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Review

The eco-toxic effects of pesticide and heavy metal mixtures towards earthworms in soil

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ABSTRACT

Earthworms are the key soil organisms, contribute to many positive ecological services that could be degraded by pesticides and other soil pollutants such as heavy metals. Chemicals usually occur as mixtures in the environmental systems which can lead synergistic effects. The assessment and characterization of soil pollutants that effects risks are very difficult due to the complexity of soil matrix, poor understanding about the fate and effects of chemical combinations like pesticide and metal mixtures in terrestrial systems, and scarcity of toxicological data on mixtures of pollutants. In this review we summarized the current studies on individual and joint effects of pesticides and metals on earthworms and indicate the mixture that cause the synergistic interactions. The review explores the methods and models used previously to evaluate the toxicity of chemical mixtures, and suggests the perspective approaches for a better knowledge of combine effects as well as research methods The summarized report indicates that pesticide and metal mixtures at all organization levels affect the earthworms negatively. Whereas, the combined pollution generated by mixtures of pesticides and metal ions could induce the DNA damage, disruption in enzyme activities, reduction in individual survival, production and growth rate, change in individual behavior such as feeding rate, and decrease in the total earthworm community biomass and density. Among the pesticides organophosphates were identified the most toxic pesticides causing the synergistic effects. The findings indicate the scarcity of toxicological data concerning the assessment of pesticide and metal mixtures at genome level; while the mechanisms causing synergism were still not sufficiently explored.

1. Introduction

Pollutants are continuously accumulated in the form of complex mixtures in the natural systems through different anthropogenic sources including industries, mining, agricultural, and waste water treatment plant (Altenburger et al., 2004). The metals and pesticides are two kinds of chemicals/toxicants globally known, and are likely to occur in most of agricultural soil ecosystems (Wang et al., 2012a). The development of intensive agriculture in many countries involves the application of various pesticides which may lead to a heavy burden on the environment especially in agriculture soil ecosystems. A variety of pesticides used in the agriculture sector to improve the annual agriculture production, however their residues increase soil contamination which may be directly or indirectly stressful for soil organisms (Choung et al., 2013). A significant portion of these pesticides may be carried out by runoff into aquatic environments or pass slowly through soil lower soil layers and ground waters (Rial-Otero et al., 2004). Beside pesticides, heavy metal pollution in soil environments has become another major challenge across the world due to their increase in geologic and anthropogenic activities. Worldwide heavy metals production has been very scaring increase and openly spread in the environment since industrial revolution (Nriagu and Pacyna, 1988). The huge contamination of metals and pesticides in soils, water and air and their imminent transfer to higher organisms through the food chain continues to be an environmental issue which may involves diverse health risks for future generation.

Earlier the scientists have been relying only on individual compounds to assess the potential risks of environmental contaminants; however assessing joint effects of chemical mixtures by considering data obtained exclusively from single chemical toxicity, tends to over or underestimate the level of the joint toxicity. Assessments that take into account of combined actions of pollutants reflect better the existent impact of environmental exposures than the assessments that evaluate toxicity of single chemicals (Schnug et al., 2014). Joint toxic effects of

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chemical mixtures on organisms and ecosystems need to be seriously addressed, however but the evaluation of every possible chemical mixture is impossible practically. Thus the usage of computer-based models is considered to be a powerful tool that may accurately predict the joint toxicity of mixtures simulated from individual compound data. The concentration addition (CA) and independent action (IA) are two classical models that are frequently used to predict the joint toxicity of different chemical mixtures. Both these models used the non-interactions approach between the mixture and individual components, which means that there is no occurrence of interactions between compounds with the mixture. But each compound can individually contribute to the joint toxicity. The joint toxicity of toxic mixtures can be similar (additive), stronger (synergistic) or weaker (antagonistic) depending of on the sum of effects from individual exposures (Warne and Hawker, 1995). Most of the studies indicate that most of the metal mixtures frequently produce synergistic effects (Cedergreen, 2014). The applications of CA and IA models are limited to predict the actions of non-interactive mixtures. To challenge this limitation, the scientists have designed the second category of model that can be applied to mixtures to show synergism or antagonism interactions between the individual compounds of the mixture. The combination index (CI)-isobologram equation is the widely used model to indicate the type of interaction between components of a certain mixture (Chou, 2006). This model facilitates quantitative prediction of chemical interactions within the mixture.

However many studies have been reported on the toxicities of single chemicals, there is a great need to improve our knowledge about the harmful effects of exposures to chemical mixtures. The pollutants are generally the same in water and soil systems, but their fate and effects on living organisms may be varied in the two ecosystems. Soil is considered a complex terrestrial ecosystem containing a large variety of organisms, mineral particles and organic matter (Cachada et al., 2016). The binding of toxic compounds to some fractions of minerals and humus can reduce their mobility and bioavailability, and thus modulating their toxicity. Moreover, the availability and toxicity of soil pollutants are influenced by the weather and age of the soil as well. Consequently, the assessment of chemical toxicity toward terrestrial organisms is more difficult than that of aquatic organisms. It is also impossible to extrapolate toxicity in soil ecosystems from studies that use aquatic organisms. Specific approaches and models are always required to evaluate the toxic effects of soil pollutants toward the terrestrial biodiversity. Approaches conducting field studies or carrying out natural or artificial soil experiments are recommended in order to explain the availability of toxicant and their relative conditions of exposures (Spurgeon et al., 2003). The most challenging issue concerning exposures to soil contaminants is to measure the level on which the living organisms can be contaminated, and evaluate the toxic effects of those contaminants at individual, population and community levels. Therefore, any study that will evaluates the individual and joint toxicity of soil pollutants must be of great importance in the ecological risk assessment.

Earthworms are among the commonly known organisms that are found in the soil ecosystems where they play a crucial role in the improvement of soil quality (Spurgeon et al., 2003). The decrease of earthworms in the soil ecosystem may reduce nutrient cycling and their availability for plant uptake (Rizhiya et al., 2007). Earthworm as bioindicators of soil pollution have been considered as the key organism for ecological risk assessment (Song et al., 2009). Pollutants including pesticides and heavy metals accumulated by earthworms may be transferred to higher trophic-level organisms through food chain. Accumulation of contaminants in earthworms may not cause significant effects to the earthworm, but may have severe impact to other organisms and humans as well (Reinecke and Reinecke, 1996). The earthworms like *Eisenia fetida* is usually improving soil structure and their vulnerability towards soil contaminants, this great role of earthworms have made them to be considered as the best biological indicators for soil contamination. Moreover, earthworm species are better to be used in the ecotoxicological laboratory-based tests for short exposures (Fourie et al., 2007).

In recent years, with the broad application of chemical compounds in agricultural ecosystem, the study on the earthworm eco-toxicology has become increasingly widespread. The researchers around the world have built up a lot of earthworms-based eco-toxicological methods to test the presence of chemical materials (OECD, 1984). Different studies about eco-toxicological mechanisms of the earthworm toward pollutants, such as heavy metals, pesticides (Stenersen, 1979) and combined pollution of different heavy metals (Spurgeon et al., 2003), metals and pesticides mixtures (Xu et al., 2006), have been reported worldwide. However, studies on the interactions between toxic effects of pesticides and heavy metals are still very limited. Therefore, in this review we summarize the current studies on single and joint effects of pesticides and metals on earthworms at genome, cellular, individual population and community levels. We also overview the interactions between pesticides and metals in earthworms and make an overall judgment of the likelihood for synergistic effects, and highlight the current knowledge gaps. Finally, we explore the methods and models used to evaluate the toxicity level of chemical mixtures, and provide perspective approaches for a better understanding of joint effects as well as research methods.

