + e (ci - NEC)}$$

All the parameters (ke , NEC and e) were simultaneous calibrated for each fungicide (included the three different ages of exposure).

6. Model calibration and statistical analyses

The differential equations were implemented in the software R Core Team (2015), and solved with the package deSolve (Karline et al., 2010). The model was fitted to the data, for all concentrations and ages of exposure for each fungicide, using the least square method. For the Swing® Gold fungicide, the calibration was performed by minimizing the difference between model simulation and experimental data points. For the copper fungicide, the model calibration was performed on growth increase between two successive data points. We chose this method for the copper fungicide because growth stopped at some tested concentration and we considered more relevant to consider the time rather the weight, at which growth stopped. For the estimation of the confidence intervals of the parameters, we used the bootstrap method (Efron, 1977).

III. Results

1. The copper fungicide

The theoretical soil concentrations were 51.0, 102.7, and 257.7 mg kg⁻¹ of copper for the Cu3.33, Cu10, and Cu30 treatments respectively. These values included the initial soil copper concentration (including the pedogeochemical background of 25.2 ± 3.8 mg kg⁻¹). The measured copper concentrations in soil after spiking were 52.0 ± 1.0, 99.2 ± 2.4 and 240.6 ± 6.5 mg kg⁻¹ of copper, showing that the spiking was correctly performed.

a. Growth pattern and analyses with the model

The copper contamination led to a drastic growth inhibition in the Cu10 and Cu30 treatments (Figure 2). We thus chose to simplify the toxicodynamic model as follows, with an infinite value for e :

$$\text{If } ci > NEC, \text{ then } \frac{d}{dt} l = 0$$

In this situation, there were only two parameters to calibrate in the model: Ke and the NEC . The parameter Ke was estimated at 1.19 and the NEC at 65 mg kg⁻¹ of copper (Table 1). These parameter values were common to the three different ages.

Table 1. Estimated parameter values and confidence intervals (CI 95%) for the copper fungicide.

Parameters	Value	CI (95%)
Ke	1.19	1.17 - 1.67
NEC copper (mg kg ⁻¹)	65.006	64.79 - 65.01
e	infinite	-

Figure 2 presents the data and the description by the model obtained for the 3 different ages of exposure and the 3 different copper concentrations tested. At 25.8 mg kg⁻¹ of copper (3.33 times the RD), the growth was not affected because the NEC was not exceeded. The growth pattern in this treatment thus corresponded to the growth pattern

provided by the model in the control. At 77.5 mg kg^{-1} of copper (10 times the RD), an inhibition of the growth was observed, appearing at different times after the beginning of the exposure for the different ages: the effects appeared immediately after the exposure for new-hatched individuals (Age 1) and after respectively 20 and 30 days after the exposure for individuals of Age 2 and 3. The models account for these differences through the dilution by growth in the toxicokinetic model and provided a good description of the data although it slightly overestimated the growth of the bigger juveniles (Age 3). At 232.5 mg kg^{-1} of copper (30 times the RD), the growth was totally inhibited right after the beginning of the exposure, whatever the age of the earthworms. Our model also accounted for this absence of difference between ages.

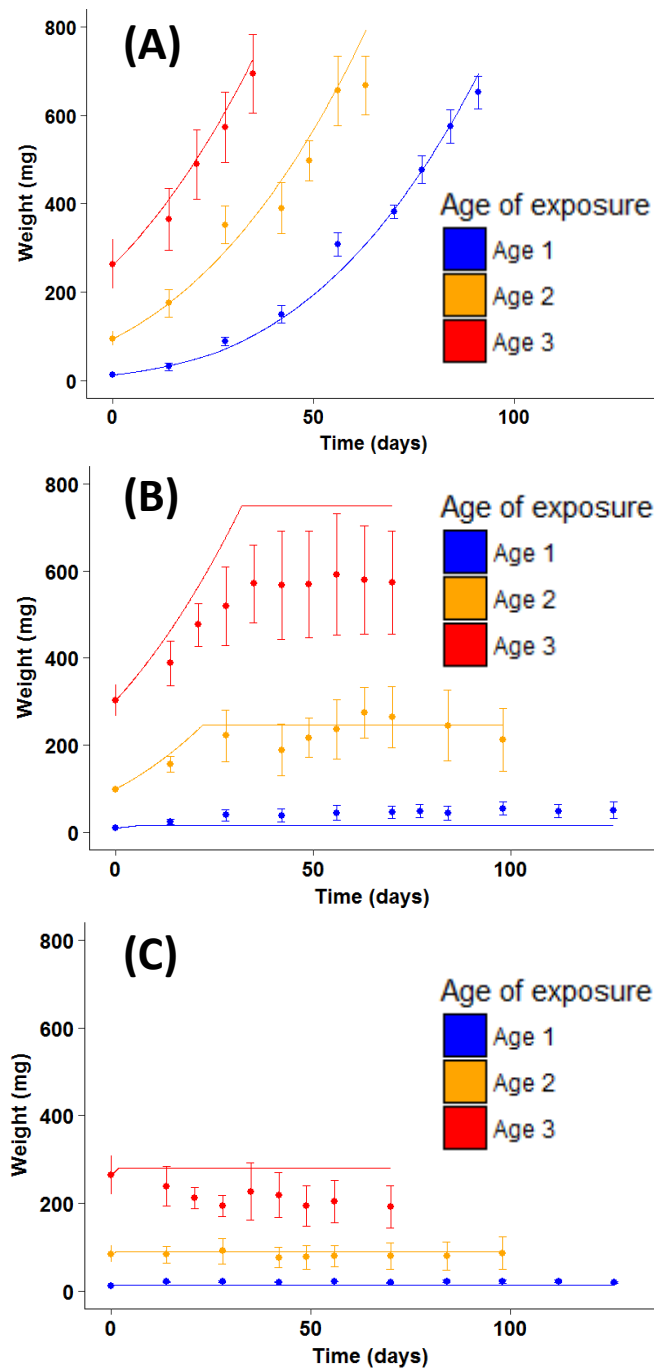


Figure.2. Growth pattern of *A. caliginosa* juveniles exposed at different ages to the Cuprafor micro[®] fungicide at **(A)** 3.33 times the RD (Recommended Dose), corresponding to 25.8 mg kg⁻¹ of copper, **(B)** 10 times the RD, corresponding to 77.5 mg kg⁻¹ of copper, and **(C)** 30 times the RD, corresponding to 232.5 mg kg⁻¹ of copper. Full lines represent the description of the observations (n=7 ± SD) by the model.

b. Copper accumulation in earthworms

The copper accumulation in earthworm significantly differed between treatments and the control (Figure 3). At 25.8 mg kg⁻¹ of copper (Cu3.33), the copper internal concentration at the end of the experiment did not differ between individuals exposed at Age 1, 2, or 3. For the individuals exposed in the Cu10 and Cu30 treatments, the copper concentration in tissues significantly decreased with increasing the age of exposed earthworms corresponding to an increase in copper accumulation with the time of exposure (Figure 3).

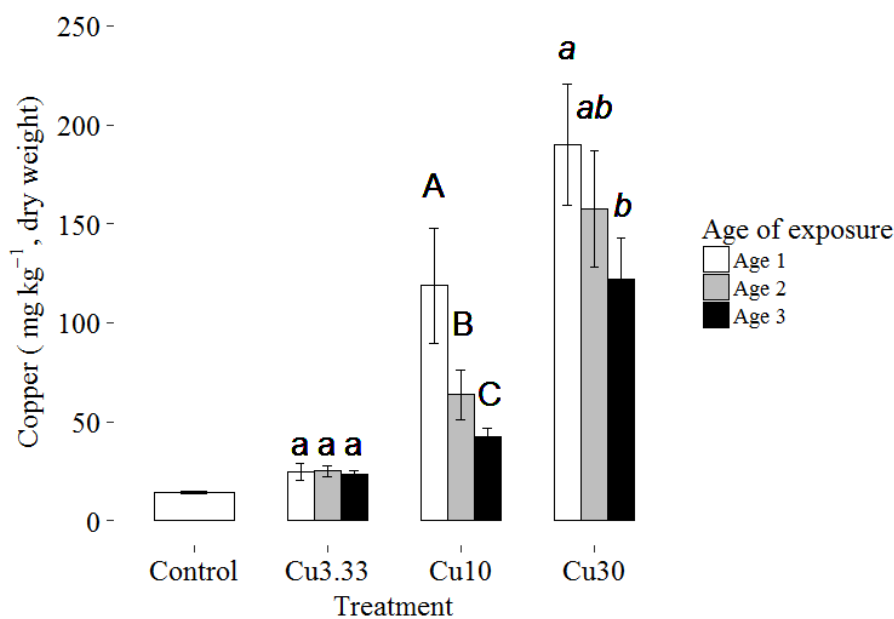


Figure 3. Copper concentration in *A. caliginosa* individuals at the end of the growth experiment in mg kg⁻¹ (dry weight). Individuals of Age 1 were exposed for on average 91 days to the Cu3.33 (25.8 mg kg⁻¹ of copper) treatment and 126 days to Cu10 (77.5 mg kg⁻¹ of copper) and Cu30 (232.5 mg kg⁻¹ of copper). Individuals of Age 2 were exposed for on average of 63 days to the Cu3.33 treatment and 98 days for the Cu10 and Cu30 treatment. Individuals of Age 3 were exposed for on average of 35 days to the Cu3.33 treatment and 70 days to the Cu10 and Cu30 treatment. Different letters mean significant differences between ages of exposure for each copper treatment.

2. The Swing[®] Gold fungicide

a. Degradation in soil over time

Less than 5% of difference was recorded for total and bioavailable concentrations of EPX and DMX between soil with earthworms and horse dung after 28 days, and soil that was contaminated at the same time but used at 28 days (for the soil renewal). This indicated that

individuals of different ages were exposed to the same total and bioavailable fungicide concentration during the soil renewal. The same results were found for soils that had waited for 56 days before being used.

The Swing[®] Gold concentration measurements allowed characterizing the exposure over time in the experiment. The total soil concentration was correlated with the bioavailable fraction of DMX and EPX, with a R^2 of 0.99 and 0.97, respectively. The choice was thus made to use the total soil concentration in the toxicokinetic model. We modeled the decrease in the total concentration with equations (among linear or exponential equations) that allowed for the best description of the exposure over the time (Figure 4). The description was correct as no significant differences were found between observed and predicted values ($P > 0.05$ with Student's t-tests) for more than 90% of the cases.

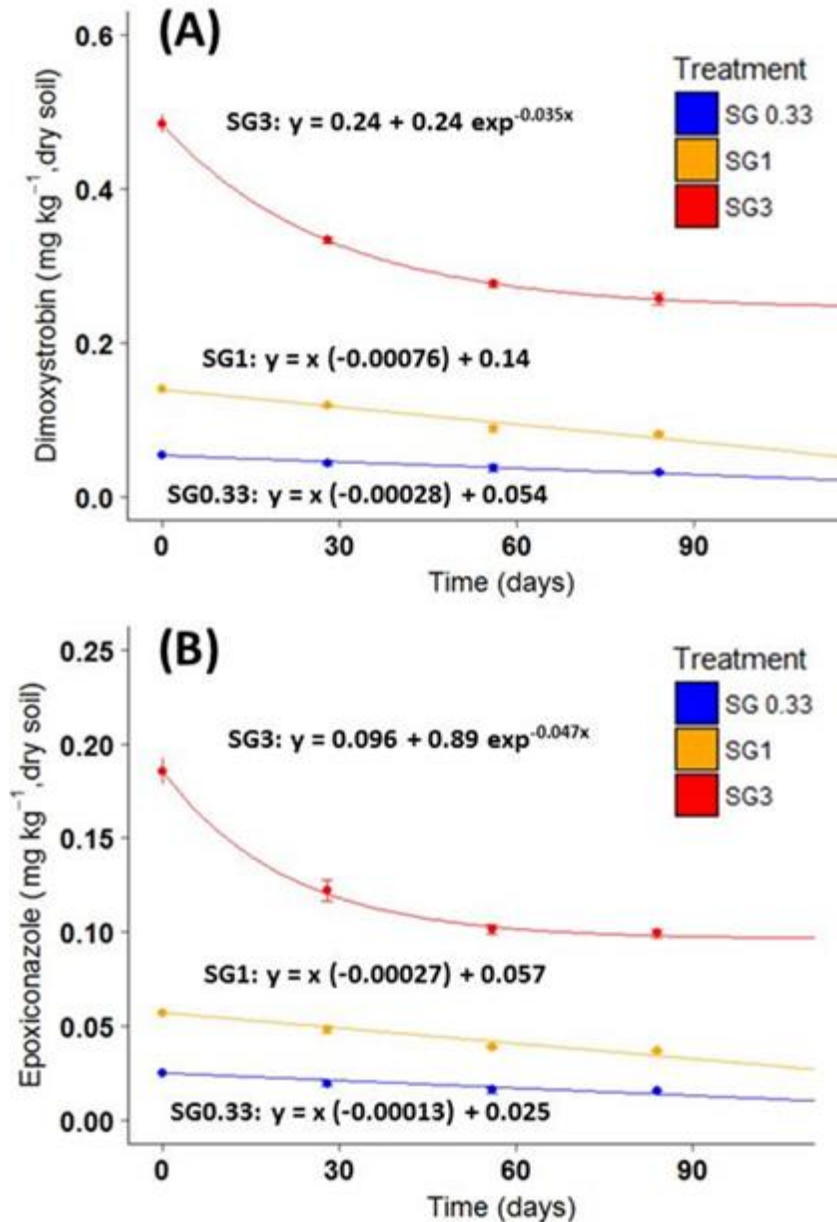


Figure 4. Total soil concentration over time for (A) dimoxystrobin (DMX) (mg kg⁻¹, dry soil) and (B) epoxiconazole (EPX) (mg kg⁻¹, dry soil) in soil spiked with the Swing[®] Gold fungicide at 0.33 (5.16 10⁻² mg kg⁻¹ of DMX and 1.94 10⁻² mg kg⁻¹ of EPX), 1 (0.155 mg kg⁻¹ of DMX and 5.81 10⁻² mg kg⁻¹ of EPX), and 3 (0.464 mg kg⁻¹ of DMX and 0.174 mg kg⁻¹ of EPX) times the RD (Recommended Dose), abbreviated SG 0.33, SG1, and SG3 respectively. Full lines represent the description of the data (n=4 ± SD) by the models.

b. Growth pattern and analyses with the model

The effects of Swing[®] Gold on the growth appeared immediately after the exposure in the SG3 treatment (Figure 5C). We thus assumed a very fast toxicokinetics and used directly the total DMX or EPX soil concentration as an internal concentration (*c_i*) in the effect model. Two parameters were thus calibrated: *e* and the *NEC*. The parameter *e* was estimated at

13.27 and 13.24 for the DMX and the EPX in the Swing® Gold formulation respectively (Table 2). The *NEC* was estimated at 0.387 mg kg⁻¹ (dry soil) and 0.128 mg kg⁻¹ of DMX and EPX in the Swing® Gold formulation respectively (Table 2). These parameter values were common to the three different ages.

Table 2. Estimated parameter values and confidence intervals (CI 95%) for the Swing® Gold fungicide.

Parameters	Value	CI (95%)
<i>Ke</i>	infinite	-
<i>NEC</i> Dimoxystrobin (mg kg ⁻¹)	0.387	0.375 - 0.402
<i>NEC</i> Epoxiconazole (mg kg ⁻¹)	0.128	0.123 - 0.143
<i>e</i> Dimoxystrobin	13.27	11.98 - 21.00
<i>e</i> Dimoxystrobin	13.24	13.22 - 44.18

Figure 5 present the data and the description by the model obtained for the 3 different ages of exposure and the 3 different Swing® Gold concentrations tested. At 0.33 and 1 times the RD (SG0.33 and SG1), the growth was not affected because the *NEC* was not exceeded. In these treatments, the growth pattern thus corresponded to the growth pattern provided by the model in the control (Figure 5A and 5B). At 3 times the RD (SG3), the growth was negatively affected just after the exposure whatever the age of exposure (Figure 5C), and during a period of 15 days corresponding to the time during which the concentration exceeded the *NEC*.

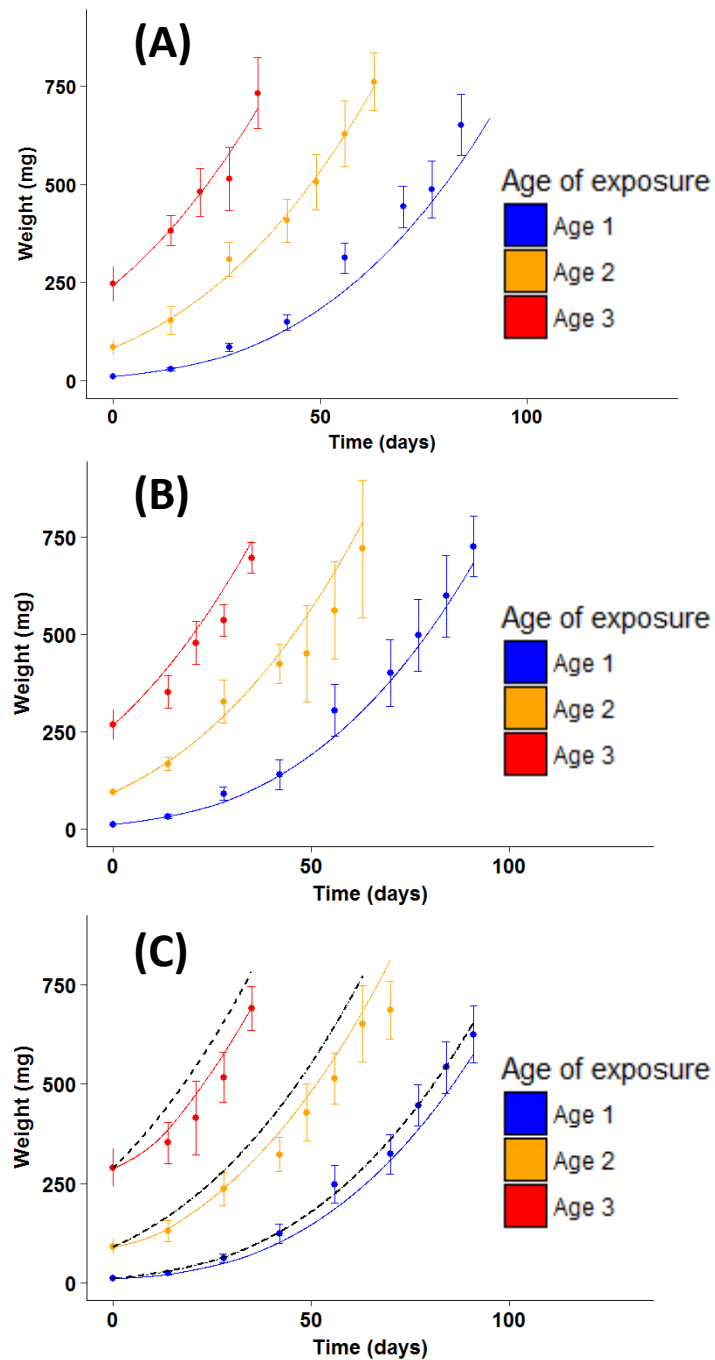


Figure 5. Growth pattern of *A. caliginosa* exposed at different ages to the Swing® Gold fungicide at (A) 0.33 times the RD (Recommended Dose), (B) 1 time the RD and (C) 3 times the RD. Full lines represent the description of observations ($n=7 \pm SD$) by the model, and the dash lines represent the description of the observations in the control treatment by the model.

c. Dimoxystrobin and epoxiconazole accumulation in earthworms

The dimoxystrobin and epoxiconazole accumulation in earthworm significantly differed between treatments and the control (Figure 6). There was no significant difference in the accumulation of dimoxystrobin or epoxiconazole between the different ages of

exposure corresponding to no difference of dimoxystrobin or epoxiconazole accumulation with the time of exposure (Figure 6).