2. Effects of heavy metals

Heavy metals released into the environment from various anthropogenic activities are toxic to soil organisms and affect the abundance, diversity, and distribution of the soil organisms. Earthworms inhabiting in the soils contaminated by heavy metals, due to their behavioral characteristic such as burrowing and feeding activities, are more susceptible to metal pollution than other groups of terrestrial invertebrates.

Exposure of earthworms to higher concentrations of heavy metals in soil ecosystems may affect cocoon production, growth and sexual development of worms, life behavior, viability and density (Andre et al., 2010). Heavy metals including zinc (Zn),lead (Pb), cadmium (Cd), mercury (Hg) and copper (Cu) are mostly bioaccumulate and results diverse effects in earthworms which may credits the earthworm as a suitable biological indicator to investigate the heavy metals pollution (Zhang et al., 2009). The most reported toxic effects of the heavy metals toward earthworm species include growth rate reduction, reproduction decline,DNA damage, pathological damage of nephridia, alterations in cytokinesis and antioxidant enzymes (Liang et al., 2011). Metals like Cd exposure may cause mortality in earthworms which ultimately lead reduction in the earthworm population size in contaminated soils. Genomic impairment in earthworm's cells due to metal is also used as early biomarker of metal effects at the individual level. Metal exposure induces very significant DNA damage in earthworms and cause alterations in the protein content especially metallothioneins and heat shock proteins (Maity et al., 2008). Cd a widely occurring metal pollutant, has been extensively studied globally and is well known to affect the physiological functions of earthworms, in terms of bioaccumulation, decrease in weight and oxidative DNA damage generation (Table 1). Multiple studies have reported metallothionein overespression in coelomocytes of *Eisenia fetida* after long-term exposure to metals particularly Cd contaminated soils (Brulle et al., 2011). However, not all heavy metals are very toxic toward earthworms. Nakashima et al. (2008) reported that among the heavy metal nickel (Ni) usually does not accumulate in the tissues of earthworm, even after long-time exposure, and did does cause any significant effects on earthworm's growth. In Table 1 we summarized the most recent reports on effects of different heavy metals including Cd, Zn, Cu, Hg, Pb and Ni towards different earthworm species; where the responses of earthworms to heavy metal exposures are the same for all earthworm species. In their study, Spurgeon and Hopkin (1996) evaluated the effects of heavy metals particularly

Table 1
Overview of the recent reports on the effects of heavy metals towards earthworm species.

Species	Metals	Toxic Effects	References
Eisenia fetida	Cd, Cu, Pb and Zn	All metals produced toxic effects on the growth and maturation of juvenile earthworms	Spurgeon et al. (1994)
Lumbricus rubellus	Pb, Zn and Cd	Only Cd induced pathological damage and increase the regulation of metallothioneins (MT)	Morgan et al. (2004)
Lumbricus rubellus	Cu and Cd	A slight decrease in weight (Cu exposures) and Cd exposures inhibited the growth of the worms	Burgos et al. (2005)
Eisenia fetida	Cd and Ni	Accumulation of Cd caused oxidative DNA damage in the earthworm. Ni was not accumulated in earthworms.	Nakashima et al. (2008)
Eisenia fetida	Cd and ciprofloxacin (CIP)	Formation of Cu-CIP complexes and once up taken by earthworms may result in changing the bioavailability, subcellular distribution, and toxicity of Cu to earthworms.	Huang et al. (2009)
Lumbricus rubellus	Pb and Zn	Accumulation of Pb and Zn in the posterior alimentary canal	Andre et al. (2010)
Drawida sp., *Allolobophora* sp., *and Limnodrilus* sp.	Hg	Bioaccumulation	Zhang et al. (2009)
Pheretima peguana	Cd and Pb	Genotoxic and affect cytokinesis of the earthworms	Muangphra and Gooneratne (2011)
Lumbricus rubellus	Pb, Zn, and Cd	A possible Cd-induced pathological damage of nephridia	Morgan et al. (2004)
Metaphire posthuma, Polypheretima elongata and Pontoscolex corethrurus	Cd	DNA damage in *M. posthuma* coelomocytes	Liang et al. (2011)
Lampito mauritii	Pb and Zn	Alterations in Antioxidant enzymes due following the exposure to Pb	Maity et al. (2008)

Cd: cadmium, Cu: copper, Hg: Mercury, Ni: Nichel, Pb: Lead, Zn: Zinc, MT: Metallothionein, DNA: Deoxyribonucleic acid.

Cd, Cu, Pb and Zn on earthworm (*Lumbricus ruberus* and *Aporrectodea rosea* and *Eisenia fetida*, and the results showed that *Aporrectodea rosea* is very sentive to toxicity of Zn than *Lumbricus ruberus)* populations. The species *Lumbricus ruberus* and *Aporrectodea rosea* were affected more by exposure to Zn at lower concentrations than *Eisenia fetia.*

3. Biological effects of pesticides and biomarker-based assessments

Generally, traditional earthworm tests are used for evaluating effects of toxic chemicals on the basis of whole-body endpoints such as survival, growth, reproduction and behavioral changes. Traditional toxicology is specifically based on physiological responses and is applied to evaluate the lethal and sub-lethal toxicity (Chen et al., 2011). In recent years, multiple studies have been suggested the use of molecular biomarkers such as gene expression profiling, DNA damage detection, enzyme activities etc., which provides new approaches for efficiently assessing the biological effects of environmental pollution (Booth and O'Halloran, 2001). Biomarker is defined as a biological response which can be related to exposures or toxic effects of the environmental chemicals (Peakall, 1994). The use of molecular biomarkers are very important because they can reveal physiological changes due to their toxicity, thus showing early-warning signs of environmental risk pollutants toward organisms (Van Gestel and Van Brummelen, 1996). Some pesticides like pirimiphos-methyl and deltamethrin cause significant alterations in enzyme activities after exposure to earthworms at lower doses than those recommended for agricultural use. Velki and Hackenberger (2013) investigated the effects of two pesticides including pirimiphos-methyl and deltamethrin on molecular biomarkers of earthworms including cholinesterase, carboxylesterase, catalase (CAT) and glutathione S-transferase activities (GST). They reported that exposures to both pesticides produced significant inhibition of cholinesterase, carboxylesterase activities and activities of CAT and GST were proportionally changed with the duration of exposure. Additionally, their results indicated that even for non-significant changes in molecular biomarkers, a toxic effect caused by exposures could be still noticed (Velki and Hackenberger, 2013).

Pesticides affect the earthworms at diverse levels of organization including individual, population and community levels. At individual level, pesticides may accumulate in the earthworm's tissues leading to changes in antioxidant enzyme activities, gene expression and DNA structure. The investigation of changes in gene expression particularly metallathionein (MT) and heat shock proteins is considered a great tool to identify toxic effects of major pollutants and to evaluate toxicity mechanisms (Brulle et al., 2011; Dittbrenner et al., 2012). At individual and population levels, pesticides can affect the reproduction growth and survival of the earthworms (Table 2). Toxicity at community level simply means the impact of pesticide pollution on earthworm diversity including biomass and abundance of the earthworm species. The Table 2 illustrates some of the available reports on the application of biological markers in different earthworm species for evaluating effects of the most used pesticides. Table 2 includes details on tested pesticide, its category or family, studied biomarker (or endpoint), test species, applied dose, resulted response and the reference of the original study. In this review we only collected different studies that evaluated the toxicity of pesticides toward earthworms using biomarker response and we tried to see what they have in common (Table 2). The effects of pesticides towards earthworms depend on the type of pesticide and its rate of application, earthworm species and age, and environmental conditions.

Insecticides and Herbicides are the highly investigated categories of pesticides with organophosphate insecticides being the most reported class. This may be related to their broad application in agricultural and urban ecological systems. The results in Table 2 indicate that organophosphate insecticides mainly induce DNA damage and make changes in the earthworm enzymatic activities particularly cholinesterase activity. In general, tested herbicides resulted in toxic effects such DNA damage and changes in antioxidant enzyme activities but at higher concentration levels. To characterize biological responses of earthworms to pesticide exposure, individuals are exposed under control laboratory conditions to one or several levels of pesticide concentrations in either artificially contaminated substrates (artificial soil or filter paper) or field-sampled soils (Table 2). The organisms used are model species or other species that have never been previously in contact with any contaminants and are not descendants of contaminated individuals. *Eisenia fetida* is the most used model species and it is internationally recommended for toxicity tests using artificial soils or filter paper contact tests (Nahmani et al., 2007). In our review over 50% of the earthworm species used in experimental tests belonged to *Eisenia* genus (Table 2). Besides using artificially contaminated substrate, biological responses can also be investigated in field-sampled organisms. More details on the pesticide effects toward earthworm species can be found in the Supplementary materials Table S1.

Table 2
Overview of studies on application of earthworm biomarkers to evaluate toxic effects of pesticides.

Pesticides	Studied biomarker	Test species	Pesticide concentration	Results	References
Insecticides					
Chlorpyrifos (Organophosphate)	Neutral red retention Time(NRRT) and Cholinesterase(ChE)	*Aporrectodea caliginosa*	28 mg/kg Natural soil test	Reduction of NRRT, inhibition of the ChE activity	Booth and O'Halloran (2001)
Chlorpyrifos-ethyl (organophosphate	Enzyme activities: GlutathioneS-transferase, Catalase and cholinesterase (ChE)	*Aporrectodea caliginosa*	Field studies in vineyards (area of 4 ha, pH = 6.7)	Modifications in GST and CAT activities, No effect on ChE activity	Schreck et al. (2012)
Pirimiphos-methyl (organophosphate)	Enzyme activities: Cholinesterase (ChE), Catalase (CAT), carboxylesterase (CES), glutathione S-transferase (GST)	*Eisenia andrei*	0.02,0.07, 0.2, 0.7 and 2 mg a.i./kg Filter paper and artificial soil (1, 3, 6, 10, 15, 21 and 28 d.)	inhibition of the AChE and CES, CAT and GST acitivies change with the exposure duration in artificial soils	Velki and Hackenberger (2013)
Deltamethrin (Pyrethroids)	Enzyme activities: cholinesterase (ChE), Catalase (CAT), carboxylesterase (CES)	*Eisenia andrei*	0.05, 0.1, 0.25 and 0.5 mg a.i./kg Filter paper and artificial soil (1, 3, 6, 10, 15, 21 and 28 d.)	inhibition of the AChE and CES, CAT and GST acitivies change with the exposure duration in artificial soils	Velki and Hackenberger (2013)
Diazinon (Organophosphate)	NRRT and Activity of Cholinesterase(ChE)	*Aporrectodea caliginosa*	60 mg/kg) Natural soil test	Diazinon induces reduction of NRRT,Inhibitioon of ChE activity	Booth and O'Halloran (2001)
Chlorpyrifos (Organophosphate)	NRRT assay, DNA strand break	*Eisenia fetida andrei*	Nominal concentrations (620 g ai/ha); 7 and 28 days in Natural soil	increased DNA damage in earthworms, alterations in cells before the appearance of damage in DNA	Casabé et al. (2007)
Chlropyrifos (Organophosphate)	Activities of cellualase superoxide dismutase (Žaltauskaitė and Sodienė, 2010 and CAT)	*Eisenia fetida*	10, 20, and 40 ppm dry soil; 1, 3, 5 and 7 days in artificial soil test	changes in enzyme activities	Wang et al. (2012b)
Organophosphorous	ChE activity	*Allolobophora chlorotica*	In situ	significant decrease in ChE activity	Denoyelle et al. (2007)
carbamate	ChE activity	*Allolobophora chlorotica*	In situ	significant decrease in ChE activity	Denoyelle et al. (2007)
Imidacloprid (Neonicotinoid)	Body mass and histopathology	*Eisenia fetida andrei, Aporrectodea caliginosa, Lumbricus terrestris*	0.2, 0.66, 2 & 4 ppm of dry weight, 1, 7 and 14 days in artificial soil test	Changes in body mass.cellur changes occurred after 24 h of exposure to the lowest concentrations	Dittbrenner et al. (2011)
Imidacloprid (Neonicotinoid)	Heat shock protein 70 (Hsp70), Avoidance behavior	*Eisenia fetida, Aporrectodea caliginosa, Lumbricus terrestris*	0.2, 0.66, 2, 4 ppm dry weight; 1, 7 and 14 days in reference test soil	*E. fetida(only)* showed a Significant avoidance behavior; Changes in Hsp70 levels	Dittbrenner et al. (2012)
Azinphos-methyl (Organophosphate)	Cholinesterase activity, NRRT assay	*Eiseni andrei*	Exposure range at 10–40 mg kg^{-1} 14 days in Artificial soil in	A higher inhibition of cholinesterase activity, Reduction of NRRT	Jordaan et al. (2012)
λ-cyhalothrin (Pyrethroids)	Enzyme activities: GlutathioneS-transferase,Catalase	*Aporrectodea caliginosa*	Field studies in vineyards (area of 4 ha, pH = 6.7)	Modifications in antioxidant activities	Schreck et al. (2012)
Fenvalerate (Pyrethroids)	Activities of cellualase superoxide dismutase (Žaltauskaitė and Sodienė, 2010 and CAT)	*Eisenia fetida*	10, 20, and 40 ppm dry soil; 1, 3, 5 and 7 days in artificial soil test	changes in enzyme activities	Wang et al. (2012b)
Chlordane (organochlorine)	Sperm count	*Lumbricus terrestris*	6.25,12.5 and 25 ppm; 16 days in artificial soil test	Significant Reduction in spermatozoa	Cikutovic et al. (1993)
Imidacloprid (Neonicotinoids)	DNA damage with Comet assay	*Eisenia fetida*	0.05, 0.1, 0.2, and 0.5 ppm pesticide solutions and Filter paper test	Induction of significant DNA damage	Zang et al. (2000)
RH-5849 (bisacylhydrazine)	DNA damage with Comet assay	*Eisenia fetida*	5, 25, 50, and 100 ppm Pesticide solutions and Filter paper test	Induction of significant DNA damage	Cikutovic et al. (1993)
Herbicides					
Atrasine (s-triazine)	DNA damage (Comet assay)	*Eisenia fetida*	0, 2.5, 5 and 10 mg/kg In artificial soil (28 days)	Induction of significant DNA damage	Song et al. (2009)
Glyphosate (Organophosphate herbicide)	Avoidance behavior,NRRT assay, DNA damage,	*Eisenia andrei*	Nominal concentration recommended for soya crops (1440 g ai/ha); 7 and 28 days in Natural soil	NRRA revealed alterations at sub-cellular levels	Casabé et al. (2007)
Acetochlor (Chloroacetamide)	DNA damage (Comet assay)	*Eisenia fetida*	5–80 mg/kg; 15,30 days in artificial tests	Higher concentrations caused DNA damage in earthworms	Xiao et al. (2006)
Acetochlor (chloroacetanilides)	Sperm count	*Eisenia fetida*	5–80 mg/kg; 15,30 days in artificial tests	Higher concentrations caused sperm decrease in earthworms	Xiao et al. (2006)
Acetochlor (chloroacetanilides)	NRRT	*Eisenia fetida*	5–80 mg/kg; 15,30 days in artificial tests	Reduction of neutral red retention time due to acetochlor pollution at concentrations more than $10 mgkg^{-1}$	Xiao et al. (2006)
Endosulfan (organochlorine insecticide)	DNA damage (comet assay)	*Eisenia fetida*	0.1–10 mg/kg; 7–28 days in artificial soil	Induction of DNA damage in earthworm	Liu et al. (2009)
Atrasine (s-triazine)	Enzymes activity: superoxide dismutase and Catalase	*Eisenia fetida*	0, 2.5, 5 and 10 mg/kg In artificial soil (28 days)	Inhibition of SOD activity. However, the herbicide stimulated CAT activity	Song et al. (2009)