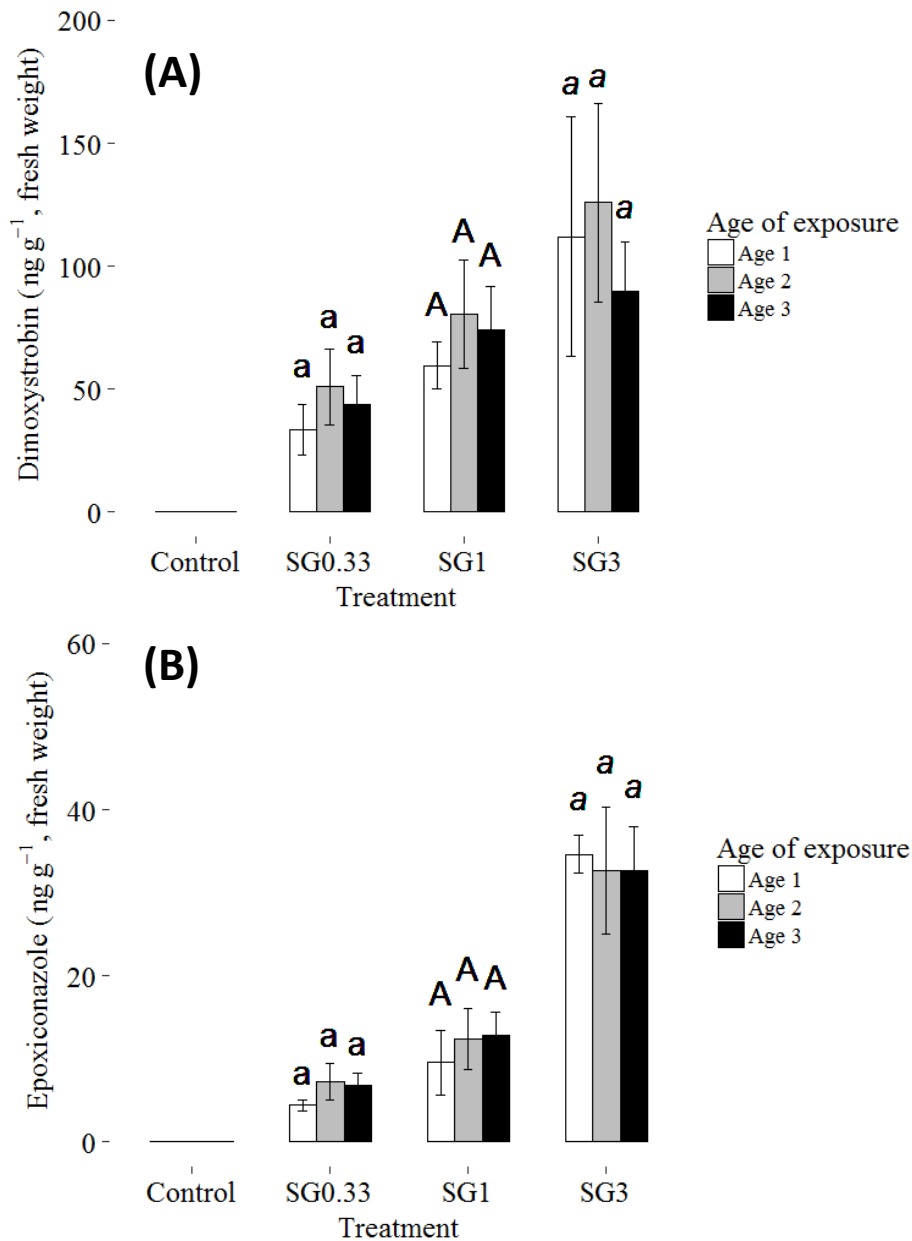


Figure 6. Concentration of dimoxystrobin (A) and epoxiconazole (B) in *A. caliginosa* individuals at the end of the growth experiment in ng g⁻¹ (fresh weight). Individual of Age 1 were exposed for on average 91 days to the SG 0.33 and SG1 treatment and 95 days to the SG3 treatment. Individual of Age 2 were exposed for on average 63 days to the SG 0.33 and SG1 treatment and 67 days to the SG3 treatment. Individual of Age 3 were exposed an average 35 days to the SG 0.33 and SG1 treatment and 38 days to the SG3 treatment. Different letters mean significant differences between ages of exposure for each Swing® Gold treatment.

IV. Discussion

Impact of the two tested fungicides on earthworm growth

We here showed that the growth pattern of one of the most representative species of earthworms in cultivated fields was highly influenced by the presence of two fungicides at environmentally relevant doses. Moreover, we highlighted and explained that the magnitude of the effects depended on the age of the individuals. Finally, we pointed out that the model proposed by Bart et al. (201x, Partie 1, Chapitre 2) as a basis for a TK-TD model can be very useful to understand impacts and provide threshold value for the effects of the tested fungicides.

The copper fungicide appeared highly harmful to earthworm growth beyond the *NEC*, estimated at 65 mg kg⁻¹ of copper, corresponding to 8.4 times the RD of the fungicide. This result is in accordance with the EC50 growth estimated at 81.8 mg kg⁻¹ of copper by Khalil et al. (1996). Helling et al. (2000) showed that copper affected the growth of *E. fetida* from 8.92 mg Cu kg substrate⁻¹ (which was urine-free cattle manure) and that earthworms exposed to 346.85 mg Cu kg substrate⁻¹ exhibited hardly any increase in weight. Ma (1984) showed that copper inhibited the growth of the earthworm *Lumbricus rubellus* at a concentration of 370 mg kg⁻¹ of copper in a sandy soil. It thus appears that copper is harmful for earthworm growth but the threshold concentration inducing impact is highly dependent on the soil characteristics and species considered (Ma and Rao 1997, UE 2008). Finally, it is important to notice that the *NEC* value estimated (i.e. 65 mg kg⁻¹) could be reached in agricultural systems because copper accumulates in soils (Brun et al., 1998). This is the case in vineyards in which copper can reach more than 100-200 mg kg⁻¹ and explain the very low density of earthworms in these agroecosystems (Paoletti et al., 1998).

The Swing[®] Gold fungicide appeared as harmfulness for earthworm growth with a *NEC* value corresponding to 2.5 times the RD. Moreover, the two active substances (i.e. DMX and EPX) are considered as persistent in the environment with DT50 (lab at 20°C) estimated at 210 and 226 days for DMX and EPX respectively (PPDB, 2018). Concerning the active substance which was responsible for the effect, it is not possible to rule. However, the data available in the literature indicated that the dimoxystrobin was more harmful to earthworms than epoxiconazole when considering the LC50 values: The European Pesticide Properties DataBase (PPDB, 2018) reported for *E. fetida* a LC50 of >500 mg kg⁻¹ and 23.65 mg kg⁻¹ for EPX and DMX respectively. Moreover, Pelosi et al. (2016) estimated a LC50 for EPX (alone in a commercial formulation of Opus[®], 125 g L⁻¹ of EPX) at 45.5 mg kg⁻¹ of EPX for

the earthworm *Aporrectodea icterica*. In this way, the NEC values estimated at 0.387 mg kg^{-1} and 0.128 mg kg^{-1} for DMX and the EPX respectively in our study are valid only in the studied commercial formulation. They are overestimated if taken separately out of the formulation, especially for the EPX.

Growth is a key life history parameter that directly influences the population dynamics in a way comparable to reproduction or survival. In the fields, earthworms are active between 3 and 7 months per year, generally in spring and autumn (Baker et al., 1992). In the present study, *A. caliginosa* individuals in the control soil took three months to become adult in optimal conditions (fed *ad libitum* soil moisture of 70% of the WHC, temperature at 15°C , Bart et al., 2018). We can thus assume that there would be no more than one or two new generations of *A. caliginosa* per year. And it is worthwhile to underline that *A. caliginosa* is an r-strategist that grow and reproduce relatively fast compared to other species of earthworms such as anecic species (e.g. *Lumbricus terrestris*, Pelosi et al., 2008). A growth delay of about ten percent, as we observed for Swing[®] Gold, could have a strong impact on the population dynamics, with adults appearing significantly later in the year at a period which could not be optimal for the reproduction. It could be obviously even more problematic with compounds such as copper that completely inhibit earthworm growth.

Relevance of the toxicity analysis with TK-TD model

The two tested fungicides had very different toxicokinetics and toxicodynamics. For the copper fungicide, we showed a drastic inhibition of growth and a slow kinetics whereas the toxicokinetics for the Swing[®] Gold fungicide was very fast with moderate effects. These conclusions came from the analysis of the data with our TK-TD model which pointed out the relevance of assuming either very fast kinetics or very strong effects. The conclusions regarding the kinetics were confirmed with the measurements of the fungicide internal concentrations at the end of the experiment.

We saw that the effects of the copper fungicide significantly depended on the age of the exposed individuals and the exposure duration. Indeed, the effects appeared earlier for small organisms (Age 1) compared to older organisms (Age 2 and 3) and that was accounted for by the dilution by growth and the influence of weight on the kinetics parameters. Thus, the difference between ages was fully explained by growth, with same parameters for

kinetics and effects. This also supports that the use of a one compartment-model was satisfactory here. In some cases, for which, for instance, uptake rate depends on exposure concentrations because of saturation of the uptake, a model with more compartments could be necessary (Steen Redeker and Blust, 2004). The difference in copper accumulation was certainly due to the longer exposure of individuals of Age 1 and 2, because low elimination rates mean that the longer the exposure the higher the accumulation. Still after more than 90 days of exposure, the plateau for accumulated concentration was thus still not reached. At 25.8 mg kg⁻¹ of copper, there is no difference in copper accumulation between the different ages, probably because the internal concentrations were close to those in the control organisms (copper is an essential metal, which is regulated by the organisms (Ridge et al., 2008)). For the Swing[®] Gold fungicide, EPX and DMX accumulation in earthworms did not differ between the different ages and times of exposure. This is coherent with a rapid kinetics, which implies no dependence between the accumulated concentration and the exposure duration. Our model, which accounts for the exposure throughout time, take into account that the two active substances of the Swing[®] Gold fungicide degraded over time, and we were able to explain the toxicodynamics. Indeed, the total concentration in soil became lower than the *NEC* after 15 days of exposure to the highest nominal concentration (SG3) and the growth was not affected anymore afterwards.

In this study, we hypothesized that the physiological mode of action of the two tested fungicides was an increase in the growth energy costs. Another possible effect on growth could have occurred through a decrease in the feeding rate. From a modeling point of view, it is tricky to assess which model would fit at best the observed data because they are very similar for small concentrations. To make the difference between these two modes of action, Péry et al. (2003) performed experiments in two feeding conditions, *ad libitum* and limited food conditions. Here, we only used *ad libitum* conditions. For copper, there are elements in the literature supporting the assumption of an increase in growth cost. First, this was the mode of action found for chironomids in Péry et al. (2003). Moreover, the increase in growth energy costs could be linked to detoxification process. Stürzenbaum et al. (1998) showed for *Lumbricus rubellus* that the production of metallothionein (MT) proteins increased 5-fold in soil contaminated with copper compared to a control soil. Brulle et al. (2007) showed the same with *E. fetida* exposed to cadmium. The MT(s) are responsible for

detoxification processes after an exposure to a metal contamination. The energy could be redirected to the production of such proteins in response to the contamination.

V. Conclusions

Earthworm growth appeared as a sensitive endpoint that should be taken into account in the ecological risk assessment of pesticide. First, the threshold values are, as for the reproduction (Neuhauser et al., 1985), lower than the LC50 value (based on survival). Moreover, growth can have a strong impact on population dynamics that determines the occurrence in the field and the related provided ecological functions. In the present study, the *NEC* for the Swing® Gold fungicide was estimated at 2.5 times the RD while the LC50 was estimated at 6.3 times the RD for *A. caliginosa* (Bart et al., 2017).

The strength of our study relies on the ability of the TK-TD model to fit, with the same parameters, the data obtained for three different ages of exposure, despite apparent differences in the toxicodynamics. These differences were fully explained by physiological considerations, relative to weight and growth in particular. Thus, our models provided No Effect Concentrations that are common to all ages of exposure and do not depend on the time of exposure. A calibration of the parameters for each age would certainly lead to a better fit of the model with the observed data, but we would expect little difference regarding toxicokinetics and toxicodynamics parameters. The use of the model permitted also to support assumptions relative to toxicokinetics and toxicodynamics and to account for the effects throughout time.

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Principales conclusions de l'article

Cet article avait pour objectif de déterminer les effets des deux pesticides étudiés (i.e le Swing® Gold et le Cuprafor micro) sur la croissance d'*A. caliginosa* à différents âges d'exposition ainsi que l'accumulation des pesticides par les individus. L'analyse des données de croissance avec le modèle bioénergétique couplé à un modèle toxicocinétique a permis de déterminer des CSE (Concentrations Sans Effet) à 65 mg kg^{-1} de cuivre, 0.39 mg kg^{-1} de dimoxystrobine et 0.13 mg kg^{-1} d'epoxiconazole (dans la formulation Swing® Gold). De plus les données ont révélé que l'effet du cuivre sur la croissance a été drastique menant à une inhibition complète de la croissance. De plus, l'apparition des effets était plus rapide chez les individus plus jeunes que chez les individus plus âgés (effets apparus plus de 20 jours après l'exposition). Ce phénomène a été pris en compte et expliqué par le modèle grâce à la dilution par la croissance qui module la toxicocinétique en fonction de la taille (= l'âge des individus). Les données de bioaccumulation du cuivre ont permis de valider que l'accumulation du cuivre était plus importante chez les individus exposés plus jeunes et plus longtemps. A l'inverse, quel que soit l'âge d'exposition, les individus exposés au Swing® Gold n'ont présenté aucune différence d'accumulation de dimoxystrobine ou d'epoxiconazole. Ce résultat conforte le choix qui a été fait dans l'analyse des effets avec le modèle, qui était de relier les effets observés directement à l'évolution des concentrations du Swing® gold dans le sol (ce qui correspond à une cinétique rapide). Ainsi, l'analyse des données avec notre modèle toxicocinétique-toxicodynamique a apporté une compréhension mécanistique. Celle-ci, commune à l'ensemble du cycle de vie, a permis de conclure que le cycle de l'énergie est affecté par les pesticides étudiés, avec des dynamiques différentes de l'apparition des effets (lentes pour le cuivre, rapides pour le Swing® gold).

Chapitre 4 – Effet des pesticides sur les fonctions écologiques fournies par les vers de terre

Ce chapitre correspond à un article intitulé “ Earthworms mitigate pesticide effects on soil microbial activity ”, en préparation pour une prochaine soumission dans la revue *Soil Biology and Biochemistry*.

Dans les trois chapitres précédents, Je me suis efforcé d'évaluer les effets des pesticides sur l'ensemble du cycle de vie d'*A. caliginosa*, en prenant en compte chaque stade de développement. Un enjeu majeur de l'évaluation du risque lié à l'utilisation des pesticides est la préservation de la qualité du sol au travers son fonctionnement écologique, pour assurer une production agricole durable. Ainsi, l'objectif de ce chapitre était d'évaluer les effets des pesticides sur une fonction clé à laquelle participent les vers de terre : la dégradation de la matière organique (MO). Pour cela, je me suis intéressé à l'activité microbienne du sol qui participe activement à la minéralisation de celle-ci. Les vers de terre sont connus pour favoriser cette activité microbienne et donc la dégradation de la MO. Ainsi, des mesures d'activités enzymatiques extracellulaires (qui proviennent de l'activité des microorganismes) et du potentiel d'activité nitrifiante (mesure direct de l'activité des bactéries nitrifiantes) reflétant les cycles biogéochimiques du carbone, de l'azote et du phosphore ont été utilisés. Ces mesures ont été réalisées en la présence et en l'absence de vers de terre, et avec différentes concentrations sublétales des deux fongicides étudiés.

Résumé: Les vers de terre sont des ingénieurs de l'écosystème qui module la disponibilité des ressources pour les autres organismes du sol. Ils sont impliqués dans la dégradation de la matière organique du sol en lien avec les microorganismes. Cette étude vise à évaluer l'influence de l'espèce de ver de terre *Aporrectodea caliginosa* sur les effets de deux fongicides sur les activités microbiennes, en microcosmes. Les fongicides étudiés étaient le Cuprafor micro® (oxychlorure de cuivre), couramment utilisé en agriculture biologique, et le Swing® Gold (époxyconazole et dimoxystrobine), un fongicide synthétique largement utilisé dans l'agriculture conventionnelle pour protéger les cultures céréalières. Des indicateurs communs de l'activité microbienne ont été mesurés: l'activité potentielle de nitrification et les enzymes extracellulaires du sol impliquées dans le cycle biogéochimique (glucosidase,

uréase, phosphatase, arylamidase). Les indices GMean et T-SQI ont été utilisés pour intégrer les réponses des activités enzymatiques. Les résultats ont d'abord mis en évidence que la présence de vers de terre augmentait les activités microbiennes, avec et sans fongicides. Dans les microcosmes sans vers de terre, les fongicides ont significativement augmenté les activités enzymatiques du sol, mis en avant par un indice GMean plus élevé que les microcosmes sans fongicide. De la même manière, les deux fongicides ont provoqué une augmentation significative du potentiel d'activité nitrifiante dans les microcosmes sans les vers de terre. A l'inverse, aucun effet des fongicides n'a été observé dans les microcosmes avec les vers de terre en terme d'indice GMean et de potentielle d'activité nitrifiante. Ces résultats suggèrent que la présence des vers de terre confère une plus grande résistance des activités microbiennes aux pesticides.

Earthworms mitigate pesticide effects on soil microbial activity

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Abstract

Earthworms are ecosystem engineers involved in soil organic matter degradation in relation with soil microorganisms, leading to the modulation of resource availability for other soil organisms. This study aims to assess the influence of the earthworms *Aporrectodea caliginosa* and two fungicides on microbial activities, in soil microcosms. The fungicides were Cuprafor micro[®] (copper oxychloride), commonly used in organic farming, and Swing[®] Gold (epoxiconazole and dimoxystrobin), a synthetic fungicide widely used in conventional farming to protect cereal crops. Common indicators of the microbial activities were measured to describe microbial response: the potential nitrification activity and soil extracellular enzymes involved in biogeochemical cycling (glucosidase, urease, phosphatase, arylamidase). To GMean and T-SQI indices were used to integrate all enzyme activities responses. Results first showed that the presence of earthworms enhanced microbial activities, with and without fungicides. In the soil with no earthworms, the fungicide treatments significantly increased soil enzyme activities leading to a higher GMean Index compared to non-treated control soils, while no fungicide effect was detected in the soils with earthworms. Similarly, both fungicide treatments induced a significant higher increase in the potential nitrification activity in soil without than with earthworms. In light of the responses of each soil enzyme activities to the presence of earthworms and to the two fungicides, our study suggests that the presence of earthworms confer a higher resistance to microbial activities against effects of pesticides.

Keywords: *A. caliginosa*, fungicide, enzyme activity, nitrification, interaction, ecotoxicology

I. Introduction

In soil, earthworms and microorganisms represent the largest part of the living biomass (Lavelle and Spain, 2001). Earthworms are ecosystem engineers (Jones et al., 1994) playing key roles in soils, thus contributing to ecosystem services (Blouin et al., 2013; Bertrand et al., 2015). It has been well established that earthworms play key roles in the dynamic of the soil organic matter (SOM) and resource availability for other soil organisms, through narrow interactions with microbial component (Bouché, 1975). Mechanical and biological activities of earthworms catalyze SOM decomposition, carbon and nitrogen mineralization, and nutrient turnover, by modulating microbial biomass and activity (Daniel and Anderson 1992; Zhang and Hendrix, 1995). For example, it has been shown that different earthworms species increased soil microbial respiration (Scheu, 1987) or soil enzyme activities related to C, N and P cycling (Dempsey et al., 2013; Tao et al., 2009), mainly in the drilosphere soil (i.e. fraction of the soil influenced by earthworms; Bouché, 1975). Moreover, soils with earthworms' burrows and casts present higher abundances and activities of different microorganisms (i.e. ammonifiers, denitrifiers, free-living aerobic and anaerobic nitrogen fixers and proteolytic bacteria) than soil with no earthworm activity (Bhatnagar, 1975; Loquet et al., 1977; Aira et al., 2003). The mucus produced by earthworms is also a trophic resource increasing the microbial activity (Martin et al., 1987). However, by ingesting microbial biomass with SOM, they can decrease the total microbial biomass but increase its active components (i.e., extra cellular enzyme activities, Zhang et al., 2000; Aira et al., 2009).