(continued on next page)

Table 2 (*continued*)

Pesticides	Studied biomarker	Test species	Pesticide concentration	Results	References
Fungicides					
Folpet (Dicarboximides),	Enzyme activities: GlutathioneS-transferase,Catalase	*Aporrectodea caliginosa*	Field studies in vineyards (area of 4 ha, pH = 6.7)	Modifications in antioxidant enzyme activities	Schreck et al. (2012)
Metalaxyl (Substituted dimethyl aniline)	Enzyme activities: GlutathioneS-transferase,Catalase	*Aporrectodea caliginosa*	Field studies in vineyards (area of 4 ha, pH = 6.7)	Modifications in antioxidant enzyme activities	Schreck et al. (2012)
Fosetyl (Phenylamide)	Enzyme activities: GlutathioneS-transferase,Catalase	*Aporrectodea caliginosa*	Field studies in vineyards (area of 4 ha, pH = 6.7)	Modifications in GST and CAT activities,	Schreck et al. (2012)
Myclobutanil (conazole)	Enzyme activities: GlutathioneS-transferase,Catalase	*Aporrectodea caliginosa*	Field studies in vineyards (area of 4 ha, pH = 6.7)	Modifications in GST and CAT activities,	Schreck et al. (2012)
mineral fungicides (copper and sulfur)	ChE activity	*Allolobophora chlorotica*	In situ	A slight reduction in ChE activity	Denoyelle et al. (2007)

CAT: Catalase, CES: Carboxylesterase, ChE: Cholinesterase, GST: GlutathioneS-transferase, NRRT: Neutral red retention Time.

4. Interactions between pesticides and heavy metals towards earthworm species

Interactions between pesticides and heavy metals have been evaluated in several studies and their synergistic mixtures are likely to occur as compared to synergistic mixtures of metals alone (Cedergreen, 2014). Information on mixtures of pesticides and metal ions are summarized in Table 3.The nature of interactions between components of each mixture are indicated. Synergistic, antagonistic and additive mixtures of pesticides and metal ions were collected from different studies. The Table 3 includes details on the test species, its phylum, the endpoint and the reference of the original study. Combinations of pesticides and heavy metals generally produce synergistic interactions, (Table 3), where 60% of investigated mixtures have synergistic interactions. The most interesting thing is that many mixtures can present a dual behavior where both synergism and antagonism were found in the same mixture depending on the dose-effect levels (Yang et al., 2015; Uwizeyimana et al., 2017). Exposures to mixtures of pesticides and metal ions may provoke adverse combined effects to soil organisms. Combined pollution generated by pesticide and metal mixtures could induce changes in genome expression in earthworms (alterations of DNA and gene expression), cause oxidative stress, reduce growth, slow sexual development, reduce cocoon production and hatchability, affect juvenile viability, cause mortality and effect the population size, abundance and species diversity in earthworms. Evaluation of effects of pesticide and metal mixtures at genome, individual, and population and community levels is very important for ecological risk assessment, because they can help to reveal the effects of stressors and toxicity mechanisms. Analysis of gene expression were used to clearly identify mechanisms of tolerance and adaptation in earthworm populations (Pauwels et al., 2013). However, studies that investigate the joint effects of pesticide and metal mixtures at the genome level through alterations in DNA structure (DNA adducts) and gene expressions are still scarce. More adverse effects of pesticide and metal mixtures toward different organisms can be found in the Supplementary materials Table S2.

5. Mechanisms causing synergistic and antagonistic interactions

Chemical mixtures in the environmental usually do not produce the same degree of toxicity towards organisms because the toxicity of the given toxicant can be influenced by many factors including target species, nature of mixture components, route and duration exposure. The components of a mixture tend to influence each other's, where some components may be interactive or non-interactive (Fig. 1). In most cases, the interactions between pesticides and metal ions increase the toxicity (synergism) like mortality rate, DNA damage, and reduction in the reproduction rate, changes in enzyme activities where some leads a decrease in the expected toxicity (antagonism) as well (Yang et al., 2015). Details on the relationship between exposures (route and duration) and effects are given in the Section 5.2.1

The most important point that can be known from Fig. 1 is to determine whether there is any interaction or no interaction taking place. By knowing this in the initial toxicological assessments, I scientist could easily define which model to be used for quantifying chemical interactions.

5.1. Non-interactions

According to categorization indicated in Fig. 1, there are two methods including Concentration Addition (CA) and Independent Action (IA) that use the model of non-interaction to predict the toxicity of chemical mixtures based on the approach of similar and dissimilar actions (Bliss, 1939). The models for similar action suppose that substances in the mixture act on the same biological site (e.g. receptor or target organ), by using similar mechanism where they differ only in

Table 3
Pesticide and metal matures.

Metal	Pesticide 1	Pesticide 2	Type of Pesticides	Test species	Phylum	Endpoint & Joint Action	References
Cd	Atrazine		Herbicide	*Eisenia fetida*	Annelida	DNA damage[SyAn]	Yang et al. (2015)
Cd	λ-cyhalothrin		Insecticide	*Eisenia fetida*	Annelida	Mortality[An]	Wang et al. (2015)
Cd	Chlorpyrifos		Insecticide	*Eisenia fetida*	Annelida	Mortality[SyAn]	Yang et al. (2015)
Cu	Methamidophos		Insecticide	*Eisenia fetida*	Annelida	Mortality[Sy]	Liang and Zhou (2003)
Cu	Acetochlor		Herbicide	*Eisenia fetida*	Annelida	Mortality[Sy]	Liang and Zhou (2003)
Cu	Glyphosate		Herbicide	*Eisenia fetida*	Annelida	Enzyme activities[An]	Zhou et al. (2013)
Ni	Chlorpyrifos		Insecticide	*Lumbricus rubellus*	Annelida	Mortality & reproduction[Ad]	Lister et al. 2011)
Cu	Carbendazim		Fungicide	*Caenorhabditis elegans*	Nematoda	Reproduction[Sy]	Jonker et al. (2004)
Ni	Prochloraz		Fungicide	*Caenorhabditis elegans*	Nematoda	Reproduction[Sy]	Martin et al. (2009)
Cd	Diuron		Herbicide	*Caenorhabditis elegans*	Nematoda	Reproduction[Sy]	Martin et al. (2009)
Cd	Siduron		Herbicide	*Eisenia fetida*	Annelida	DNA damage[SyAn]	Uwizeyimana et al. (2017)
Mixtures including more than two pesticides							
Cd	λ-cyhalothrin	Imidacloprid	Insecticides	*Eisenia fetida*	Annelida	Mortality[An]	Wang et al. (2015)
Cd	Imidacloprid	Atrazine	Insecticide, Herbicide	*Eisenia fetida*	Annelida	Mortality[Sy]	Wang et al. (2015)
Cd	Phoxim	Butachlor	Insecticide, Herbicide	*Eisenia fetida*	Annelida	Mortality[Sy]	Wang et al. (2015)
Cd	Butachlor	λ-cyhalothrin	Herbicide. Insecticide	*Eisenia fetida*	Annelida	Mortality[Ad]	Wang et al. (2015)
Cd	Chlorpyrifos	Atrazine	Insecticide, Herbicide	*Eisenia fetida*	Annelida	Mortality[An]	Wang et al. (2015)
Cd	Chlorpyrifos	Avermectin	Insecticide, Wormicide	*Eisenia fetida*	Annelida	Mortality[Sy]	Wang et al. (2015)
Cd	Atrasine	Butachlor	Herbicides	*Eisenia fetida*	Annelida	Mortality[Sy]	Wang et al. (2015)

Cd: Cadmium; **Cu:** Copper; **Ni:** Nickel; **Sy**: Synergism; **An**: Antagonism; **Ad**: Additive; **SyAn**: Synergysm and Antagonism (double synergistic and antagononistic bevavior).

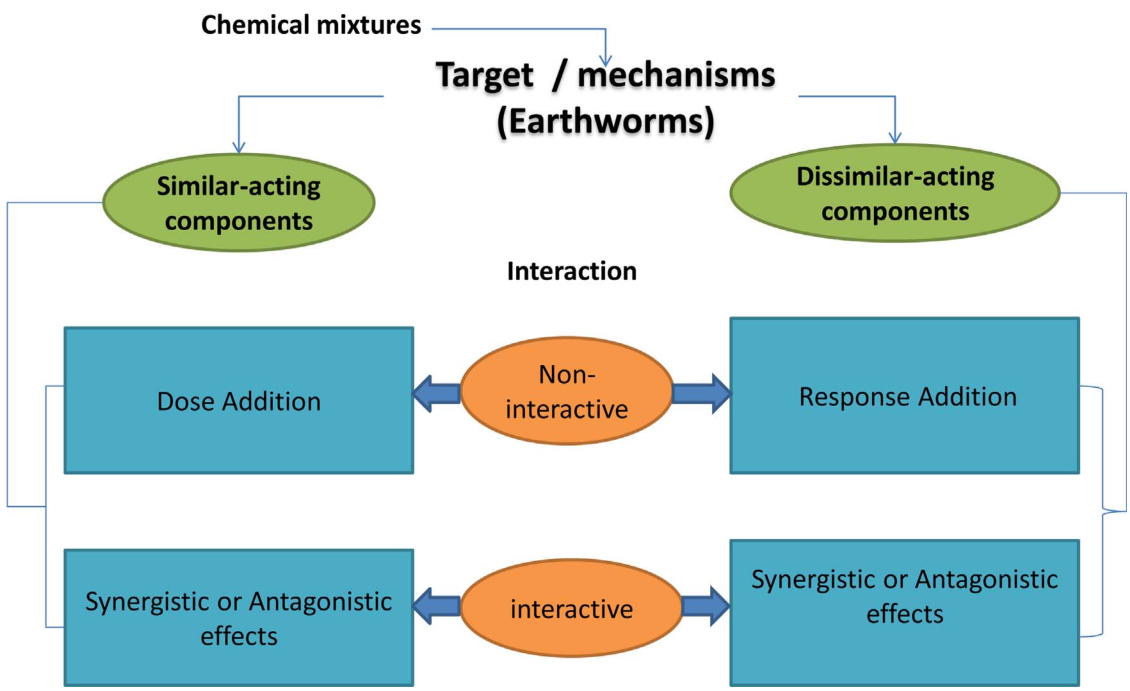

Fig. 1. Characterization of combined toxic actions between two or multiple components in chemical mixtures.

their effectiveness. Each substance contributes to the combined toxicity in a proportion to its concentration, where their relative toxic effects are supposed to be constant at all dose levels (Fig. 2a). The combined effect would be equal to the sum of doses from all the contributing substances. Many studies related to risk assessment of mixtures, frequently work on the hypothesis that a mixture of compounds will produce additive toxic effect (Altenburger et al., 2013). Models of dissimilar actions assume that mixture substances contribute to a common result, but using different mechanisms (Fig. 2a). Also, the site of action may not be necessarily the same among the components in a mixture. Thus, the action of one substance will not affect the toxicity of another substance (Altenburger et al., 2013).

5.2. Interactions

In some mixtures the combined toxicity becomes different from the expected one by using the hypothesis of concentration addition or independent action. In these mixtures, substances interact with each other to produce total toxicity, which may be stronger (synergistic) or weaker (antagonistic) than the predicted ones (Fig. 2b). Many studies revealed that mixtures of pesticides and metal ions usually cause synergistic toxicity due to interaction with each other in mixtures (Cedergreen, 2014).

5.2.1. Relationship between exposures and effects

Chemical interactions can take place before absorption process, particularly in the toxicokinetic and toxicodynamic process (Fig. 3).