The use of pesticides in agroecosystems may impair biodiversity and biological activity in cultivated soils (Bengtsson et al., 2005; Hole et al., 2005; Pelosi et al., 2014). Earthworms are often used as soil biological indicators (Spurgeon et al., 2003) and the impact of pesticide on earthworms have been deeply documented (Pelosi et al., 2014). Pesticide application can also impact soil microorganism activities, but with much more contrasted results (decrease or increase) depending on the active compound and the microbial activity (Wainwright et al., 1978; Chen et al., 2001; Niemi et al., 2009), with possible outcomes for processes mediated by microorganisms (Munoz-Loez et al., 2013).

The interactions between soil microbial communities and earthworms towards soil processes have been widely investigated and earthworms and soil microorganisms are known to be affected by pesticides. However, the impact of pesticide on the outcomes of their interactions (i.e. microbial functions), have been poorly documented. Sanchez-Hernandez et al. (2018) recently explored the impact of an insecticide commercial formulation on soil enzymes under the influence of *Lumbricus terrestris*. An increase in global soil enzyme activities was observed after the application of chlorpyrifos, underlying that earthworms can increase the microbial activity even under pesticide pressure.

Here we aim at assessing the consequences of the presence of earthworms on soil microbial activities in soil contaminated by two commercial formulations of fungicide (Cuprafor micro[®], made of copper oxychloride, and Swing[®] Gold, made of epoxiconazole and dimoxystrobin) earthworms. In this study, we make the assumption that earthworms modulate the microbial responses to fungicides because they modify the habitat of microorganisms.

II. Material and methods

1. Soil and earthworms

The soil used for all experiments was sampled from the top 0–20 cm in a permanent grassland in Versailles (48°48' N, 2°5' E) where no chemicals had been applied for more than 20 years. It was a Luvisol (FAO soil classification) and its main physical characteristics were: pH 7.5, organic matter 32.6 g kg⁻¹, C/N 12.7, 29% sand, 48% silt, and 23% clay (See Bart et al., 2017 for more detail). The soil was air-dried and passed through a 2-mm sieve.

Mature individuals of *Aporrectodea caliginosa* s.s were collected by hand-sorting from an agricultural field in Estrées-Mons (49°52' N 3°01'E). Their weight ranged from 600 to 1000 mg. They were stored in the soil used for the experiments at 15±1 °C, 24 h darkness for at least 10 days before the experiments.

2. Pesticides

Swing® Gold (BASF Agro SAS, dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹) is an organic synthetic fungicide. The Recommended Dose (RD) was calculated as 1.16 10⁻³ mL kg⁻¹ of dry soil according to a soil density of 1.29 and considering that the active compounds of this fungicide are almost always found in the top 10 cm of soil (McDonald et al., 2013; Chabauty et al., 2016). Based on the LC50 estimated at 6.3 times the RD for *A. caliginosa* (Bart et al., 2017), we tested 0.33, 1 and 3 times the RD of this commercial formulation. Cuprafor micro® (Quimicas del Valles, 50% copper oxychloride) is a metal based fungicide, allowed in organic management. The RD was calculated as 15.5 mg kg⁻¹ (corresponding to 7.75 mg kg⁻¹ of copper) of dry soil according to a soil density of 1.29 and considering that the active compounds of this fungicide are almost always found in the top 5 cm of soil (Couto et al. 2015). Based on literature review (PPDB, 2017; Ma 1984; Spurgeon et al., 2004; Bart et al., 2017) and taking into account that copper can accumulate in soils, we tested 3.33, 10 and 30 times the RD which correspond to the addition of 25.8, 77.5, and 232.3 mg kg⁻¹ of copper.

3. Experimental design

Soil microcosms were built up with five replicates for all conditions. Each microcosm corresponded to a 1 L plastic vessel, with a removable perforated cover for gas exchange, filled out with 500 g of dry soil and 24 g of horse dung (dry mass) as food resource for earthworms, corresponding to a feeding of 6 g ind⁻¹ month⁻¹ as suggested in Lowe and Butt, 2005 and in Bart et al., 2018 for *A. caliginosa*. The soil moisture was adjusted at 70% of the maximum water holding capacity (mWHC) using the fungicide solutions or tap water for controls. The food moisture was also adjusted at 70% mWHC and mixed with the soil. Four individuals of *A. caliginosa* were introduced in each vessel and incubation was run for 28 days of incubation in a climate room at 15 ± 1 °C. Earthworms were weighted at day 0 and at the end of the experiment (day 28). The soil moisture content was controlled once a week. A similar set design of microcosms was performed without earthworms.

4. Microbial activity

Soil extracellular enzymatic activities, involving in the cycle of carbon (C), nitrogen (N) and phosphor (P) (Burns et al., 2013) and potential nitrification activity were selected as microbial indicators because of their importance in SOM mineralization and nutrient cycling and given the recognized sensitivity of nitrification to pesticides (Crouzet et al., 2016). The potential nitrification activity (PNA) and soil extracellular enzymes (β -D-glucosidase (GLU), phosphatase (PHOS), urease (URE) and arylamidase (ARN)) was assessed at day 28 after the fungicide treatments, in each microcosm.

PNA was determined in accordance to Peterson et al. (2012), with some modifications specified in Corbel et al., 2015. Briefly, 4 g of fresh soil were sampled and mixed with 25 mL of MilliQ water and $(\text{NH}_4)_2\text{SO}_4$ at a final concentration of 1mM. Samples were incubated at 25 °C under continuous stirring (150 rpm). After 2.5 h and 45 h, 1 ml was sampled and centrifuged at 13 000 g for 5 min and supernatants were stored at -20°C until analyses of the ions nitrate (NO_3^-) and nitrite (NO_2^-) following the colorimetric method according to the Griess reaction . The supernatants were transferred in microplate and the Griess solution was added (HCl, 0.5M, vanadium chlorure III (Sigma-Aldrich 208272) at 1 g L⁻¹, sulfanilamide (Sigma-Aldrich S9251) at 2.5 g L⁻¹ and N-(1-naphthyl)-ethylenediaminedihydrochloride (Sigma-Aldrich 222488) at 0.25 g L⁻¹), and then incubated for 1.5 h at 60°C. The optic densities were thus determined at 540 nm with a microplate reader (SAFAS Xenius, Monaco). Results were then expressed in Potential Nitrification Activity (PNA) as the rate of production of $\text{N-NO}_3^- + \text{N-NO}_2^-$ between 45h and 2h30.

Soil extracellular β -D-glucosidase (GLU), phosphatase (PHOS), urease (URE) and arylamidase (ARN) were mesuerd according to the ISO standard (ISO, 2018). Three aliquotes of 4 g of fresh soil were sampled in each microcosm and mixed with 25 mL of MilliQ water and incubated for 10 min at ambient temperature under continuous stirring (250 rpm). An aliquot of soil solution (125 μL) was then incubated in a 96-well micro plate with substrates: 4-nitrophenyl- β -D-glucopyranoside (0.05 M, 1 h at 37°C) for GLU, 4-nitrophenylphosphate (0.05 M, 30 min at 37°C) for PHOS, urea (0.4 M, 3 h at 25°C) for URE and L-leucine β -naphthylamidehydrochloride (0.008 M, 1 h at 37 °C) for ARM. After the incubation period,

reactions were stopped with a CaCl₂ solution (0.5 M) and Tris–HCl (0.1 M, pH 12) for GLU, PHOS and URE. For ARN, reactions were stopped with ethanol 96% (v/v). The microplate was then centrifuged at 3000 g for 5 min and an aliquot of 0.200 mL of each well was used to evaluate the enzymatic activity. For GLU and PHOS the para-nitrophenol released was measured at 405 nm with a microplate reader (SAFAS, Monaco) and the concentration of the product released was compared with para-nitrophenol standards (Sigma). For URE activity, the ammonium ion NH₄⁺ released was determined at 610 nm with an HACH reagent (Loveland, CO, USA) and the concentration of the product released was compared with NH₄Cl standards (Sigma). Finally, for ARN activity, the β-naphthylamine released was determined at 540 nm with p-diméthylaminocinnamaldéhyde (DMCA) and the concentration of product released was compared with β-naphthylamine standards (Sigma). Results were then expressed in mU g⁻¹ dry soil, representing 10⁻³ micromoles of product hydrolysed (as chromophore release) per minute and per g of equivalent dry-soil.

5. Data analysis

The impact of pesticide on soil enzyme activities was assessed using two common enzymatic indices in soil biochemistry. These indices can be used as proxies of the response of the whole microbial activity. The geometric mean index (GMean) (Hinojosa et al., 2004): Where y_i is the enzyme activity, n is the number of soil enzymes. High GMean values mean high microbial functional diversity of soil (Lessard et al., 2014).

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

The treated-soil quality index (T-SQI) as proposed by Mijangos et al. (2010), where m is the reference soil (mean value of enzyme activity, set to 100%), and n is the mean value for each enzyme activity in soils contaminated with the two fungicides, as percentages of the reference soil which is the control soil with or without earthworms. The T-SQI takes into account i) the magnitude and direction (increase or inhibition) of changes caused by a stressor (here the two tested fungicides) on soil enzyme activities compared with those from

a control or reference soil (first Σ of numerator) and (ii) the maintenance of the evenness among the enzyme activities shown by the reference soil (second Σ of numerator) (Mijangos et al., 2010; Muñoz-Leoz et al., 2013).

$$T-SQI = 10^{\log m + \frac{\sum_{i=1}^n (\log n_i - \log m) - \sum_{i=1}^n |\log n_i - \log \bar{n}|}{n}}$$

All the statistical analyses were performed with the software R CoreTeam (2015). The analyses of biomass change between day 0 and day 28 were performed using the non-parametric Wilcoxon-Mann-Whitney tests. When the normality and homoscedasticity conditions were satisfied, each microbial activity and indices were analyzed using a two ways ANOVA for testing the effect of fungicide concentrations, of the presence of earthworms, and of the interaction of both factors. Then, a Tukey test was performed to assess the difference between pesticide treatments in soils with and without earthworms. When the normality and homoscedasticity conditions were not satisfied, the non-parametric adonis test was used, followed by the kruskalmc (multiple comparison) test (adjusted p-values based on Bonferroni's corrections were applied) to assess the difference between pesticide treatment in soil with and without earthworms.

III. Results

1. The copper fungicide

There was no significant difference in earthworm weight between the beginning and the end of the experiment whatever the copper fungicide concentration tested.

The responses of enzyme activities in control soil and Cu-treated soil with or without earthworms are presented in Table 1. The presence of earthworms significantly increased all enzyme activities. Considering the treatments with earthworms, a significant effect of the Cu treatment was measured on two enzyme activities (i.e. PHOS and ARN) but the stronger consistent effect was the 20% decrease in phosphatase activity in the Cu30 treatment compared to the control soil. Without earthworms, the differences between copper

treatments were higher than with earthworms since three enzyme activities (GLU, PHOS, ARN) differed between the control and Cu treatments. The PHOS activity still decreased (by 25%) when increasing Cu concentrations. The ARN activity still increased when increasing Cu concentration, to reach +70% of the value in the control, in the Cu30 treatment. The GLU activity was significantly increased in all Cu treatment compared to the control but decrease in the Cu10 and Cu30 treatment compared to the Cu3.33 treatment (Table 1).

Table 1. Enzyme activities in soil with or without earthworms exposed to a control soil and the same soil spiked with different concentrations of Cuprafor[®] micro (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper, abbreviated Cu3.3, Cu10 and Cu30 respectively). Different letter means significance differences between treatments. " t" is for treatment effect, "E" for earthworm effect and "(txE)" for the interaction of both factors.

Enzymatic activities	Treatment								Effects		
	With earthworms				Without earthworms				E	t	txE
	Control	Cu 3.33	Cu 10	Cu 30	Control	Cu 3.33	Cu 10	Cu 30			
β-D-glucosidase	24.4 ± 0.9A	24.3 ± 1.8A	23.5 ± 2.6A	24.5 ± 0.9A	10.9 ± 1.0a	17.7 ± 0.8b	13.0 ± 0.7c	14.6 ± 1.2d	***	***	***
Phosphatase	33.8 ± 2.4A	34.9 ± 2.5A	30.8 ± 3.9AB	26.9 ± 1.0B	20.7 ± 1.3a	19.6 ± 1.3a	17.1 ± 1.4b	15.5 ± 1.6b	***	***	ns
Urease	30.1 ± 2.1A	31.9 ± 2.0A	29.7 ± 2.6A	28.3 ± 2.1A	17.6 ± 2.8a	21.2 ± 2.6a	19.7 ± 1.4a	20.5 ± 2.4a	***	ns	ns
Arylamidase	13.6 ± 0.7AB	12.4 ± 0.5B	12.4 ± 1.1AB	13.7 ± 0.2A	6.3 ± 0.4a	8.2 ± 0.6b	8.9 ± 0.3b	10.7 ± 1.8b	***	***	***

The effect of earthworms on the PNA was significant only in the control without Cu (Figure 1). In the soil with earthworms, statistical analyses did not revealed any effect whatever the Cu dose, compared to control, but the PNA in the Cu30 treatment was higher than in the Cu3.33 and Cu10 treatments. Now considering the soil without earthworms, the PNA activity was significantly increased by +37%, +40% and +57% in the Cu3.33, Cu10, and Cu30 treatment, respectively, when compared to the control. The magnitude of increase in PNA between the control and the Cu30 treatment was strongly higher in soil without earthworms (56.8 ± 11%) than in soil with earthworms (12.3 ± 5%) (Wilcoxon test, p = 0.012).

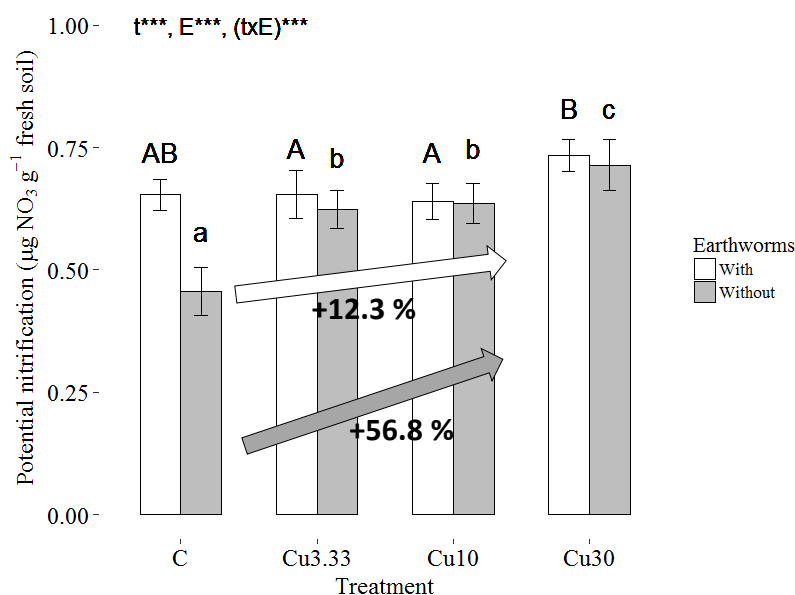


Figure 1. Potential nitrification in $\mu\text{g NO}_3 \text{g}^{-1}$ (fresh soil) in soil with or without earthworms exposed to a control soil and the same soil spiked with different Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg^{-1} of copper, abbreviated Cu3.3, Cu10 and Cu30 respectively). Different letter means significance differences between treatments. t is for treatment effect, E for earthworm effect and (txE) for the interaction of both factors. The arrows showed the increase between the control treatment and the Cu30 treatment.

There was a significant effect of the copper (Cu) treatment, earthworm effect, and interaction effect of both factors on the GMean index (Figure 2 A). Indeed, the presence of earthworms promoted the soil microbial activities in all treatments. When considering only the set of soil microcosms with earthworms, there was no effect of the Cu treatment on the GMean index, while in set of soil microcosms without earthworms, there was a significant increase in the GMean index with increasing Cu concentration.

Significant effects of Cu treatments and earthworms were observed, but no significant effect of the interaction of both factors on the T-SQI index (Figure 2 B). In the set of soil microcosms with earthworms, increasing copper concentrations significantly decreased the T-SQI index. By contrast, without earthworms, there was no effect of Cu concentrations on the T-SQI index.

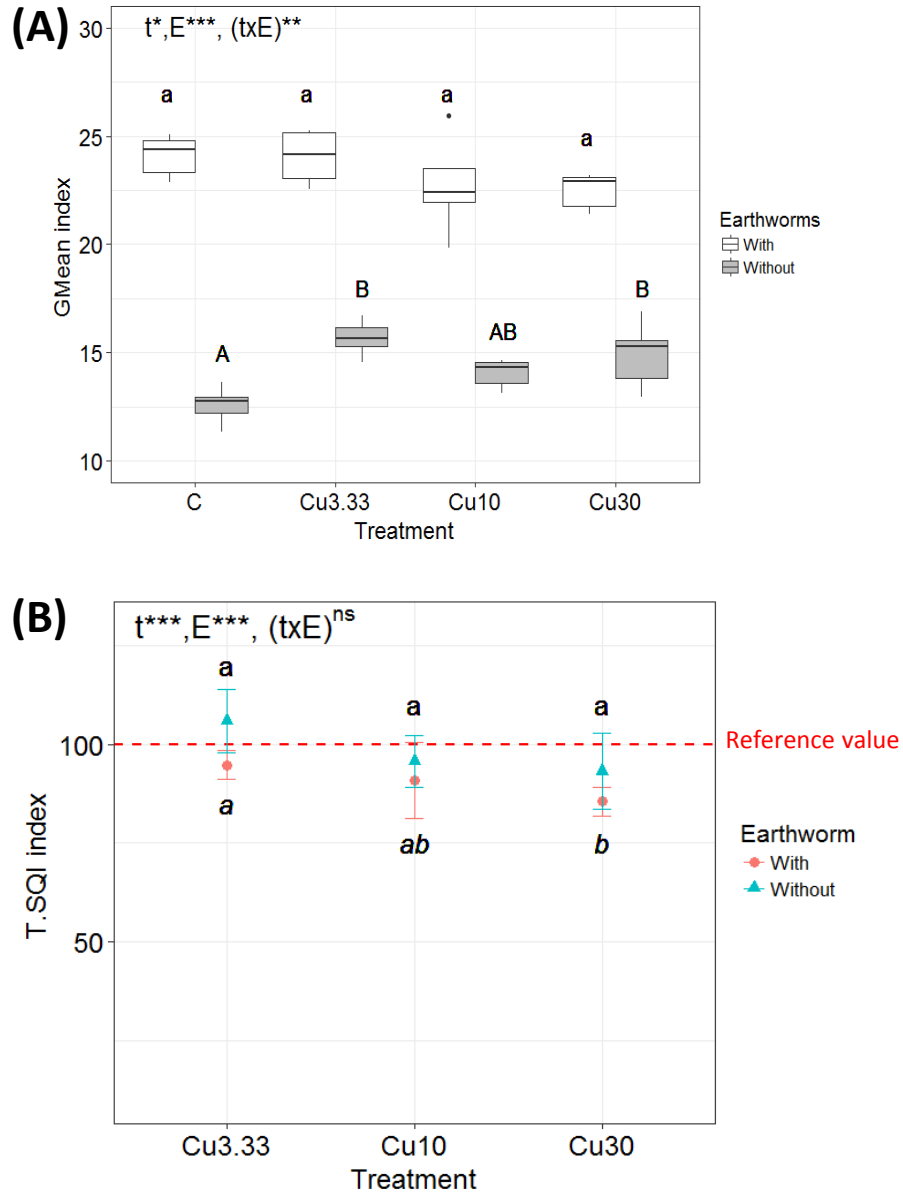


Figure 2. Mean values ($n=5, \pm SD$) of **(A)** the geometric mean (GMean index) and **(B)** the treated-soil quality index (T.SQI) of soil enzyme activities (β -D-glucosidase, phosphatase, urease and arylamidase) in soil with and without earthworms, exposed to a control soil and the same soil spiked with different s of Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper, abbreviated Cu3.3, Cu10 and Cu30 respectively). Different letter means significance differences between treatments. “t” is for treatment effect, “E” for earthworm effect and “(txE)” for the interaction of both factors. In **(B)**, dotted horizontal line shows the reference value which is the control soil, with or without earthworm, set to 100%.