Interactions between chemical compounds can alter biological processes that are important for the resultant effect of a compound towards the exposed organism (Cedergreen, 2014). These processes include bioavailability, absorption and internal distribution, binding at the target, metabolism and excretion mechanisms. Synergistic and

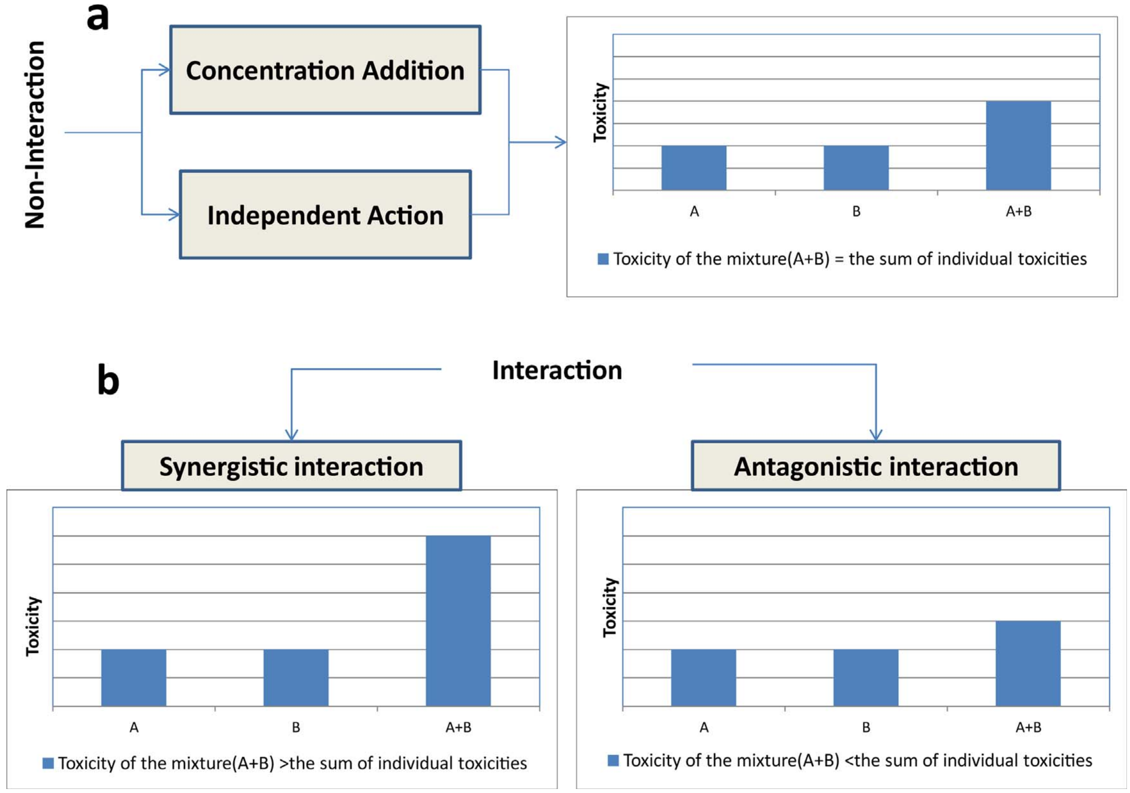

Fig. 2. Schematic representation of the reference models used to quantify the combined toxicological responses.

antagonistic effects are caused by the interactions that happen around one or more of these processes. Toxicokinetic interactions happen when there is any alteration in the process of absorption, internal distribution, metabolization or excretion of a toxic substance in a chemical mixture (Binderup et al., 2003). Chemical interactions may occur outside the exposed organism, in which one compound influences the availability of other. These types of interactions are mostly found in metal/metal mixtures where alteration in ion speciation outside the organism is the main mechanism that causes synergistic interactions (Binderup et al., 2003). Some herbicides like atrazine can potentially react with metal ions and form complexes that may influence absorption in the resulting mixture, and thus reduce the toxicity of the mixture. Atrazine has been reported to potentially interact with Cd ions (Cd^{2+}) generating anhydrous and hydrated complexes, which affect the absorption process of these two compounds. The interactions between atrazine and Cd outside of the target organism might reduce the absorption process resulting in the antagonistic effects (Wang et al., 2012a).

One compound can affect the absorption rate of other through competition at biological ligands or inhibition of transport proteins. After absorption, majority of substances pass through the metabolization process where they are further bio-transformed. Metabolization can enhance or reduce the toxicity of absorbed compounds. If toxicity is caused by the parent substance and its metabolites are not toxic, then the metabolization will result in reduction of toxicity. On the other hand if metabolites of a substance are more toxic, metabolization will increase toxicity. Toxicodynamics occurs at the target organ or site of action where the exposure is translated into toxic effect. At the target organ different processes may be involved in determining the mechanisms that causes biological effects of a chemical (Rider and LeBlanc, 2005). These processes may involve inhibition of antioxidant enzymes, damage of DNA through binding of toxicants to proteins.

6. Summary

In ecotoxicological test model organisms are usually chosen from the species that are easy to handle in the laboratory conditions. The earthworm *Eisenia fetida* is prolific, cheap, commercially available earthworm specie that is very easy to handle during experiments due to its simple laboratory conditions. (OECD, 1984). Consequently, most toxicological studies have used test species from the genus of *Eisenia* including *Eisenia fetida* and *Eisenia Andrei* (Sanchez-Hernandez, 2006). The present review provides a summary of existing knowledge on the interactions between effects of pesticides and heavy metals towards earthworms. By assessing the bibliographic review concerning effects of pesticides at infra-individual level, and then looking into biological responses resulting from the interactions between pesticides and metals, we realized that evaluating toxic effects of exclusively individual pesticides can lead to underestimation of effects caused by those pesticides when they are combined with other chemicals in complex mixtures. In the environment, chemicals are often in form of mixtures resulting additive or either synergistic or antagonistic effects. One

Fig. 3. Relationship between chemical exposure and their biological effects.

typical example is the combination of atrazine and Cd which produces antagonistic response (less toxic) toward *Eisenia fetida*. Where in contrast, both chemicals at the individual level are very toxic to earthworm *Eisenia fetida*, which might give a wrong assumption to understand their toxicity based on the effects evaluated only from the individual substances. The previous study Wang et al. (2012a,b) assessed DNA damage in coelomocytes of *Eisenia fetida* worms induced by a mixture of atrazine and Cd. The individuals of *Eisenia fetida* were exposed in the artificial soils containing atrazine concentrations 0, 0.5, and 2.5 mg/kg and Cd concentrations Cd; 0, 0.03, 0.3, and 3.0 mg/kg. Their results revealed that both chemicals (at individual atrazine and Cd) could induce a significant DNA damage in earthworm coelomocytes. Their findings about DNA damage in earthworms by individual atrazine were consistent to another study conducted by Song et al. (2009), that used atrazine concentrations ranging from 0.5 to 10 mg/kg. The results of combined toxic effect revealed that the combination of atrazine and Cd reduced their individual toxicities (Wang et al., 2012a). Thus, the interaction between atrazine and Cd is reported to be antagonistic, i.e. the combined effect is lower than additive. This was explained by the fact that atrazine has a potential to interact with cadmium ions (Cd^{+2}) resulting in Atrasine-Cd complex (Meng and Carper, 2000). Another case is that of joint toxicity of acetochlor and Cu toward earthworms *E. fetida* (Liang and Zhou, 2003). Both acetochlor and Cu have been proven to be toxic towards earthworms when applied individually; however their mixture showed an interesting behavior: dual antagonistic and synergistic interaction. Cu at lower concentrations was found to weaken the capacity of acetochlor for causing toxic effects, but at higher concentrations, Cu enhanced acetochlor's toxicity toward earthworms resulting a synergistic effects (Liang and Zhou, 2003). Liang and Zhou (2003) in their study got similar findings to those of Jonker et al. (2004) concerning the determination of combined effects of the mixture copper-carbendazim on the reproduction of *Caenorhabditis elegans*. The effect of copper-carbendazim mixture on reproduction was noticed synergistic at low dose levels and antagonistic at high dose levels. Therefore, we clearly conclude that the combined effect of pollutants depend on their available concentration in the ambient environment.