2. The Swing® Gold fungicide

There was no significant difference in earthworm weight between the beginning and the end of the experiment whatever the Swing® Gold fungicide concentration tested.

The responses of enzyme activities in control and soil treated with the Swing® Gold fungicide, with or without earthworms, are presented in table 2. The presence of earthworms significantly increased all enzyme activities, as previously observed with the Cu treatment. Considering the set of soil microcosms incubated with earthworms, a significant effect of the Swing® Gold treatment was measured on two enzyme activities: the PHOS and URE. They respectively increased by 17% and 12 % in the SG3 treatment compared to the control. In set of soil microcosms without earthworm, the Swing® Gold treatment had a significant effect on two other enzyme activities: the GLU and ARN increased by 58% and 24%, respectively, in the SG3 treatment compared to the control.

Table 2. Enzyme activities in soil with or without earthworms exposed to a control soil and the same soil spiked with different Swing® Gold concentrations (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively). Different letter means significance differences between treatments. " t" is for treatment effect, "E" for earthworm effect and "(txE)" for the interaction of both.

Enzymatic activities	Treatment								Effects		
	With earthworms				Without earthworms				E	t	txE
	Control	SG 0.33	SG 1	SG 3	Control	SG 0.33	SG 1	SG 3			
β-D-glucosidase	24.4 ± 0.9A	25.6 ± 1.2A	25.0 ± 1.4A	23.7 ± 0.6A	10.9 ± 1.0a	13.2 ± 1.0b	12.1 ± 1.4ab	17.2 ± 1.9c	***	***	***
Phosphatase	33.8 ± 2.4A	38.9 ± 1.8B	38.0 ± 1.5B	37.9 ± 2.1B	20.7 ± 1.3a	23.5 ± 2.1a	20.2 ± 0.7a	20.3 ± 2.4a	***	***	*
Urease	30.1 ± 2.1A	32.3 ± 0.7AB	33.5 ± 2.1BC	35.2 ± 1.2C	17.6 ± 2.8a	19.1 ± 2.5a	18.7 ± 2.0a	21.5 ± 0.6a	***	***	ns
Arylamidase	13.6 ± 0.7A	12.8 ± 0.7A	13.2 ± 0.5A	12.8 ± 0.5A	6.3 ± 0.4a	7.7 ± 1.3b	7.4 ± 0.5ab	7.8 ± 0.3b	***	ns	**

The PNA was significantly affected by the presence of earthworms (Figure 3). In the soil with or without earthworms, there was a significant effect of the SG treatments with increases of the PNA with the increase in Swing® Gold concentrations. The magnitude of increase in PNA between the control and the SG3 treatment was strongly higher in soil without earthworms ($73.4 \pm 11.4\%$) than in soil with earthworms ($32.4 \pm 8.2\%$) (Wilcoxon test, $p = 0.008$).

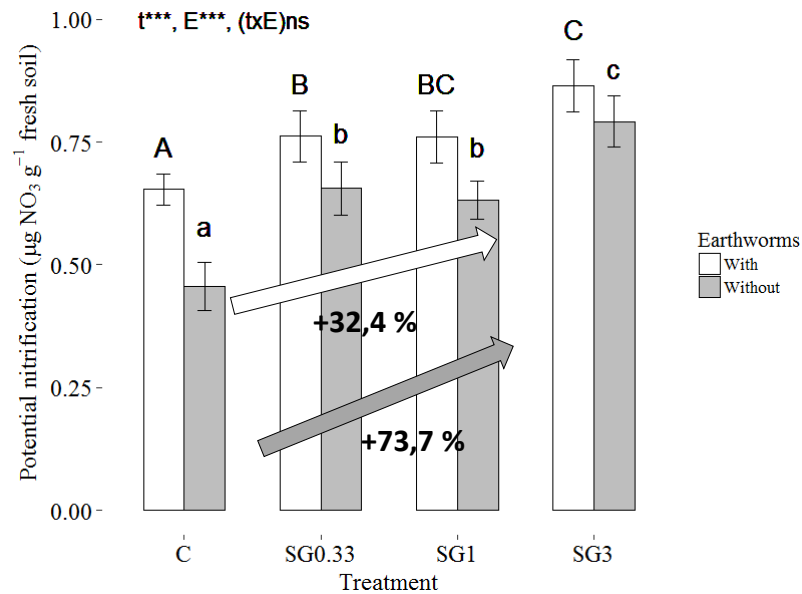


Figure 3. Potential nitrification in $\mu\text{g NO}_3 \text{ g}^{-1}$ (fresh soil) in soil with or without earthworms exposed to a control soil and the same soil spiked with different Swing[®] Gold concentrations (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively). Different letter means significance differences between treatments. "t" is for treatment effect, "E" for earthworm effect and "(txE)" for the interaction of both factors. The arrow showed the increase between the control treatment and the SG3 treatment.

There was significant effects of the Swing[®] Gold treatment, earthworm, and interaction between these factors on the GMean index (Figure 4 A.). Indeed, the presence of earthworms promotes the soil microbial activities in all conditions. There was no effect of the Swing[®] Gold treatment when considering only the set of soil microcosms with earthworms, while considering only the set of soil microcosms without earthworm, there was significant increases of the GMean indices with the increase in Swing[®] Gold concentrations.

There was a significant effect of the earthworms on the T-SQI index (Figure 4 B). However, there was no effect of the different Swing[®] Gold concentration, whatever the presence or absence of earthworms in soils.

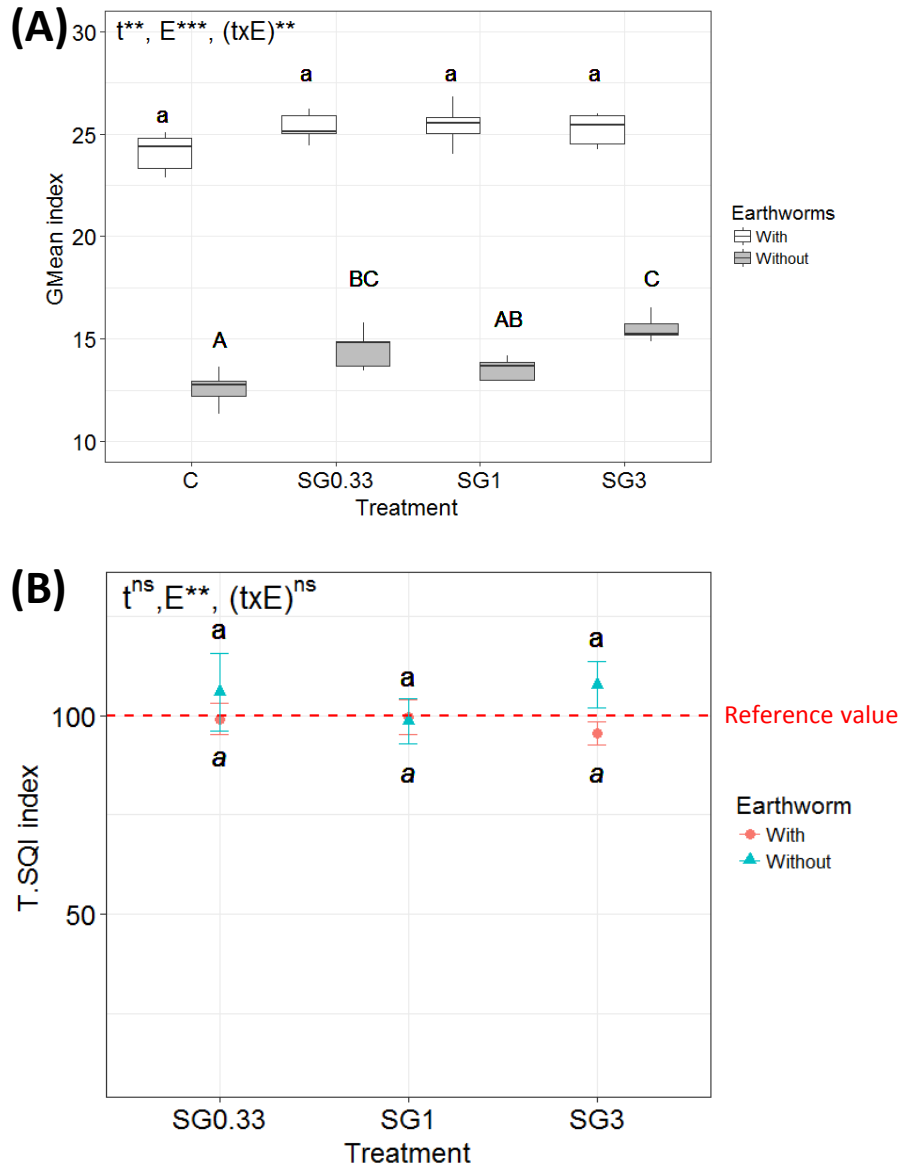


Figure 4. Mean values ($n=6, \pm SD$) of **(A)** the geometric mean (GMean index) and **(B)** the treated-soil quality index (T.SQI) of soil enzyme activities (β -D-glucosidase, phosphatase, urease and arylamidase) in soil with or without earthworms exposed to a control soil and the same soil spiked with different Swing® Gold concentrations (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively). Different letter means significance differences between treatments. "t" is for treatment effect, "E" for earthworm effect and "(txE)" for the interaction of both factors. In **(B)** Dotted horizontal line shows the reference value which is the control soil, with or without earthworm, set to 100%.

IV. Discussion

The absence of impact of fungicide concentrations, noticed on earthworm biomass during the time of exposure, validate the sublethal concentrations chosen for this experiment. Nevertheless, biomass do not inform on impacts of pesticides on earthworms

behavior in soil (Capowiez et al., 2006; Dittbrenner et al., 2011a). So that, possible effects of treatments on earthworm movement or feeding activity, that are key parameters related to influence on microbial communities (Edwards and Bohlen 1996), cannot be excluded.

In soil without earthworms, the results showed that the Swing® Gold and copper fungicide treatments did not impact the T-SQI index, but they both increased the Gmean index and the PNA, highlighting an overall increase in microbial activity with fungicide treatments. This result was already found by other authors for other pesticides. For example, Chen et al. (2001) showed that N mineralization rates were significantly higher in the fungicide captan-treated soils (field application rate) than in untreated soil as we found that the. Haney et al. (2002) reported that Roundup Ultra (composed of isopropylamine salt of glyphosate), applied at the recommended dose, significantly stimulated soil microbial activity as measured by C and N mineralization, as well as soil microbial biomass. In the same way, Burrows and Edwards (2000) reported that the fungicide carbendazim increased the rates of nitrification. Overall, the two fungicides, at high doses, induced increases of many microbial activities, except for the PHO activity which was the only one decreasing with increasing the copper fungicide, with and without earthworms. All these results, including ours, highlighted that, at doses from several fold to tens folds field rates, several fungicides can increase the microbial activity. One assumption is that the fungicides killed or inhibited the activity of certain groups of fungi. On short periods of time, dead fungal biomass might be used as food resources by the living microorganisms that, along with the decrease in the competition for other resources, can lead to a flush of bacterial activity. Another assumption is that the fungicides would kill or inhibit the soil micro-fauna, such as protozoa or nematodes which are predatory for microorganisms, thus turning off the top-down regulation of the microbial biomass and activity.

For copper, such a negative effect was expected for microbial activities, as the Cu^{2+} free ion is recognized as highly toxic for microorganisms, especially bacteria (Boyd et al., 2005; Giller and Witter, 2009). Phosphatase activity was found to be significantly decreased in soil treated with 150 and 450 mg Cu kg^{-1} and appeared more sensitive to Cu than β -glucosidase which was less affected (Wyszkowska et al., 2010). In our experiment, the β -D-glucosidase was not affected and even increases with increasing Cu concentrations, but only

in soils without earthworms. Similarly, the urease and arylamidase increased or remain stable under both fungicide pressures depending on the presence of earthworms. Wightwick et al. (2013) showed that Cu (hydroxide) did not impact urease activity even at 156 mg kg^{-1} of copper which supported our result which showed no change in the urease activity, even at the highest tested concentration (232.5 mg kg^{-1} of copper), with or without earthworms. However, it must be noted, that in numerous studies, Cu impacted soil microbial activities several soil microbial activities (extracellular enzymes or respiration or nitrification) (Sokhn et al., 2001; Burges et al., 2015). However, in these studies, Cu was spread as a salt in solution, whilst we used a Cu-oxide form in a commercial formulation comprising an unknown adjuvant. Different fate and bioavailability in soils of these Cu forms (Kiaune and Singhasemanon, 2011), could explain these different effects. In addition, it might be hypothesized that adjuvants present in the commercial formulation might act as an available source of N- and C-compounds for N-mineralizing microorganisms (Crouzet et al., 2010; Mijangos et al., 2010).

Concerning the Swing[®] Gold fungicide, Jastrzębska and Kucharski (2007), in an experiment with a commercial fungicide made of the same active substances (dimoxystrobin and epoxiconazole), showed negative effects of these fungicides on the activity of dehydrogenases and urease in soil, but only at 100-fold the recommended dose. These results and ours, underlying that fungicides based on a mixture of dimoxystrobin - epoxiconazole did not negatively affects microbial activities (i.e. phosphatase, β -D-glucosidase, urease and arylamidase activity) at realistic doses, and even increased the microbial activity.

Our study highlighted that the presence of earthworms increased all enzyme and nitrification activities, even in soils treated with fungicides. Overall, this result suggests that earthworm behavior was not significantly modified in SG or Cu treated soils, as earthworm effects on activities were similar in all soils. The stimulation of microbial activities by earthworms was already observed (Scheu 1987; Binet et al., 1998; Aira et al., 2003). As ecosystems engineers (Jones et al., 1994), earthworms improve the microbial activity through their casting and burrowing activity, in natural soils without contaminants. However, our study, along with that of Sanchez-Hernandez et al. (2018), is the first to show that this ability is preserved in soil treated with pesticides. This stronger tolerance of

microbial activity to pesticides provided by the presence of earthworms could be explained by the ecosystem engineer role of earthworms providing favorable micro-sites for microbial communities. In addition, to provide more resources for the development of microbial activity, earthworms can increase the degradation of pesticides. Indeed, Akhouri et al. (1997) showed that under conventional tillage, atrazine degradation was greater in the casts of earthworms than in the surrounding soil. Liu et al. (2011) also highlighted that the earthworms *Aporrectodea caliginosa* stimulated abundance and activity of phenoxyalkanoic acid herbicide degraders. Consequently, due to earthworm activity, microbial activity could be less exposed to pesticides and could have more resource available for their development.

In conclusion, the results of the present study highlighted (i) the suitability of the soil biological indexes to summarize the overall impact of pesticide on the microbial activity under laboratory conditions. Then, ii) the PNA was a very sensitive indicator of fungicide effect on soil mediated microbial process of N-cycling. Finally our results also highlighted iii) the importance of biotic interactions in the response of some proxies of soil functions (i.e. microbial activities) to pesticides. Indeed, earthworms mitigate pesticides effect on microbial activities.

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Principales conclusions de l'article

Dans cette étude, en l'absence de vers de terre, l'augmentation des concentrations des fongicides a entraîné une augmentation des activités microbiennes, ce qui n'a pas été le cas lors de la présence des vers de terre. De plus, les activités microbiennes responsables de la dégradation de la matière organique ont été significativement plus élevées lors de la présence des vers de terre dans les sols sans, mais aussi avec les deux fongicides testés. Il apparaît ainsi que les vers de terre sont capables i) d'augmenter les activités microbiennes, même dans un sol contaminé, mais également ii) d'atténuer les effets des pesticides sur celles-ci. Ainsi, pour maintenir un certain niveau de dégradation de la matière organique, la présence des vers de terre est essentielle.

Discussion générale et perspectives

Les différents résultats présentés dans cette thèse permettent de poser de premiers jalons pour aller vers une amélioration de l'évaluation du risque liée à l'utilisation des pesticides, avec (i) la proposition d'une espèce modèle de ver de terre et (ii) le développement d'un modèle bioénergétique associé à cette espèce, qui a pour vocation de servir à l'analyse de données écotoxicologiques. Les différents résultats expérimentaux ont apporté des connaissances nouvelles sur les effets de deux formulations commerciales de fongicides, largement utilisées en Europe, sur le cycle de vie d'*A. caliginosa*. Dans cette évaluation des effets, les différents stades de vie de l'espèce ont été pris en compte (cocons, juvéniles et adultes). De plus, nous avons analysé les effets sur la croissance à l'aide du modèle bioénergétique, couplé à un modèle toxicocinétique, permettant l'estimation de concentrations sans effet (CSE) et une compréhension mécanistique des effets. Il reste cependant encore des recherches à mener pour modéliser les effets sur l'ensemble du cycle de vie, à l'aide du modèle bioénergétique. Enfin, cette modélisation des effets au niveau individuel pourra servir à l'extrapolation au niveau de la population, à l'aide d'outils de modélisation. De plus, les résultats obtenus sur l'impact des vers de terre sur les activités microbiennes (responsables de la dégradation de la matière organique), en condition d'exposition aux pesticides, pourront servir de sorties fonctionnelles à un modèle de dynamique des populations.

I. Vers des améliorations en évaluation du risque

Le rapport de l'EFSA « Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms » (EFSA, 2017) a suggéré des critères de sélection pour les tests réalisés dans le cadre de l'évaluation du risque liée à l'utilisation des pesticides pour les invertébrés du sol. Les principaux sont la faisabilité, la reproductibilité et un coût financier acceptable. De plus, les tests doivent être écologiquement pertinents, c'est-à-dire être sensibles aux perturbations (avec une réponse des organismes) et présenter un réalisme écologique (en termes d'exposition). Enfin, les tests doivent être représentatifs de l'écosystème à protéger, ce qui inclut la pertinence des organismes utilisés (ils doivent être présents dans l'écosystème à protéger).

Les travaux de thèse présentés dans la première partie ont permis de mettre en évidence qu'*A. caliginosa* remplit plusieurs de ces critères. Tout d'abord, cette espèce est représentative des agroécosystèmes de toute la zone tempérée (Bart et al., 2018). Ensuite, la mise en place d'un élevage optimisé est possible et permet un approvisionnement suffisant en individus de tous les stades de vie de l'espèce (cocons, juvéniles et adultes), ce qui rend les tests faisables pour un coût abordable. Concernant la reproductibilité des tests, les critères sont encore à affiner, notamment concernant la variabilité inter-individuelle en fonction de l'origine des individus. En effet, la classification taxonomique d'*A. caliginosa* reste aujourd'hui discutée. Il existe un complexe d'espèces (i.e. un groupe d'espèce dans lequel la séparation exacte entre espèces est difficile ou cryptique), ce qui peut être un frein pour l'acceptation de l'espèce comme modèle. Cependant le même genre de considération existe actuellement pour beaucoup des vers de terre, connus pour présenter une grande plasticité phénotypique (Decaëns et al., 2013). C'est par exemple le cas pour l'espèce *Eisenia fetida* pour laquelle on distingue deux sous-espèces : *fetida* et *andrei* (Latif et al., 2017). Il apparaît ainsi que, dans un objectif de reproductibilité, il sera nécessaire de bien identifier l'espèce utilisée *via* les critères habituels (Sims et Gerard, 1999), ou *via* l'utilisation d'outil de biologie moléculaire, tel que le barcoding moléculaire (Decaëns et al., 2013). Une étape importante a été franchie pour faire progresser l'évaluation du risque puisque la proposition d'ajout d'une annexe a été déposée pour les trois normes internationales ISO 11268-1; 11268-2 et 17512-1. Cette annexe, déposée par Céline Pelosi, est actuellement en discussion et se base sur la synthèse bibliographique réalisée dans le cadre de cette thèse sur *A. caliginosa*. Elle propose ainsi l'utilisation de cette espèce pour la réalisation des tests, en expliquant les changements à apporter aux protocoles utilisés.

De plus, toujours dans un objectif de reproductibilité, l'utilisation d'un sol standardisé est nécessaire puisque les caractéristiques de celui-ci auront un effet direct sur la toxicité des substances pour les organismes (Amorim et al., 2005). Cependant, le sols artificiel OECD ou LUFA qui sont actuellement recommandés pour être utilisés avec *E. fetida* ne conviennent pas à *A. caliginosa* (Bart et al., 2018). Il pourrait donc s'avérer pertinent de proposer l'élaboration un sol standardisé de type limono-argileux, qui conviendrait pour des expérimentations avec *A. caliginosa* et d'autres espèces présentes en agroécosystèmes. Celui-ci devrait être testé dans le cadre d'un essai circulaire pour que l'espèce et les tests

associés soit acceptés comme références en évaluation du risque sur le compartiment « sol », et, plus généralement, en écotoxicologie terrestre.

Un critère important qui figure dans le rapport de l'EFSA (2017) est le développement de tests écologiquement pertinents. La possibilité d'une extrapolation dans le temps et/ou à des niveaux d'organisation supérieurs (population) serait un élément important de cette pertinence. C'est dans cet objectif que le modèle bioénergétique a été développé (Partie 1, chapitre 2). L'étude du cycle de vie a permis de comprendre le mode de gestion de l'énergie chez *A. caliginosa* et d'en déduire la modélisation adaptée permettant de faire le lien entre l'acquisition d'énergie (*via* la quantité de nourriture disponible) et les paramètres du cycle de vie (croissance et reproduction). Le cycle de vie peut ainsi être divisé en deux périodes. Au cours de la première période, l'essentiel de l'énergie fournie par la nourriture est investie dans la croissance. Puis, au cours de la seconde période, l'énergie est accaparée pour la fabrication des cocons, c'est-à-dire la fonction de reproduction. Il a été mis en évidence que la transition entre ces deux périodes pouvait être approchée en considérant une biomasse qui fait la transition entre le stade juvénile (période 1) et le stade adulte (période 2).

Le modèle proposé est suffisant pour évaluer les effets des pesticides au niveau individuel au laboratoire et estimer des valeurs seuils en écotoxicologie (les concentrations sans effets: CSE, utilisable en évaluation du risque), dans des conditions constantes de température et d'humidité du sol. Cependant, dans un objectif d'extrapolation au niveau de la population et/ou de se placer dans des conditions réalistes de terrain où ces deux paramètres (i.e. température et humidité) varient au cours du temps, il serait nécessaire d'améliorer notre modèle. La température et l'humidité sont connues pour influencer fortement le cycle de vie d'*A. caliginosa* (Eriksen-Hamel et Whalen, 2006) et des vers de terre en général (Berry et Jordan, 2001). Cette influence pourrait se traduire en termes de rapidité d'assimilation de la nourriture (paramètre a du modèle bioénergétique). Moreau-Valancogne et al. (2013) ont proposé un modèle décrivant l'influence de la température (Figure 1) et de l'humidité sur le développement et la croissance de plusieurs espèces de vers de terre dont *A. caliginosa*.

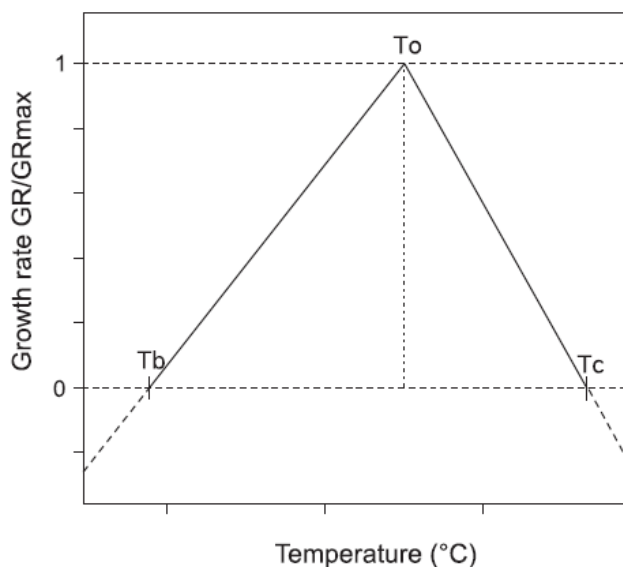


Figure 1. Modèle théorique proposé par Moreau-Valancogne et al. (2013) pour relier la croissance et la température (à humidité constante). T_o correspond à l'optimum de croissance, T_b et T_c correspondent aux limites inférieures et supérieures au-delà desquelles la croissance est nulle.

En se basant sur le modèle théorique proposé par ces auteurs, le paramètre a de notre modèle pourrait être multiplié par un facteur multiplicatif, de 0 à 1, qui correspondrait à l'influence de la température. Pour l'humidité, un modèle identique serait aussi envisageable, sur la base de la publication de Moreau-Valancogne et al. (2013).

II. Effets des pesticides sur le cycle de vie d'*A. caliginosa*

Dans les agroécosystèmes, les populations de vers de terre sont composées de cohortes d'individus de stades de vie différents. Par conséquent, des cocons, ainsi que des juvéniles et des adultes de tout âge coexistent dans les sols. Lors de l'application d'un pesticide au champ, celui-ci peut atteindre le sol et sa concentration sera amenée à évoluer au cours du temps en fonction des conditions pédoclimatiques et du pesticide considéré (Arias-Estévez et al., 2008). Ainsi, les vers de terre sont susceptibles d'être exposés à tous leurs stades de vie, et à des concentrations qui varient au cours du temps. Pour évaluer les effets sur les populations de vers de terre à partir d'expérimentation de laboratoire, il est donc important de prendre en compte ces deux aspects. C'est ce qui a été fait au travers des différentes expériences réalisées dans cette thèse, à commencer par la prise en compte de l'ensemble des stades de vie, comme illustré dans le cas du Swing® Gold (Figure 2).

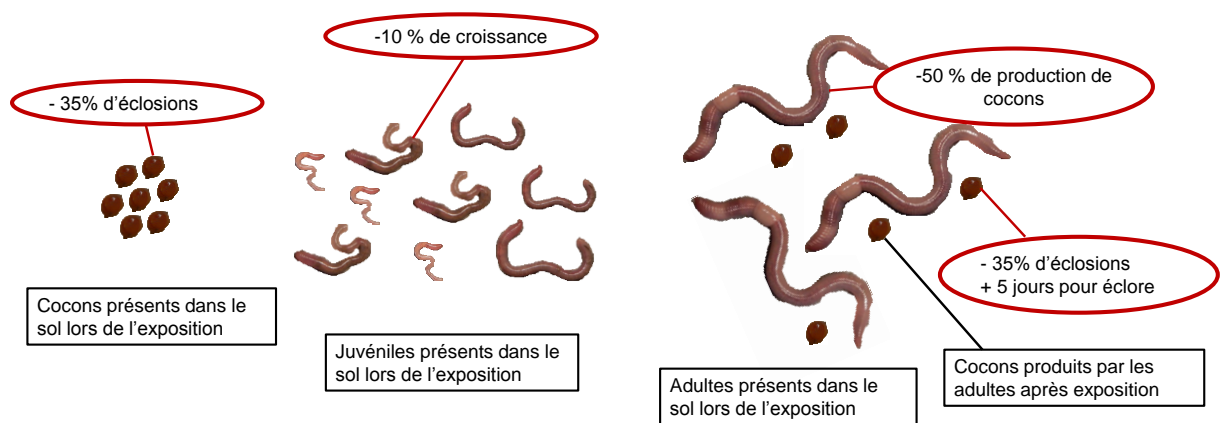


Figure 2. Effets du Swing® Gold à 3 fois la dose recommandée sur les différents stades de vie d'*A. caliginosa*.

Les différents stades de vie ont présenté des différences de sensibilité notables. Ainsi, la biomasse des individus exposés dans du sol contrôle et dans du sol traité au Swing® Gold à 3 fois la dose recommandée (DR) n'a pas été affectée. Cependant, la reproduction a été fortement affectée à cette dose, *via* une baisse conjointe de la production de cocons et de la qualité des cocons produits (i.e. réduction du pourcentage d'éclosion et augmentation du temps d'éclosion). La croissance des individus juvéniles exposés à cette même concentration a diminué (mesure de la biomasse) comparé au sol non contaminé. Ces résultats sont cohérents avec la gestion de l'énergie, telle que décryptée lors de la calibration du modèle bioénergétique. L'énergie est une cible du toxique, qui va affecter les fonctions où celle-ci est allouée prioritairement. Au stade adulte, l'énergie est dirigée prioritairement vers la reproduction (Partie 1, Chapitre 2), et c'est sur cette fonction que les effets ont été observés au stade adulte. A l'inverse, au stade juvénile, toute l'énergie est allouée à la croissance et c'est sur celle-ci que les effets ont été observés.

Les cocons « naifs » (i.e. produits par des individus non exposés) sont apparus plus résistants que les autres stades de vie (juvénile et adulte), puisque, par exemple, le cuivre n'a pas affecté leur succès ou temps d'éclosion, même après l'ajout de 232.5 mg Cu kg⁻¹. Cette absence de sensibilité peut s'expliquer par le fait que la pénétration des pesticides dans les cocons est certainement réduite comparé aux individus juvéniles et adultes qui ingèrent du sol et donc directement les pesticides. Cependant, nous n'avons pas mesuré les concentrations en pesticides dans les cocons et nous ne pouvons donc pas donner

davantage d'éléments sur ce point. De plus, il est à noter que, chez les vers de terre, les cocons constituent des formes de résistance face aux perturbations environnementales (e.g. inondation, sécheresse, variation de température) (Holmstrup et al., 1990). Ceci explique certainement en partie pourquoi l'exposition aux pesticides au stade cocon a moins de conséquences écotoxicologiques que l'exposition au stade adulte. Pourtant, Holmstrup et al. (1998) ont montré que les membranes des cocons étaient perméables au cuivre (Figure 3), pouvant donc être potentiellement affectés.

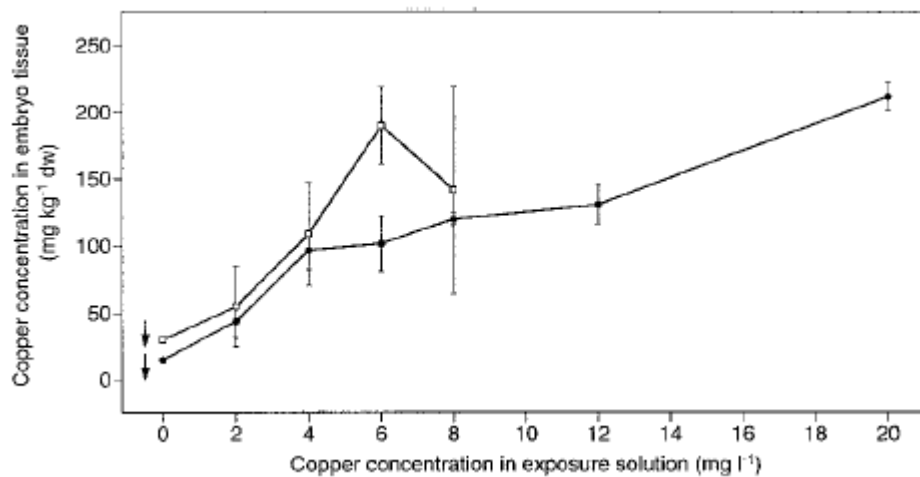


Figure 3. Concentration en cuivre dans les tissus d'embryon après 14 jours d'exposition dans des solutions aqueuses de $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ à différentes concentrations pour deux espèces de vers de terre. Point noirs : *A. caliginosa*, points blancs : *Dendrobaena octaedra*. (Source : Holmstrup et al., 1998).

Nous avons donc tenté d'exposer des cocons « naifs » dans une solution d'eau contaminée (semi-immersion) avec le fongicide cuivré à différentes concentrations (Annexe 3). Les doses recommandées du sol ont été converties en mg L^{-1} de cuivre, en considérant que le cuivre était 100% en solution (en prenant en compte 80% de la CRE de notre sol). Nous avons constaté une réduction du taux d'éclosion de 46% (effets significatifs) et une augmentation du temps d'éclosion de 10 jours (effets significatifs) à partir de 475 mg L^{-1} de cuivre (Figure 4).

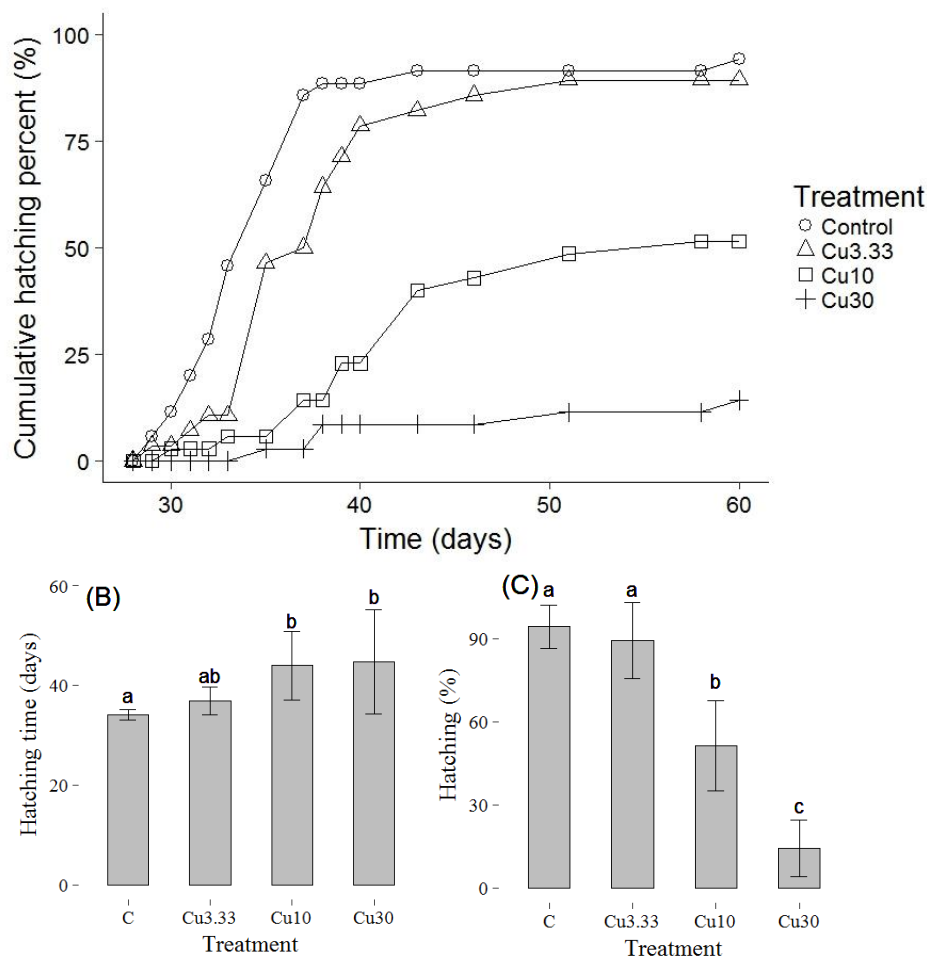


Figure 4. Dynamique d'éclosion (A) des cocons d'*A. caliginosa* produits sur une période de 7 jours dans un sol non contaminé et exposés à des solutions aqueuses contaminées par différentes concentrations de Cuprafor micro® (3.33, 10 and 30 fois la dose recommandée, correspondant à 157, 475 et 1567 mg L⁻¹ de cuivre abrégés Cu3.3, Cu10 et Cu30 respectivement)

Il est à noter cependant que les concentrations testées n'étaient pas représentatives des concentrations en cuivre présentes dans une solution de sol contaminé en cuivre. En effet, dans un sol de vigne contaminé en cuivre à une concentration de 118 mg kg⁻¹, Navel (2011) a mesuré une concentration de cuivre dans la solution du sol de 0.4 mg L⁻¹. La disponibilité environnementale du cuivre, qui correspond à la quantité de contaminant présent dans le sol pouvant être libérée de la phase solide vers la phase aqueuse, est donc bien inférieure aux concentrations totales du sol. Ces faibles concentrations en cuivre pourraient expliquer la résistance des cocons dans les sols contaminés en cuivre.

Concernant le Swing® Gold, des effets ont été observés pour les cocons exposés dans le sol contaminé au Swing gold à 3 fois la DR. À cette concentration, la disponibilité environnementale de la dimoxystrobine (DMX) et de l'époxiconazole (EPX) est de 0.12 et 0.089 mg kg⁻¹ respectivement ce qui correspond à 15-20% de la concentration totale du sol

pour la DMX et 30-40% pour l'EPX. Ces substances sont donc très mobiles et présentes dans la solution du sol, ce qui suggère qu'un transfert dans les cocons est possible et cela expliquerait les effets observés. Aucune information n'est disponible dans la littérature scientifique sur l'accumulation dans les cocons de pesticides organiques, tels que la DMX ou l'EPX (i.e. les substances actives du Swing® Gold).

De la même manière que pour le cuivre, nous avons tenté d'exposer des cocons « naifs » dans des solutions contaminées avec le Swing® Gold. Les concentrations des solutions ont été calculées de la même manière que pour le cuivre et correspondaient à 0.156, 0.474 et 1.421 mg L⁻¹ de DMX et 0.059, 0.18 et 0.53 mg L⁻¹ d'EPX. Après 70 jours d'exposition, aucun cocon n'a éclos quelle que soit la concentration testée, et l'expérimentation a donc été arrêtée. La concentration la plus faible (0.156 mg L⁻¹ de DMX et 0.059 mg L⁻¹ d'EPX) est proche de la disponibilité environnementale à 3 fois la DR (i.e. 0.12 mg kg⁻¹ de DMX et 0.089 mg kg⁻¹ d'EPX), pour laquelle des effets sur le taux et temps d'éclosion ont été observés. Il semble donc pertinent de considérer la disponibilité environnementale comme concentration d'exposition. Afin d'évaluer plus finement les effets du Swing® Gold, il serait nécessaire de (i) tester des concentrations inférieures en solution pour déterminer à partir de quel seuil des effets apparaissent mais aussi (ii) de vérifier s'il existe bien un transfert des substances actives dans les cocons, qui expliquerait les effets observés.

Les données obtenues dans cette thèse sur les cocons n'ont pas été analysées à l'aide d'outils de modélisation. L'utilisation d'un modèle toxicocinétique-toxicodynamique (TK-TD) est à envisager pour faire le lien entre effets observés (en termes de temps et taux d'éclosion) et la cinétique du contaminant dans la solution du sol, qui semble être la principale voie d'exposition des cocons. Concernant le Swing® Gold, les concentrations totales et la disponibilité environnementale sont corrélées (cf Partie 2, Chapitre 3), l'utilisation des concentrations totales serait donc envisageable dans le cadre d'une modélisation toxicocinétique. Pour le cuivre, il serait nécessaire de vérifier que la concentration dans la solution de sol est corrélée à la concentration totale du sol, ce qui est souvent le cas pour un sol donné à humidité et température constante (Navel, 2011). L'acquisition de données complémentaires est cependant nécessaire (tester des concentrations d'exposition plus faibles et plus resserrées pour pouvoir mieux analyser les effets), pour aboutir à un modèle robuste permettant de décrire les effets et de fournir des valeurs seuils de toxicité pour les cocons.

Les analyses des données de toxicité à l'aide d'outils de modélisation, permettant de décrire les effets au cours du temps et d'estimer des concentrations sans effet (CSE), mériteraient aussi d'être utilisées pour analyser l'effet des pesticides sur la reproduction. Cependant, contrairement à la croissance, les expérimentations sur la reproduction n'ont pas permis d'utiliser le modèle bioénergétique pour analyser les données puisque les expérimentations réalisées n'ont pas fourni de séries temporelles de données. Il aurait pour cela été nécessaire de suivre la production des cocons au cours du temps, afin de relier les effets (la baisse de la production de cocons) à un modèle toxicocinétique de l'accumulation des pesticides chez les individus adultes.

Des expérimentations restent ainsi à mener pour pouvoir modéliser les effets des pesticides sur l'ensemble du cycle de vie suite à une exposition aux pesticides en s'intéressant en premier lieu à la reproduction. Cette modélisation des effets des pesticides sur l'ensemble des stades de développement faciliterait ensuite l'extrapolation des effets au niveau de la population *via* un modèle de dynamique des populations.