Pesticides have many groups but all groups of pesticides are not likely to cause synergistic effects when combined with heavy metals. Mixtures of organophosphate insecticides and heavy metals are among the compounds that causing synergistic effects. The mode of action of organophosphate pesticides such as chlorpyrifos is to inhibit cholinesterase activity. In the study conducted by Yang et al. (2015), the combination of Chlorpyrifos and Cd in adult *Eisenia fetida* individuals led to dual antagonistic/synergistic effects in artificial soil and a moderate synergism in filter paper. Chlorpyrifos is among the most utilized pesticides in the world for both crop protection and pest control (UEP Agency, 2002). This suggests that the utilization of pesticides should be done with much attention, where knowledge of other major pollutants in the surrounding environment should not be ignored before any pesticides application.

Classical models i.e., the concentration addition and independent action were broadly used by many scientists in the measurement of mixture's toxicities (Teuschler, 2007). The concentration addition or dose addition model was built on the concept of that constituents of a given chemical combination act in a similar way. The concept of independent action or response addition model considers that the constituents of a given chemical combination act dissimilarly. These two models can only be applied in the mixtures with non-interaction between the components of mixture.

The combination index (CI)-isobologram model is another potential concept that has been proven to be efficient in determining the interactions of two or multiple components in a given mixture. CI-model is a broadly used method in toxicology to examine interactions between the contaminants and expose the type of toxicological interactions of multiple pollutants in exposed organisms (Chou and Talalay, 1984). This model can be defined on the median-effect law, that demonstrates a clear dose-effect relationship of the combined chemicals (Chou, 1976). Although, there are different models that available to quantify combined responses of chemical mixtures, the researchers still have a great challenge to identify the mechanisms behind synergistic effects caused by the chemical combinations such as those made of pesticides and metal ions. The current models, including CA, IA and CI models can only predict combined effects of mixtures, but they don't define the mechanisms behind combined responses. In this review we have collected eighteen mixtures of pesticides and metals, all of those studies in which none of them could explain well the existent mechanism behind synergistic or antagonistic effects. Among few exiting reports on the toxicity of pesticide and metal mixtures, the discussion about the mechanisms by which antagonistic or synergist effects happen is still based on speculations, which suggest further studies to define those mechanisms that cause synergism or antagonism in chemical mixtures.

The mechanisms that cause synergistic effects of pesticides and metal ions should be of priority in future studies of chemical mixtures. By understanding the mechanisms that cause synergistic effects in chemical mixtures may help to prevent mixtures to reach the level of causing synergistic effects in the ecosystems. Cedergreen (2014) investigated six processes including bioavailability of the toxicant, its uptake by organism, transportation within organism's body, biotransformation, binding at the targeted site and excretion that can be affected by interactions between chemicals. He showed that theses selected processes act like a bridge between chemical interactions and the resulting joint toxicity toward organisms. However, to understand which process is affected by the interactions between pesticides and heavy metals is a key point in getting the right mechanism behind synergistic effects caused by chemical interactions.

Toxic endpoints of traditional tests such as survival, reproduction, growth and development have been mainly investigated in the earthworms compare to bioindicator species. Biomarkers have been used extensively to assess the effects of individual pesticides towards earthworms. There is a big gap in toxicological assessments of chemical mixtures using endpoints based on biomarkers such as gene and protein expression, DNA damage (comet assay), enzymes activities, Neutral Red Retention Time (NRRT) and Activity of Cholinesterase (ChE). Responses at the infra-individual level "biomarker responses" may serve as early warning of individual and population level because biomarker responses are observed at the similar or lower concentrations than those causing an adverse effects on cocoon production and cocoon viability (Booth and O'Halloran, 2001). But the biomarkers useful in the risk assessments should be linkage between biomarker responses and effects at the population level (Van Gestel and Weeks, 2004). However to our knowledge there are few studies that used earthworm's biomarkers for investigating ecological toxicity of pesticide- metal mixtures. As in soil ecosystem chemical pollution is frequently caused by complex mixtures of contaminates rather than single pollutants. The occurrence of a joint toxicity from pesticides and heavy metals is inevitable because of their huge input into most soil environmental systems. Earthworm's biomarkers have been well used to reveal the toxic effects caused by individual chemicals but we still lack enough information to understand the effects of chemical mixtures such as those made of pesticides and metal ions which are frequently formed in the environment especially in the agriculture ecosystems. Future, studies might be encouraged to investigate the potential synergistic effects from combined pollution of mixtures of pesticides and metals, and to understand the mechanisms that cause those synergistic responses to achieve a clear understanding about what is happening in agriculture systems or other environmental systems where mixtures of pesticides and metals are likely to occur; and to help the decision makers in establishing strong regulations for remediation/protection of the environment.

7. Conclusion

We conclude that synergistic interactions between pesticides and

heavy metals are evident. Some pesticides such as atrazine were found to enhance the toxic effects of Cd toward earthworms and exhibit a joint effect larger than predicted. Thus, in more than 50% of the reviewed studies, the synergistic interactions between pesticides and metals were reported. Antagonistic interactions were also detected where some mixtures showed to cause synergistic effects at lower concentrations and antagonistic effects at higher concentrations. Among the pesticides Organophosphates were recorded the most prominent chemicals causing synergistic effects when combined with heavy metals; where in risk assessment perspective, it could be argued that occurrence of synergistic interactions is the most significant and of concern.

We also addressed a gap in the knowledge to determine the real joint effects of mixtures formed by pesticides and metal ions on agricultural systems. The other challenge is to design good methodologies needed to obtain the knowledge and extrapolate it to pesticides applied in the cropping systems, i.e. designing strong tests based on short-term and standard laboratory experiments that are able to predict the real field effects of pesticides., these two challenges cannot be overcome due to lack of enough experiments to evaluate the combined effects of the same combinations of pesticides and metals on the same earthworm species at different organization levels (from infra-individual level to population level). Our findings revealed that previously more studies have been conducted based on molecular biomarkers to investigate the effects of individual pesticides, however very few studies are available to evaluate the biochemical marker responses for combined pesticides and heavy metals. We also concluded the lack of many studies conducted on other earthworm's species mostly found in agriculture soils. Moreover, the combined effects of pesticide and metal mixtures and their long-term exposures to earthworms are insufficiently explored, where the responses of contaminant mixtures are very hard to predict compared to individual contaminants. Moreover, we suggest that the chronic exposures of earthworm species to pesticide/heavy metal mixtures could improve the strategies to manage the application of some pesticides. We also suggest to investigate the impact of agricultural practices on earthworm populations and to elaborate pesticides that are more favorable to them and the functions they provide.

Conflict of interest

Authors report grants from National Natural Science Foundation of China, personal fees and non-financial support from CAS-TWAS President's fellowship program, during the conduct of the study; grants from National Natural Science Foundation of China, outside the submitted work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.etap.2017.08.001.