III. Vers l'extrapolation au niveau de la population et le lien avec le fonctionnement du sol

La cible des pesticides est l'organisme, et les effets observés au niveau des populations sont les conséquences des effets sur les organismes (Lin et al., 2005). Il est donc possible, à partir de données obtenus au laboratoire, au niveau des organismes, d'extrapoler les effets des pesticides au niveau de la population. C'est l'objectif envisagé au-delà de ce travail de thèse.

Parmi les familles de modèles de changement d'échelle de l'individu à la population, les modèles individu-centrés (IBM: Individual Based Model) sont à ce jour les plus pertinents pour expliciter une dynamique de population à partir d'informations obtenues au niveau individuel. L'approche individu-centrée a pour objectif de faire émerger les propriétés d'un système (population, communauté) à partir de la représentation des propriétés des éléments le constituant (les individus) et de leurs interactions (Grimm, 1999; Lomnicki, 1999). Les individus sont donc considérés comme des entités uniques et discrètes possédant

des caractéristiques propres qui changent au cours de leur cycle de vie (Grimm et Railsback, 2005). Ainsi, dans le cadre de ces modèles, la dynamique de population émerge des comportements des individus et des interactions entre eux et l'environnement (dans notre cas, le sol). La connaissance des effets des pesticides au niveau de l'individu peut donc permettre, grâce à ces modèles, d'envisager les effets au niveau populationnel et ainsi de lier ces deux niveaux d'organisation.

L'utilisation d'un modèle bioénergétique de type DEB est une base solide pour être utilisé dans le cadre d'une modélisation individu-centrée. Le couplage DEB-IBM permet d'aboutir à des modèles réalistes qui peuvent servir à prédire les effets de toxiques sur les populations, à partir des données obtenus au niveau individuel (Johnston et al., 2014; Beaudouin et al., 2015). Le modèle bioénergétique que nous avons développé pourrait être utilisé pour la mise en place d'un modèle de dynamique des populations de type IBM. Dans cet objectif, la première étape consisterait à pouvoir simuler une dynamique de population en condition contrôle et de valider le modèle avec un jeu de données externes, obtenues par exemple lors d'expérimentations en mésocosmes. Ceux-ci, placés en extérieurs, pourraient correspondre à des bacs (de quelques mètres carrés et quelques dizaines de centimètres de profondeurs), remplis d'un sol défauné qui serait inoculé à l'aide de cocons (provenant d'un élevage d'*A. caliginosa* par exemple). Un apport de matière organique serait appliqué régulièrement comme apport de nourriture et incorporé dans le sol. Des prélèvements réguliers (4 fois par an par exemple), permettraient de suivre l'évolution des populations. Ainsi, pour simuler une dynamique de population en condition contrôle, des expérimentations et calibrage de modèles sont encore nécessaires.

Dans un second temps, il s'agirait d'extrapoler les effets des pesticides au niveau de la population. De nombreuses données produites dans cette thèse sont déjà utilisables, tel que les analyses réalisées sur la croissance (Partie 2, Chapitre 3) et les tests d'évitement (Partie 2, chapitre 1). Les tests d'évitement sont en effet essentiels, puisque dans le cadre d'une modélisation IBM, les individus interagissent avec l'environnement et la présence de pesticides dans les sols en fait partie. Ainsi, l'évitement d'une zone contaminée entraînera des mouvements d'individus (migration) dans le modèle, et les individus ne subiront pas (ou moins longtemps) les effets des pesticides. La modélisation de l'effet des pesticides sur la reproduction et sur les cocons, comme discuté dans la partie précédente, reste à développer

pour pouvoir extrapoler les effets des pesticides au niveau individuel sur l'ensemble du cycle de vie, puis au niveau de la population.

Un aspect important de l'évaluation du risque est de préserver le fonctionnement de l'écosystème et notamment les fonctions écologiques du sol, assurant la durabilité des productions agricoles. Dans le cadre de l'utilisation des pesticides, un enjeu majeur est ainsi de caractériser les effets sur les fonctions écologiques du sol remplies par les vers de terre, telles que la dégradation de la matière organique et la structuration physique du sol. Ainsi, il serait pertinent de pouvoir incorporer dans un modèle de dynamique de population, de type DEB-IBM, des modules prenant en compte ces aspects fonctionnels. Les expérimentations du chapitre 4 de la partie 2 pourraient soutenir cette incorporation. Les résultats ont permis de faire le lien entre la présence de vers de terre et la minéralisation de la matière organique par les microorganismes.

Une approche simple pour ajouter une sortie fonctionnelle au modèle de dynamique des populations serait de prendre en compte une relation quantitative entre l'abondance des vers de terre et l'activité des microorganismes. Une sortie pertinente serait par exemple un indice de type Gmean (regroupant différentes activités enzymatiques du sol). Dans cette hypothèse, l'effet des pesticides sur les individus (croissance reproduction) se traduirait par une baisse des abondances au cours des générations et *in fine* par une baisse de l'indice Gmean dans les sorties du modèle.

La structuration physique du sol par les vers de terre est une autre fonction écologique clé qu'il serait intéressant d'incorporer dans un modèle DEB-IBM. Pour se faire, des pistes intéressantes existent dans la littérature, avec notamment le modèle « SWORM » (pour Simulated Worms) développé par Blanchart et al. (2009), qui décrit et quantifie l'effet des vers de terre sur l'agrégation et la porosité du sol. Leur modèle, de type agent-centré, considère que les vers de terre sont les agents et que leurs déplacements modifient les propriétés du sol en termes de structure et de dynamique des matières organiques. Ce type de modèle serait une base pertinente pour amender un modèle DEB-IBM dans lequel l'abondance des vers de terre serait corrélée à ces fonctions.

Pour conclure, la recherche fait face à de grands défis pour améliorer l'évaluation du risque liée à l'utilisation de pesticides. La modélisation est désormais un outil incontournable

pour : (i) analyser des données écotoxicologiques et fournir des valeurs seuils (les concentrations sans effet : CSE), (ii) extrapoler les effets sur les individus à des niveaux d'organisations supérieurs (population, communauté), mais aussi (iii) faire le lien avec les fonctions écologiques fournies par ces ingénieurs de l'écosystème. Pour répondre aux défis auxquels fait face l'évaluation du risque, la modélisation individu-centrée est la piste à privilégier puisque ces modèles présentent un grand réalisme. Cependant ils nécessitent de nombreuses données, qui ne peuvent être acquises que par des expérimentations adaptées. Il est donc nécessaire de maintenir un effort expérimental pour la production de données écotoxicologique. Ces expérimentations doivent être pensées pour répondre aux objectifs des modèles, ce qui sous-entend d'évaluer les effets au cours du temps et de prendre en compte la dynamique des pesticides dans les sols.

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Annexes

Annexe 1 : Article 7. Effets des pesticides sur la reproduction et l'activité alimentaire des enchytréides

Cette annexe correspond à un article intitulé «How to assess the feeding activity in ecotoxicological laboratory tests using enchytraeids? » publié dans la revue *Environmental Science and Pollution Research* (short communication). <https://doi.org/10.1007/s11356-018-1701-3>

Les enchytréides sont, au même titre que les vers de terre, des annélides oligochètes. Taxonomiquement très proches, ces organismes du sol sont également impliqués dans des fonctions similaires, à des échelles différentes. (Dégradation de la matière organique, structuration physique du sol, ..). Ces organismes sont moins sensibles aux facteurs du milieu et aux activités anthropiques que les vers de terre, les enchytréides pourraient donc constituer un relai fonctionnel dans les milieux où les vers de terre sont moins présents ou absents (Pelosi et al., 2016c). Au début de ma thèse, il était question de travailler en parallèle sur les enchytréides et les vers de terre. Il est cependant rapidement apparu que le développement d'un modèle de type DEB sur les enchytréides serait trop chronophage et qu'il ne serait pas possible de travailler sur les deux groupes simultanément. En effet, les enchytréides sont plus petits et les mesures de poids ou de taille sur ceux-ci sont très délicates. Or, ces données sont nécessaires à la mise en place d'un modèle DEB. Il a donc été préférable de renoncer à développer un modèle sur les enchytréides pour se focaliser sur les vers de terre. Différents travaux avaient cependant été initiés sur ces organismes, comme illustré dans le chapitre 1 de la Partie 2. Ces travaux avaient pour objectif de répondre aux questions suivantes:

- (1) Existe-il une différence de sensibilité aux pesticides entre les enchytréides et les vers de terre en ce qui concerne la reproduction?
- (2) Si les enchytréides sont de potentiels relais fonctionnels, les fonctions écologiques fournies par ces organismes peuvent-elles être affectées par les pesticides?

Pour répondre à la question (1), il m'a été donné l'opportunité d'encadrer un étudiant de BTS (Alexis Vincent). Je lui ai confié la réalisation des tests de reproduction des enchytréides à l'automne 2016, sous ma supervision. Ces essais furent infructueux, principalement en

raison d'une mauvaise humidification des cosmes, mais riches d'enseignement pour un potentiel second essai. En parallèle de ces essais, et afin de répondre à la question (2), j'ai travaillé à la mise en place d'un protocole en laboratoire pour évaluer l'effet des pesticides sur l'activité alimentaire des enchytréides *via* l'utilisation des bait-lamina (mesure indirecte d'une fonction écologique: la dégradation de la matière organique).

Sur la base des protocoles expérimentaux rédigés, j'ai recruté et encadré un stagiaire de Master 1, Sacha Roudine, pour mener ces deux expérimentations. Ces travaux font l'objet des résultats présentés dans cette annexe.

Résumé : L'évaluation des risques liés à l'utilisation des pesticides sur la faune du sol est réglementée et nécessaire pour protéger la durabilité des agroécosystèmes. Les enchytréides sont reconnus comme des bioindicateurs du stress chimique dans ces systèmes. En conditions de laboratoire, le test de reproduction s'est avéré sensible pour évaluer l'effet des pesticides sur ces organismes. Cependant, il ne permet pas d'évaluer les effets sur les fonctions écologiques dans lesquelles les enchytréides sont impliqués. L'objectif de cette étude était (i) d'explorer la faisabilité du test bait-lamina avec des enchytréides dans des conditions de laboratoire, et (ii) de comparer sa sensibilité avec le test de reproduction. Des individus de l'espèce *Enchytraeus albidus* ont été exposés à deux formulations commerciales de fongicides largement utilisés en Europe. L'EC50 reproduction pour le Swing® Gold (50 g L⁻¹ époxiconazole et 133 g L⁻¹ dimoxystrobine) et le Cuprafor micro® (oxychlorure de cuivre 50%) ont été respectivement estimées à 1,66 ± 0,93 fois la dose recommandée et > 496 mg kg⁻¹ de cuivre. Cependant, aucun effet n'a été mis en évidence sur l'activité alimentaire des enchytréides et le test bait-lamina s'est donc révélé moins sensible que le test de reproduction pour les fongicides testés. Malgré cela, cette étude a mis en évidence que le test bait-lamina était réalisable dans des conditions de laboratoire. Rapide, facile à mettre en œuvre et bon marché, ce test est un outil intéressant pour évaluer l'effet des pesticides sur l'activité alimentaire des organismes du sol. Cependant, il mériterait d'être amélioré pour pouvoir être utilisé dans le cadre d'une exposition plus longue, *via* l'addition répétée de bait-lamina, afin d'augmenter sa sensibilité.

How to assess the feeding activity in ecotoxicological laboratory tests using enchytraeids?

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Abstract

The risk assessment of pesticides on soil fauna is an issue to protect agroecosystem sustainability. Enchytraeids are recognized as relevant soil bioindicators of chemical stress in agroecosystems. In laboratory, the reproduction test was found to be sensitive to reveal chemical impacts on enchytraeids. However, it does not allow to assess the impacts on ecological functions in which enchytraeids are involved. The objective of this study was (i) to explore the feasibility of the bait-lamina test with enchytraeids under laboratory conditions, and (ii) to compare its sensitivity with the Enchytraeid Reproduction Test. We exposed individuals of *Enchytraeus albidus* to two commercial formulations of fungicides widely used in Europe. The EC50 reproduction for the Swing[®] Gold (50 g L⁻¹ epoxiconazole and 133 g L⁻¹ dimoxystrobin) and the Cuprafor micro[®] (50% copper oxychloride) were respectively estimated at 1.66 ± 0.93 times the recommended dose and $> 496 \text{ mg kg}^{-1}$ of copper. However, no impact was found on the feeding activity of enchytraeids. The bait-lamina test thus appeared less sensitive than the Enchytraeid Reproduction Test to the tested fungicides. Despite that, this test which is achievable under laboratory conditions and allows assessing indirect effects of pesticides, is quick, cheap and easy to perform. It would deserve to be used to explore longer-exposure effects through the repeated addition of bait-lamina sticks.

Keywords: *Enchytraeus albidus*, Bait-lamina test, soil ecotoxicology, Pesticide, Risk assessment

Introduction

Since many decades, the massive use of pesticides in agroecosystems has led to a decrease in biodiversity and soil biological activity (Tilman et al. 2001; Hole et al. 2005). Enchytraeids are soil annelid Oligochaeta involved in the soil structure dynamics, organic matter decomposition and nutrient cycling (Didden, 1990; Maraldo et al. 2011). They are recognized as relevant indicators of soil biological activity, anthropic activities and physicochemical stresses in terrestrial ecosystems (Didden and Römcke 2001; Jänsch et al. 2005; Pelosi and Römcke 2016). Despite their ecological importance and their use in standardized tests (ISO 16387 2014; OECD 220 2016), the toxicity of pesticides to enchytraeids remains poorly documented (Römcke et al. 2017). The assessment of chemical impacts on mortality and reproduction can provide key information on population dynamics that determine the provision of ecological functions. For enchytraeids, reproduction is currently recognized as the most sensitive endpoint (Römcke et al. 2017). In the same time, the effects of pesticides on the ability of enchytraeids to degrade soil organic matter are currently unknown. The bait-lamina test can be used to assess the impact of soil contaminants on the feeding activity of soil dwelling organisms *in situ* (ISO 18311 2016). Kratz (1998) highlighted the potential of such test in ecotoxicological laboratory experiments. The feeding activity could be indirectly linked to an ecological function of soil organisms: the organic matter degradation.

Helling et al. (1998) assessed the feeding activity of enchytraeids, collembolans and microorganisms with the bait-lamina test in a laboratory experiment and highlighted the high feeding rate of enchytraeids and collembolans compared with the feeding rate of microorganisms. However, as far as we know from the scientific literature, this method has never been used to measure the consequences of pesticide use on the feeding activity by enchytraeids. We thus tested the effects of two commercial formulations of fungicides on the enchytraeid species *Enchytraeus albidus* (Henle, 1837) in a natural soil in order (i) to assess the feasibility of the bait-lamina test under laboratory conditions as presented in Helling et al. (1998) and (ii) to compare the sensitivity of this test with the Enchytraeid Reproduction Test (ERT).

Material and methods

Soil and pesticides

For a better representativeness of field conditions, a natural soil was preferred over an artificial soil for bait-lamina and reproduction tests. The soil was a Luvisol (FAO soil classification) sampled in a permanent grassland in Versailles, France (48°48'31''N, 2°05'26''E). The grassland has not been treated with pesticides for more than 20 years. The soil was collected from the soil layer 0–20 cm, air-dried, sieved at 2 mm and frozen at -80°C for 48h to exterminate soil macro-meso invertebrates. The main soil physical and chemical properties were as follows ($n=7 \pm SE$): pH: 7.5 ± 0.2 , soil bulk density: 1.29; organic matter content: $32.6 \pm 1.7 \text{ g kg}^{-1}$, C/N ratio: 12.7 ± 0.3 , sand: $29\% \pm 1.3$, silt: $48\% \pm 1.5$, and clay: $23\% \pm 0.6$ (see Bart et al. 2017 for more details).

We used two commercial formulations of fungicides. The first one was the Swing[®] Gold (BASF Agro SAS, composed of dimoxystrobin at 133 g L^{-1} and epoxiconazole at 50 g L^{-1}) with the French RD at 1.5 L ha^{-1} for wheat crop (E-phy 2017b). Taking into account the same soil bulk density as earlier and the main concentration of this pesticide in the top 10 cm of the soil (McDonald et al. 2013; Chabauty et al. 2016), the Swing[®] Gold at the RD was calculated as $1.16 \cdot 10^{-3} \text{ mL kg}^{-1}$ of the dry soil. As Bart et al. (2017) estimated a LC50 for this fungicide of 11 times the RD for *E. albidus*, the following sublethal concentrations were used in both experiments: 1, 3 and 9 times the RD, named as SG1, SG3 and SG9, respectively. The second fungicide was the Cuprafor micro[®] (Quimicas del Valles, 50% copper oxychloride) with the French Recommended Dose (RD) at 10 kg ha^{-1} for potato crops and vineyards (E-phy 2017a). Taking into account the soil bulk density (1.29) and the main concentration of copper in the top 5 cm of the soil (Couto et al. 2015), the Cuprafor micro[®] at the RD was calculated as 15.5 mg kg^{-1} of the dry soil which correspond to 7.75 mg kg^{-1} of copper. In an OECD artificial soil, Amorim et al. (2005) estimated the LC50 for *E. albidus* to be $> 320 \text{ mg kg}^{-1}$ of copper. Based on this study, the following sublethal concentrations were used for both bait-lamina and reproduction experiments: 16, 32 and 64 times the RD named as C16, C32 and C64, which correspond to 124, 248 and 496 mg kg^{-1} of copper, respectively. The dry soil was spiked with aqueous solutions of the fungicides, and the soil moisture was concomitantly adjusted to 75% of the water holding capacity.

Reproduction test

The reproduction test was conducted according to the standard ISO 16387 (2014), but by using the previously described natural soil. Ten mature individuals collected from a laboratory culture (INRA Versailles-Grignon) were introduced in one vessel (75 mL) containing 20 g of soil (dry mass equivalent). Soil moisture content was checked and adjusted weekly with tap water. For food supply, 50 mg of finely rolled oats were mixed with the soil at the beginning of the experiment and 25 mg were then added every week until the fourth week. Five replicates per treatment were performed. The vessels were stored in a climate room ($18 \pm 0.5^\circ\text{C}$, 24h darkness). After three weeks, adults were removed from the soil using a binocular microscope and the mortality was assessed. After six weeks, juveniles were fixed with ethanol (99.8% v/v) and colored with 300 μL of Bengal red (1% solution in ethanol). This procedure ensures the observation and counting of juvenile enchytraeids using a binocular microscope (ISO 16387 2014).

Bait-lamina test

The feeding activity of enchytraeids was assessed with the bait-lamina test (ISO 18311 2016) adapted for laboratory conditions (Helling et al. 1998). We used PVC sticks (18 cm long, 6 mm width and 1.5 mm thick) purchased from Terra Protecta (GmbH, Berlin, Germany). A stick contained 16 apertures which were 5 mm apart from each other (Fig. 1). As presented in the ISO guideline 18311 (2016), apertures were filled with a mixture of cellulose (70%), crushed wheat bran and sieved at 500 μm (25%) and activated charcoal carbon (5%).

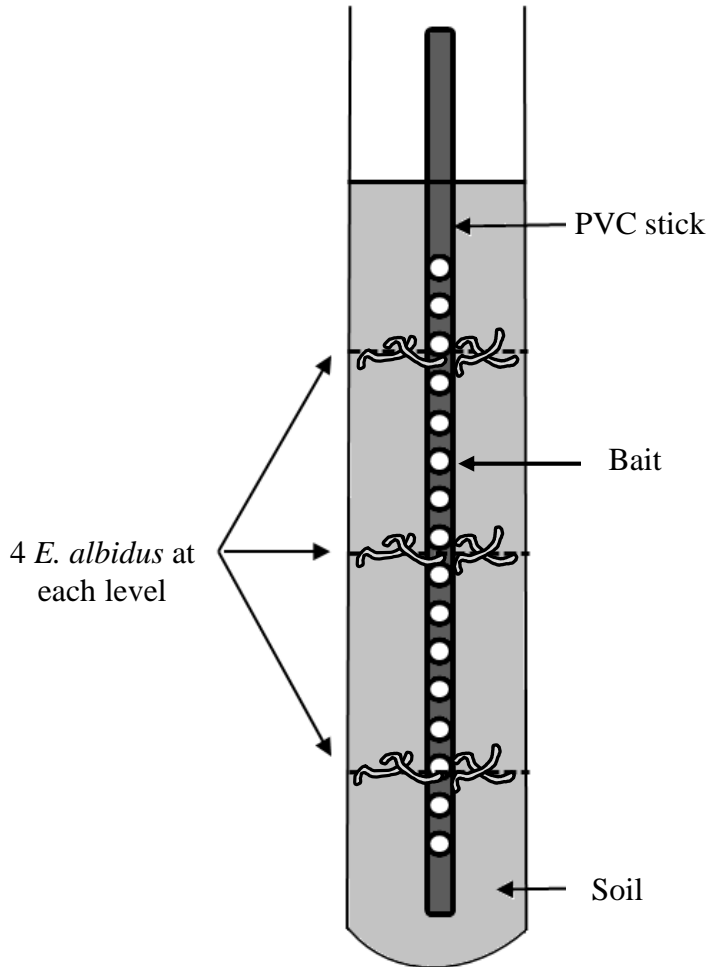


Fig. 1 Illustration of the experimental device using a glass tube filled with soil, PVC bait-lamina stick and 12 mature *Enchytraeus albidus*.

Twelve mature *E. albidus* from a laboratory culture (INRA Versailles-Grignon) were inoculated (corresponding to a density of 21 000 individuals m^{-2}) in a glass tube (20 cm high, 3 cm in diameter) with 50 g of dry soil adjusted at 75% of the water holding capacity. The tubes were filled with 4 layers of soil successively allowing a vertical distribution of enchytraeids in the tube at 3 levels (4 individuals per level) as suggested by Helling et al. (1998) (Fig. 1). Five replicates were used per treatment, including a control. One stick was added in each tube. After five days of exposure in a climate room (18 ± 0.5 °C, 24 h darkness), the sticks were removed from the soil and fed (i.e., disappearance of at least half of the organic bait material) and not fed apertures were directly counted according to the ISO 18311 (2016). The number of fed apertures was used to calculate the feeding rate expressed as bait consumed (%) per stick. The mortality was assessed as in the ERT.

Statistical analysis

All data were analyzed with the statistical software R Core Team. The R package “ezec” (Zhian N Kamvar 2016) was used to estimate the effective concentration that induced 50% decrease of the reproduction (EC50 reproduction). Because normality and homoscedasticity conditions were not satisfied (tested by the Shapiro–Wilk test), non-parametric Wilcoxon tests were used to compare the control treatment to those treated with pesticides in both experiments. A Kruskal-Wallis test was used to analyze the vertical distribution of bait consumption to test that the feeding activity was homogeneous along the profile.

Results and discussion

The reproduction test

In the control treatment, no enchytraeid mortality was observed and we found on average 68.2 juveniles (Fig. 2). In SG1 and SG3 treatments, corresponding to 1 and 3 times the RD of Swing® Gold, no mortality was recorded. However, the number of juveniles was significantly lower in the SG3 treatment (1.4 juveniles on average) than in control treatments (Figure 1A). In the SG9 treatment, a mortality of 90% was recorded and no juveniles were found (Fig. 2 A). This mortality rate is higher than expected as Bart et al. (2017) estimated a LC50 of 11 times the RD after 14 days in the same soil and with the same commercial formulation of fungicide. This could be explained by the incubation temperature which was higher in the present study (18°C, close to the $20 \pm 2^\circ\text{C}$ recommended by the ISO 16387, 2014) compared to the study of Bart et al. 2017 (i.e. 15°C). For instance, De Silva et al. (2009) showed that “the effects of pesticides (chlorpyrifos and carbofuran) on the earthworm *Eisenia andrei* survival, growth and reproduction may be higher at higher temperature”. Actually, a higher temperature may have increased the environmental bioavailability of pesticides (Harmsen, 2007) and thus their toxicity towards animals. The high mortality rate could explain the absence of reproduction in the SG9 treatment. Finally, the EC50 reproduction was estimated at 1.66 ± 0.93 times the RD. In the EFSA Scientific Report 46, 1-82 (2005), a NOEC value in a reproduction test with *Eisenia fetida* was estimated at $<0.0887 \text{ mg kg}^{-1}$ for the dimoxystrobin in a formulation such as Swing® Gold, which would correspond

to 0.6 times the RD in our experimental conditions. Our results on enchytraeids strongly support the conclusion that this fungicide is harmful to soil oligochaete reproduction.

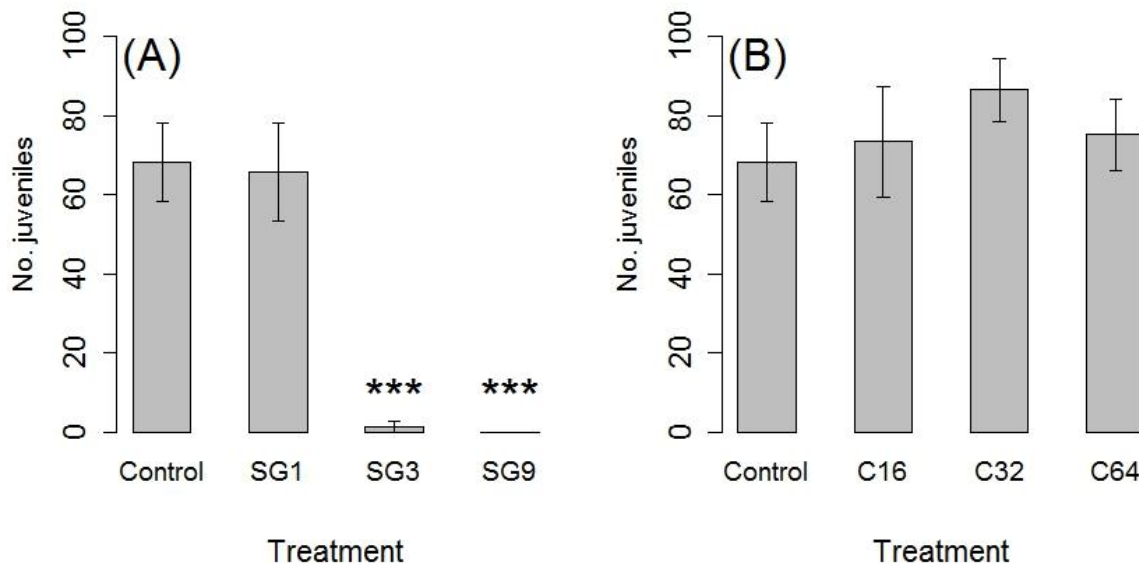


Fig. 2 Juveniles produced by 10 mature *Enchytraeus albidus* exposed for 21 days in a control soil and in soils contaminated with Swing Gold® (A) at 1, 3 and 9 times the recommended dose (RD) (abbreviated SG1, SG3 and SG9 respectively), and in soils contaminated with Cuprafor micro® (B) at 16, 32 and 64 times the RD (abbreviated C16, C32 and C64 respectively) which corresponded to 124, 248 and 496 mg kg⁻¹ of copper. Results are expressed in number of juveniles (n=5 ± SE). *** means significant differences between treatments and the control at p-value < 0.001.

Whatever the concentration of the copper fungicide tested, no mortality was observed and the reproduction of *E. albidus* was not significantly different between treatments (Fig. 2 B). The EC50 reproduction was > 496 mg kg⁻¹ of copper. This value is in accordance with Amorim et al. (2005) who found for *Enchytraeus albidus* an EC50 reproduction value > 320 mg kg⁻¹ with copper chloride in an OECD standardized soil. At the same time, Amorim and Scott-Fordsmand (2012) reported an EC50 reproduction value of 251 mg kg⁻¹ with copper-salt (CuCl₂) in a natural sandy soil. Finally, Amorim et al. (2005) found an EC50 reproduction value of 97 mg kg⁻¹ with copper chloride in a LUFA 2.2 standard sandy soil (Speyer, Germany). Thus, copper ecotoxicity towards enchytraeid's reproduction appears to depend on the copper species and the soil used in the experiments that once again drive the bioavailability of contaminants to living organisms. The high soil organic matter content in the soil of our experiment (i.e. 32.6 g kg⁻¹) may have decreased the copper bioavailability (Ma and Rao 1997) for enchytraeids and thus alleviate its ecotoxicological effects.

Bait-lamina test

In the same line as the ERT performed with the Swing[®] Gold, a mortality rate of 92% and no feeding activity was observed in the SG9 treatment (Fig. 3 A). However, no impact of the Swing[®] Gold at 3 RD (SG3 treatment) was found on the feeding activity compared with the control. The feeding activity of adult enchytraeids was thus not affected in a short-term by the Swing[®] Gold fungicide.

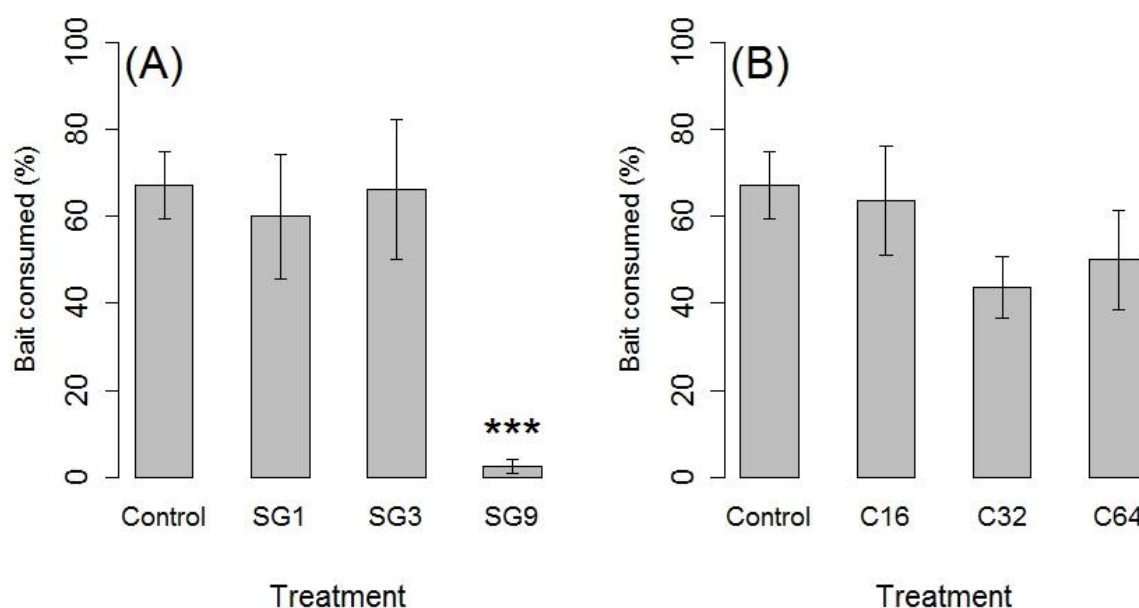


Fig. 3 Average feeding activity of 12 mature *Enchytraeus albidus* exposed for 5 days in a control soil and in soils contaminated with Swing Gold[®] (A) at 1, 3 and 9 times the recommended dose (RD) (abbreviate SG1, SG3 and SG9 respectively), and in soils contaminated with Cuprafor micro[®] (B) at 16, 32 and 64 times the RD, abbreviate C16, C32 and C64 respectively which correspond to 124, 248 and 496 mg kg⁻¹ of copper. Results are expressed in bait consumed (%). (n=5 ± SE). *** means significant differences between treatments and the control at p-value < 0.001.

The Cuprafo micro[®] fungicide induced no mortality and no impact on the feeding activity was recorded whatever the concentration tested (i.e., 16, 32 and 64 times the RD corresponding to 124, 248 and 496 mg kg⁻¹ of copper respectively) (Fig. 3 B). As for the reproduction test, the relatively high soil organic matter content may have decreased the copper bioavailability to enchytraeids, thus explaining why the feeding activity was not affected.

Feasibility, sensitivity and ecological relevance of the bait-lamina test under laboratory conditions

Larink (1994) had proposed the bait lamina test as a tool for testing the feeding activity of animals in contaminated soils under natural conditions. After several other studies (e.g., Andre et al. 2009; Filzek et al. 2004; Kula and Römbke 1998), a guideline was published by the International Standardization Organization (ISO) and recently updated (ISO 18311, 2016). We tested this method under laboratory conditions and the statistical analysis performed revealed that the feeding activity of enchytraeids, through the bait consumption, was homogeneous along the soil profile in the glass tube. This result validated the experimental design (i.e., vertical distribution of enchytraeids at 3 levels with 4 individuals per level) proposed by Helling et al. (1998). This bait-lamina test adapted under laboratory conditions was easy to perform, quick and cheap, making it relevant as a tool to be used in risk assessment. However, our results highlighted that the bait-lamina test was less sensitive than the ERT on a short time step (i.e. 5 days). The test could thus be adapted to properly assess pesticide impacts on an ecological function provided by enchytraeids (i.e. organic matter consumption) in laboratory. Considering the effects observed on the reproduction and thus the potential negative impacts on the population dynamics, a longer period of exposure could be tested to increase the sensitivity and the relevance of the bait-lamina test under laboratory conditions. However, given that after 5 days, the sticks were consumed at $67 \pm 8\%$ in the control treatment, a longer exposure will quickly lead to a 100% consumption. A longer exposure would thus imply the successive addition of several sticks in the tube in order to assess the feeding activity over time, thus allowing for a better assessment of the risk linked to the use of pesticides. Furthermore, this test could be proposed for standardization and submitted to a ring test procedure. For this purpose and for more repeatability, the soil could be an OECD soil.

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Principales conclusions de l'article

Les expérimentations menées dans cette étude ont permis de mettre en évidence une diminution de la production de juvéniles par les enchytréides *E. albidus* à 3 fois la dose recommandée de Swing® Gold. L'hypothèse selon laquelle les enchytréides seraient plus résistants que les vers de terre aux pesticides n'a donc pas été vérifiée dans ce cas puisque la reproduction d'*Aporrectodea caliginosa* avait été affectée aux mêmes concentrations (cf. Partie 2, Chapitre 2). Cependant, les enchytréides sont apparus plus résistants au fongicide à base de cuivre que les vers de terre. En effet la reproduction des enchytréides n'a pas été affectée, même à 496 mg kg^{-1} alors que les vers de terre avaient produits moins de cocons à 232.5 mg kg^{-1} de cuivre.

Par ailleurs, il semblerait que les enchytréides puissent maintenir leur rôle fonctionnel en contexte contaminé puisque les pesticides testés n'ont eu aucun effet sur l'activité alimentaire aux concentrations testées. Cependant, il est important de noter que ce test a été réalisé sur un temps court (5 jours). Ce test pourrait être adapté pour évaluer l'effet d'une exposition plus longue (de l'ordre de 42 jours, correspondant à la durée du test de reproduction).

Annexe 2 : Supplementary materiel Article 1 (Partie 1, Chapitre 1)

Table S1. List of references for mapping *A. caliginosa* in Fig. 1.

Code	Country / Region	References
1	Kamchatka	Shekhovtsov et al., 2014
2	Bangladesh	Makin et al., 2014
3	United States	McDaniel et al., 2013
4	Greece	Solomou et al., 2012
5	Algeria	Kherbouche et al., 2012/ Baha 1997
6	India	Najar and Khan, 2011
7	Uzbekistan	Rakhmatullaev et al., 2010
8	Argentina	Falco and Momo, 2010/ Falco et al., 2015
9	Iran	Ezzatpanah et al., 2010/ Omrani et al., 2005
10	Romania	Brinzea 2010
11	Belgium	Valckx et al., 2009/ Tiho and Josens, 2000
12	South Korea	Hong and Kim, 2009
13	Russia	Geras'kina 2009
14	Netherlands	Eijsackers et al., 2009/ Zorn et al., 2005/ Marinissen 1992
15	Ireland	Curry et al., 2008/ Cotton and Curry, 1982
16	Iceland	Rundgren 2007
17	Estonia	Ivask et al., 2007/ Ivask et al., 2000
18	Canada	Eriksen-Hamel and Whalen, 2007
19	New Zealand	Bithell et al., 2005/ Francis et al., 2001
20	Finland	Raty and Huhta, 2004/ Pitkanen and, Nuutinen 1997
21	Poland	Makulec 2004
22	Israel, Jordan, Lebanon, Syria	Pavlicek et al., 2003
23	United States	Deibert and Utter, 2003
24	Pakistan	Abdul et al., 2003
25	Sweden	Lagerlof et al., 2002
26	Germany	Judas 1990/ Poier and Richter, 1992/ Ammer and Ammer, 1998
27	Australia	Abbott 1985
28	Tajikistan	Belyuchenko and Podarueva, 1990
29	Denmark	Hendriksen 1991
30	Australia	Baker et al., 1992/ McKenzie and Dexter, 1993/ Baker et al., 1999
31	Tasmania	Garnsey 1994/ Dalby et al., 1998
32	Slovakia	Kovac et al., 1996
33	Australia	Baker et al., 1997/ Mele and Carter, 1999
34	Scotland	Boag et al., 1997
35	Norway	Sveistrup et al., 1997
36	Libya	Bennour and Nair 1997
37	France	Cannavacciuolo et al., 1998/ Bouche 1972/ Beaumelle et al., 2014
38	United States	Holmstrup et al., 1999
39	South Africa	Owojori et al., 2009
40	China	Yu et al., 2005
41	Mexico	Rosas-Medina et al., 2010
42	Spain	Perez-Losada et al., 2009
43	Italy	Carpene et al., 2006
44	England	Hutcheon et al., 2001
45	Egypt	Nasr and Ei Badawy, 2015
46	Turkey	Szedetjesi et al., 2014
47	Morocco	Nejmeddine et al., 1997
48	Belarus	Shekhovtsov et al., 2014
49	Ukraine	Krivolutzkii et al., 1992

Table S2. Effects of metals on life history traits and behaviour of *A. caliginosa* under laboratory conditions.

Reference	Studied parameters	Origin	Development stage	Metals	Form	Method of addition	Main results
Bart et al. (2017)	Mortality, biomass and avoidance behaviour	Collected	Adult	Cu	Oxychloride	Mixed	LC50 > 5000 mg/kg, EC50 avoidance : 51.2 mg/kg. Significant decrease in biomass from 50 mg/kg.
Eijsackers et al. (2005)	Mortality and biomass	Collected	NA	Cu	Oxychloride	Mixed	Decrease in biomass with addition of 60 mg/kg (effective concentration : 102 mg/kg) and increase in mortality (67%) after 4 week related to increase in copper body content.
Friis et al. (2004)	Mortality, estivation	Collected	Adult	Cu	Chloride	Mixed	At 150 mg/kg, copper increase mortality during drought demonstrating synergetic effects of drought and copper. Decrease in development of estivation cells for worms exposed to copper compared with worm not exposed.
Holmstrup et al. (1998)	Cocoons viability	Cultured	Cocoon	Cu	Chloride	Aqueous solution	Desiccation and copper exposure combined had synergistic effects on survival rate of embryos. At full saturation, NOEC : 12 mg/L, whereas at 97% relative humidity, NOEC : only 6 mg/L.
Ma (1988)	Cocoons production	NA	Adult	Cu	NA		EC10 : 27-70 mg/kg, EC50: 68 mg/kg.
Martin (1986)	Cocoons production, growth and mortality	Collected	Adult and juvenile	Cu	Sulphate	NA	For 7 days of exposure, NOEC mortality : 500 mg/kg and NOEC growth : 100 mg/kg. For 14 days of exposure, NOEC cocoon production : 50 mg/kg.
Khalil et al. (1996a)	Growth	Cultured	Juvenile	Cd, Cu and Zn	Sulphate salts	Mixed	EC50 growth: 81.8, 68.4 and 461 mg/kg for Cu, Cd and Zn, respectively.
Khalil et al. (1996b)	Mortality and cocoon production	Cultured	Adult	Cd, Cu and Zn	Sulphate salts	Mixed	LC50 : 540, 640 and 3610 mg/kg after 8 weeks of exposure and EC50 cocoon production : 34.4, 185.8 and 826 mg/kg for Cd, Cu and Zn respectively after 8 weeks of exposure. Decrease in LC50 with time of exposure (2-8 weeks).
Nahmani et al. (2005)	Burrow systems	Collected	NA	Zn, Cu, Pb and Cd	Contaminated soil	Contaminated soil	Metal pollution principally affected the shape of the burrow system (higher branching rate in the polluted soil).
Van rhee (1975)	Cocoons production, biomass	NA	NA	Cu, Zn	NA	Mixed	At 110 mg/kg of Cu, Decrease in cocoons production. At 1100 mg/kg Zn, Decrease in biomass and cocoons production.
Khalil (2013)	Mortality, reproduction and behaviour	Collected	Adult	As	Inorganic sodium arsenate	NA	96-h LC50 : 147 mg/kg. Avoidance behaviour at 240 and 400 mg/kg. Decrease in biomass between 110-400 mg/kg after 25 days of exposure.
Langdon et al. (2005)	Mortality and biomass	Collected	Adult	Pb	Pb nitrate	Mixed	After 28 days, LC50 : 2747 mg/kg and EC50 for biomass loss : 1208 mg/kg. Mortality increased with time exposure.
Maleri et al. (2007)	Mortality, biomass and reproduction	Collected	Adult	Cr, Co, Mg and Ni	Contaminated soil	Contaminated soil	Slower biomass gain in the contaminated soil. Automization of the tail observed at higher concentration. Decrease in cocoons production.
Qiu et al. (2011)	Mortality	Collected	Adult	Cd and Zn	Acetate salts	Mixed	LC50 : 344 and 1499 mg/kg for Cd and Zn respectively.
Spurgeon et al. (2000)	Mortality, biomass and cocoons production	Collected	Adult	Zn	Salt	Mixed	LC50 : 1695 mg/kg. EC50 for biomass change : 417 mg/kg. EC50 for cocoon production : 442 mg/kg.

For Origin: cultured (in laboratory) or collected (in the field). Method of addition: mixed (into the soil). LC50: Lethal concentration for 50% of exposed individuals. NOEC = No Observed Effect Concentration. NA= Not Available. EC_x: Effective concentration.

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Annexe 3 : Photothèque



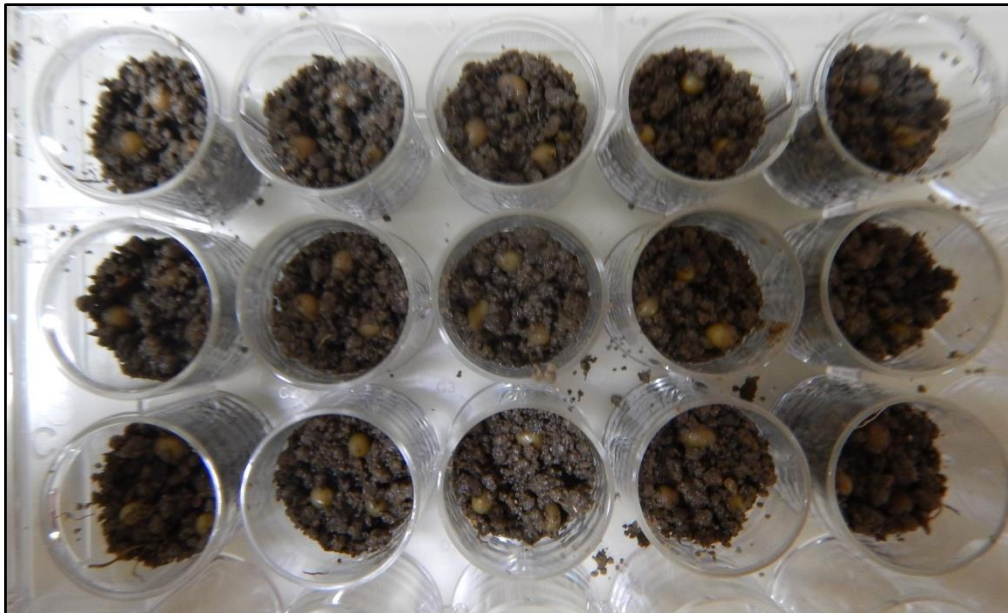
Enchytraeus albidus (Henle, 1837)



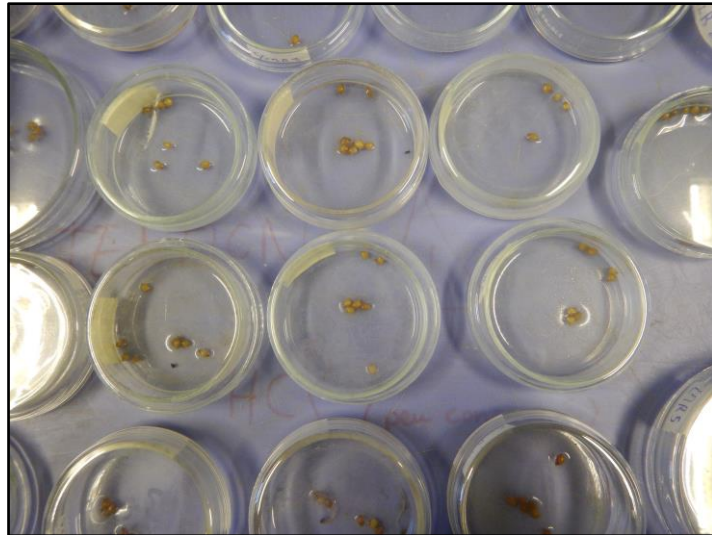
Essai d'évitement avec *Aporrectodea caliginosa* (ISO, 17515)



Tests d'évitement avec *Enchytraeus albidus*



Incubation de cocons d'*Aporrectodea caliginosa* dans des sols contaminés

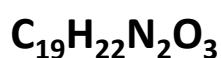
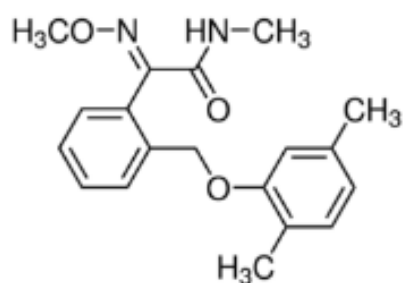


Incubation de cocons d'*Aporrectodea caliginosa* en solutions contaminées

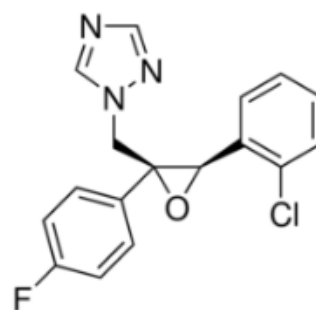


Cosmes de l'expérience de croissance, stockés en chambre climatique à 15 ± 1 °C

Annexe 4 : Principes actifs du Swing® Gold



Poids moléculaire: 326.39



Poids moléculaire: 329.76

Figure 1 : Formules chimiques de la dimoxystrobine (à gauche) et de l'époxiconazole (à droite).

Tableau 1. Caractéristiques de la dimoxystrobine et de l'époxiconazole (Source: PPDB, 2018)

	Dimoxystrobine	Epoxiconazole
Famille chimique	Strobilurine	Triazole
Origine	Synthétique	Synthétique
activité biologique	Fongicide	Fongicide
Mode d'action	Inhibiteur de la respiration	Inhibiteur de la biosynthèse des stérols
DT50 (laboratoire)	210	226
DT50 (champs)	22.9	120
Mobilité dans le sol	Modérément mobile (+)	Assez mobile (++)
Risques pour la santé humaine avérés	Perturbateur endocrinien	---
Risques pour la santé humaine possibles	Cancérogène/ Effet sur le développement et la reproduction	Cancérogène/ Perturbateur endocrinien/ Effet sur le développement et la reproduction

Annexe 5 : Valorisations des activités de thèse

Publications dans des revues internationales à comité de lecture directement liées à la thèse

Bart S, Laurent C, Péry ARR, Mougin C, Pelosi C (2017) Differences in sensitivity between earthworms and enchytraeids exposed to two commercial fungicides. *Ecotox Environ Safe*. 140, pp 177-184. <https://doi.org/10.1016/j.ecoenv.2017.02.052>

Bart S, Roudine S, Amossé J, Mougin C, Péry ARR, Pelosi C (2018) How to assess the feeding activity in ecotoxicological laboratory tests with enchytraeids? *Environ Sci Pollut Res*. <https://doi.org/10.1007/s11356-018-1701-3>

Bart S, Amossé J, Lowe CN, Péry ARR, Mougin C, Pelosi C (2018) *Aporrectodea caliginosa*, a relevant earthworm species for a posteriori pesticide risk assessment: Current knowledge and recommendations for culture and experimental design. *Environ Sci Pollut Res*. <https://doi.org/10.1007/s11356-018-2579-9>

Bart S, Barraud A, Amossé J, Péry ARR, Mougin C, Pelosi C. Assessing pesticide effects on earthworm reproduction by exposing adults and cocoons. Soumis à *Ecological Indicators*

Bart S, Pelosi C, Péry ARR. Towards a better understanding of the life cycle of the earthworm *Aporrectodea caliginosa*: new data and energy-based modelling. Soumis prochainement à *Environmental Science and Technology*

Bart S, Pelosi C, Néliu S, Lamy I, Péry ARR. An energy-based model to analyse growth data of earthworms exposed to two fungicides. Soumis prochainement à *Environmental Science and Technology*

Bart S, Pelosi C, Barraud A, Péry ARR, Cheviron N, Mougin C, Crouzet O. Earthworms mitigate pesticide effects on soil microbial activity. En préparation pour une soumission prochaine dans la revue *Soil Biology and Biochemistry*

Publications dans des revues internationales à comité de lecture lors de participation à des travaux annexes à la thèse

Amosse J, **Bart S**, Péry ARR, Pelosi C 2018. Short-term effect of fungicides on enchytraeid and earthworm diversity and community patterns in field conditions. *Ecotoxicology* 27(3): 300-312. <https://doi.org/10.1007/s10646-018-1895-7>

Amosse J, Boros G, **Bart S**, Péry ARR, Pelosi C (2018) First record of terrestrial Enchytraeidae (Annelida: Clitellata) in Versailles palace's park, France. *Opuscula*. <http://dx.doi.org/10.18348/opzool.2017.S2.53>

Amosse J, **Bart S**, Péry ARR, Pelosi C. Time-dependent effects of two fungicides on enchytraeid and earthworm community structure under field conditions. En préparation

Communications orales à des congrès internationaux (le nom de l'auteur ayant présenté est souligné)

Bart S, Laurent C, Amosse J, Péry ARR, Pelosi C (2016) Avoidance behaviour and survival of two annelid oligochaetes exposed to two fungicides. *12th International Symposium on Enchytraeidae (Hongrie)*.

Bart S, Amosse J, Mougin C, Péry ARR, Pelosi C (2018) A biology-based model to analyze growth data of earthworms exposed to copper at different development stages. *SETAC Europe 28th annual meeting (Rome, Italie)*.

Amossé J, Pery ARR, **Bart S**, Pelosi C (2016) A field study to assess pesticide effects on oligochaetes and soil functioning. *12th International Symposium on Enchytraeidae (Hongrie)*.

Amossé J, Thiel P, **Bart S**, Pery ARR, Pelosi C (2018) Time-dependent effects of two fungicides and their mixture on enchytraeid and earthworm communities under field conditions. *SETAC Europe 28th annual meeting (Rome, Italie)*.

Amossé J, Thiel P, **Bart S**, Pery ARR, Pelosi C (2018) Time-dependent effects of two fungicides and their mixture on enchytraeid and earthworm communities under field conditions. *13th International Symposium on Enchytraeidae (Versailles, France)*.

Communications orales à des congrès nationaux (le nom de l'auteur ayant présenté est souligné)

Bart S, Barraud A, Amosse J, Péry ARR, Pelosi C (2017) Impacts of two commercial fungicides on *Aporrectodea caliginosa* (Savigny, 1826). 47^{ème} congrès du Groupe Français des Pesticides (GFP, Nancy).

Bart S, Amosse J, Mougin C, Péry ARR, Pelosi C (2017) Recommandations pour l'utilisation d'*Aporrectodea caliginosa* s.s pour l'évaluation du risque pesticide. 6^{ème} Séminaire du réseau d'écotoxicologie terrestre et aquatique. Alixan (France)

Amossé J, **Bart S**, Péry ARR, Pelosi C (2017) Suivi au champ des effets de fongicides sur les populations d'enchytréides et de vers de terre. 47^{ème} congrès du Groupe Français des Pesticides (GFP, Nancy)

Amossé J, **Bart S**, Péry ARR, Pelosi C (2017) Suivi au champ des effets de fongicides sur les populations d'enchytréides et de vers de terre. 6^{ème} Séminaire du réseau d'écotoxicologie terrestre et aquatique. Alixan (France)

Communications par l'affiche à des congrès (le nom de l'auteur ayant présenté est souligné)

Bart S. Amosse J, Péry ARR, Pelosi C (2017) Impacts de deux fongicides sur la croissance d'*Aporrectodea caliginosa*. (SEFA, Lille). Avec présentation orale lors de la session mon poster en 180 secondes.

Bart S. Amosse J, Péry ARR, Pelosi C (2017) C'est l'histoire d'un ver. *Journées scientifiques de l'unité ECOSYS*.

Bart S. Amosse J, Péry ARR, Pelosi C (2017) A worm story. *Journées des doctorants ABIES (Paris)*.

Nélieu S, Delarue G, Amosse J, **Bart S.** Pelosi C (2018) Dissipation in soil and bioavailability to earthworm of two fungicides: comparison of laboratory and field experiments. *SETAC Europe 28th annual meeting (Rome, Italy)*.

Thiel P., **Bart S.** Pelosi C, Amosse J (2017) Effects of a fungicide on oligochaetes and soil organic matter dynamics under controlled conditions. *13th International Symposium on Enchytraeidae (Versailles, France)*.

Récompenses

Prix du meilleur poster lors des journées des doctorants de l'école doctorale ABIES (2017)

Prix du meilleur poster lors des journées scientifiques de l'unité ECOSYS (2017)

Liste des congrès nationaux et internationaux

- 12th International Symposium on Enchytraeidae (Tihany, Hongrie) 2016.
- 47^{ème} congrès du Groupe Français des Pesticides (GFP, Nancy) 2017.
- Colloque de la Société Française d'Ecotoxicologie Fondamentale et Appliquée (SEFA, Lille, France) 2017.
- 6^{ème} Séminaire du réseau d'écotoxicologie terrestre et aquatique (Alixan, France) 2017.
- SETAC Europe 28th annual meeting (Rome, Italie) 2018.
- 13th International Symposium on Enchytraeidae (Versailles, France) 2018.

Formations (sélection)

- Communiquer sur sa thèse : l'exposé oral efficace. Paris (3 jours)
- EIDI - Ecole Interdisciplinaire de l'Université Paris-Saclay (pour les doctorants financés par l'Initiative Doctorale Interdisciplinaire). Les Gâtines. (1 semaine)
- Initiation à la modélisation de phénomènes dynamiques (1 semaine)
- Analyse de sensibilité, métamodélisation et optimisation de modèles complexes INRA, Réseau MEXICO. La Rochelle (1 semaine)

- Utilisation avancée du logiciel R pour les statistiques. ANASTAT. Versailles (3 jours)

Enseignement

2016 et 2017 : Ecotoxicologie terrestre : impact des pesticides sur les annélides du sol. 3H.

Master 2 Toxicologie de l'environnement (Université du Maine)

2017 et 2018 : Ecotoxicologie terrestre : impact des pesticides sur les annélides du sol. 3H30.

Master 2 Ecophysiologie et écotoxicologie. (Université Pierre et Marie Curie)

2017 et 2018 : La modélisation mathématique pour analyser et prédire le devenir et les effets des substances chimiques. 2H. Master 2 Ecophysiologie et écotoxicologie. (Université Pierre et Marie Curie)

2017 : TD de Bioclimatologie. Echanges biosphère-atmosphère : application au cas de l'azote réactif. 3H (2 x 1h30). Cours ingénieur AgroParisTech, 1ère année.

Encadrement

Stagiaires de Master 2 : Céline Laurent (2016) et Alexandre Barraud (2017)

Stagiaire de Master 1 : Sacha Roudine (2017)

Stagiaire de BTS : Alexis Vincent (2016)

Animation diverses

Présentations des activités de recherche, lors de la journée de l'unité ECOSYS (2017), à des étudiants (en 2016, 2017 et 2018) et lors des journées portes ouvertes (2017).

Membre du comité d'organisation du 13^{ème} Symposium international sur les Enchytréides (ISE13) à Versailles (4 au 6 juin 2018).

Responsabilités et expertises

Membre élu au Conseil Scientifique et Pédagogique (CSP) de l'école doctorale ABIES lors des 3 années de thèse (2016, 2017 et 2018).

Relecture d'articles pour les revues « Ecotoxicology and Environmental Safety » et « Environmental Science and Pollution Research ».

Titre : Impacts comportementaux, démographiques et fonctionnels des pesticides sur les annélides oligochètes du sol

Mots clés : Vers de terre; Pesticide; Evaluation du risque; modélisation bioénergétique; TK-TD.

Résumé : L'évaluation du risque liée à l'utilisation des pesticides pour les invertébrés du sol est aujourd'hui réalisée à l'aide de l'espèce modèle *Eisenia fetida* via l'utilisation de tests ecotoxicologiques standardisés (ISO et OCDE) sur la mortalité ou la reproduction. Ces deux points mériteraient d'être révisés puisque (i) l'espèce modèle est rarement présente dans les agroécosystèmes où les pesticides sont majoritairement utilisés, (ii) les tests réalisés sont peu réalistes et leur analyse statistique de type ad hoc ne permet aucune extrapolation dans le temps ou à des niveaux d'organisation supérieurs. Ainsi, une nouvelle espèce modèle a été proposée: *Aporrectodea caliginosa*. De plus, dans l'objectif d'analyser les effets ecotoxicologiques des pesticides sur cette espèce, un modèle bioénergétique a été proposé et calibré. Ensuite, les effets ecotoxicologiques de deux formulations commerciales de pesticide, couramment utilisées en

Europe, ont été évalués sur l'ensemble du cycle de vie (mortalité, comportement, reproduction, croissance). Les données de croissance ont été analysées à l'aide du modèle bioénergétique couplé à un modèle toxicocinétique. Ces analyses ont apporté une compréhension mécanistique des effets et ont permis l'estimation de valeurs seuils (les concentrations sans effet, CSE), indépendantes du temps d'exposition. Enfin, dans un objectif d'évaluation intégrée de l'usage des pesticides, il a été mis en évidence que la présence des vers de terre atténuait les effets des pesticides sur les communautés microbiennes des sols, responsables de la dégradation de la matière organique. L'ensemble des résultats pourra être utilisé, au-delà de la thèse, pour calibrer un modèle de dynamique des populations spatialisé de type individu centré (IBM). Ce type de modèle pourra être utilisé en évaluation du risque, pratiquée par les industriels et les agences d'évaluation.

Title : Behavioral, demographic and functional impacts of pesticides on soil annelid Oligochaeta.

Keywords : Earthworms; Pesticide; Risk assessment; energy-based modelling; TK-TD.

Abstract : Pesticide risk assessment for soil invertebrates is currently performed using the earthworm model species *Eisenia fetida* through the use of standardized ecotoxicological tests (ISO and OECD) on mortality or reproduction. These two points should be revised since (i) the model species is rarely found in agroecosystems where pesticides are used, (ii) the tests performed are unrealistic and their statistical analysis does not allow any extrapolation in time or at higher organizational levels (population). Thus, after a thorough reading of the literature, a new model species has been proposed: *Aporrectodea caliginosa*. In addition, in order to analyze the ecotoxicological effects of pesticides on this species, an energy-based model has been calibrated. Then, the ecotoxicological impacts of two commercial pesticide formulations, commonly used in Europe, were assessed on the

entire life cycle (mortality, behavior, reproduction, growth). The growth data were analyzed with the energy-based model coupled with a toxicokinetic model. These analyzes provided a mechanistic understanding of the effects and the estimation of non-time-dependent threshold values (The no effect concentrations, NEC). Finally, for the purpose of an integrated pesticide risk assessment, it has been demonstrated that earthworms mitigate pesticide effects on soil microbial activity, involved in the soil organic matter degradation. All of the results are intended to be used, beyond the thesis, for the calibration of a spatialized population dynamics model with an Individual-based model approach (IBM). This model could be use in risk assessment, as performed by industrials and risk-assessment agencies.