References

Altenburger, R., Walter, H., Grote, M., 2004. What contributes to the combined effect of a complex mixture? Environ. Sci. Technol. 38 (23), 6353–6362.
Altenburger, R., et al., 2013. Simplifying complexity: mixture toxicity assessment in the last 20 years. Environ. Toxicol. Chem. 32 (8), 1685–1687.
Andre, J., et al., 2010. Metal bioaccumulation and cellular fractionation in an epigeic earthworm (*Lumbricus rubellus*): the interactive influences of population exposure histories, site-specific geochemistry and mitochondrial genotype. Soil Biol. Biochem. 42 (9), 1566–1573.
Binderup, M.-L., et al., 2003. Combined Actions and Interactions of Chemicals in Mixtures: The Toxicological Effects of Exposure to Mixtures of Industrial and Environmental Chemicals.
Bliss, C., 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. 26 (3), 585–615.
Booth, L.H., O'Halloran, K., 2001. A comparison of biomarker responses in the earthworm *Aporrectodea caliginosa* to the organophosphorus insecticides diazinon and chlorpyrifos. Environ. Toxicol. Chem. 20 (11), 2494–2502.
Brulle, F., et al., 2011. Gene expression analysis of 4 biomarker candidates in *Eisenia fetida* exposed to an environmental metallic trace elements gradient: a microcosm study. Sci. Total Environ. 409 (24), 5470–5482.
Burgos, M.G., et al., 2005. Cu and Cd effects on the earthworm *Lumbricus rubellus* in the laboratory: multivariate statistical analysis of relationships between exposure, biomarkers, and ecologically relevant parameters. Environ. Sci. Technol. 39 (6), 1757–1763.
Cachada, A., et al., 2016. Risk assessment of urban soils contamination: the particular case of polycyclic aromatic hydrocarbons. Sci. Total Environ. 551, 271–284.
Casabé, N., et al., 2007. Ecotoxicological assessment of the effects of glyphosate and chlorpyrifos in an Argentine soya field. J. Soils Sediments 7 (4), 232–239.
Cedergreen, N., 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9 (5), e96580.
Chen, C., et al., 2011. Multilevel ecotoxicity assessment of polycyclic musk in the earthworm *Eisenia fetida* using traditional and molecular endpoints. Ecotoxicology 20 (8), 1949.
Chou, T.-C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.
Chou, T.-C., 1976. Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. J. Theor. Biol. 59 (2) 253IN1255-254276.
Chou, T.-C., 2006. Theoretical basis: experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58 (3), 621–681.
Choung, C.B., et al., 2013. The ecological effects of a herbicide-insecticide mixture on an experimental freshwater ecosystem. Environ. Pollut. 172, 264–274.
Cikutovic, M., et al., 1993. Sperm count in earthworms (*Lumbricus terrestris*) as a biomarker for environmental toxicology: effects of cadmium and chlordane. Environ. Pollut. 81 (2), 123–125.
Denoyelle, R., et al., 2007. Cholinesterase activity as a biomarker of pesticide exposure in *Allolobophora chlorotica* earthworms living in apple orchards under different management strategies. Environ. Toxicol. Chem. 26 (12), 2644–2649.
Dittbrenner, N., et al., 2011. Sensitivity of *Eisenia fetida* in comparison to *Aporrectodea caliginosa* and *Lumbricus terrestris* after imidacloprid exposure. Body mass change and histopathology. J. Soils Sediments 11 (6), 1000–1010.
Dittbrenner, N., et al., 2012. Stress protein response (Hsp70) and avoidance behaviour in *Eisenia fetida*, *Aporrectodea caliginosa* and *Lumbricus terrestris* when exposed to imidacloprid. J. Soils Sediments 12 (2), 198–206.
Fourie, F., Reinecke, S., Reinecke, A., 2007. The determination of earthworm species sensitivity differences to cadmium genotoxicity using the comet assay. Ecotoxicol. Environ. Saf. 67 (3), 361–368.
Huang, R., et al., 2009. Accumulation: subcellular distribution and toxicity of copper in earthworm (*Eisenia fetida*) in the presence of ciprofloxacin. Environ. Sci. Technol. 43 (10), 3688–3693.
Jonker, M.J., et al., 2004. Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. Environ. Toxicol. Chem. 23 (6), 1529–1537.
Jordaan, M.S., Reinecke, S.A., Reinecke, A.J., 2012. Acute and sublethal effects of sequential exposure to the pesticide azinphos-methyl on juvenile earthworms (*Eisenia andrei*). Ecotoxicology 21 (3), 649–661.
Liang, J., Zhou, Q., 2003. Single and binary-combined toxicity of methamidophos, acetochlor and copper acting on earthworms *Esisenia foelide*. Bull. Environ. Contam. Toxicol. 71 (6), 1158–1166.
Liang, S.-H., et al., 2011. Cadmium-induced earthworm metallothionein-2 is associated with metal accumulation and counteracts oxidative stress. Pedobiologia 54 (5), 333–340.
Lister, L., et al., 2011. Modelling the joint effects of a metal and a pesticide on reproduction and toxicokinetics in Lumbricid earthworms. Environ. Int. 37 (4), 663–670.
Liu, W., et al., 2009. Assessment of the genotoxicity of endosulfan in earthworm and white clover plants using the comet assay. Arch. Environ. Contam. Toxicol. 56 (4), 742–746.
Maity, S., et al., 2008. Antioxidant responses of the earthworm *Lampito mauritii* exposed to Pb and Zn contaminated soil. Environ. Pollut. 151 (1), 1–7.
Martin, H.L., et al., 2009. Measurement and modeling of the toxicity of binary mixtures in the nematode *Caenorhabditis elegans*—a test of independent action. Environ. Toxicol. Chem. 28 (1), 97–104.
Meng, Z., Carper, W.R., 2000. Effects of hydration on the molecular structure of metal ion-atrazine dimer complexes: a MOPAC (PM3) study. J. Mol. Struct. 531 (1), 89–98.
Morgan, A., et al., 2004. Differential metallothionein expression in earthworm (*Lumbricus rubellus*) tissues. Ecotoxicol. Environ. Saf. 57 (1), 11–19.
Muangphra, P., Gooneratne, R., 2011. Toxicity of commercial neem extract to earthworms (*Pheretima peguana*). Appl. Environ. Soil Sci. 2011.
Nahmani, J., Hodson, M.E., Black, S., 2007. Effects of metals on life cycle parameters of the earthworm *Eisenia fetida* exposed to field-contaminated: metal-polluted soils. Environ. Pollut. 149 (1), 44–58.
Nakashima, T., et al., 2008. 8-Hydroxydeoxyguanosine generated in the earthworm *Eisenia fetida* grown in metal-containing soil. Mutat. Res. 654 (2), 138–144.
Nriagu, J.O., Pacyna, J.M., 1988. Quantitative assessment of worldwide contamination of

air, water and soils by trace metals. Nature 333 (6169), 134–139.
OECD, T.N., 1984. Earthworm, Acute Toxicity Tests. OECD Guidelines for the Testing of Chemicals. Organization for Economic Co-operation and Development, Paris.
Pauwels, M., et al., 2013. Using biomarkers in an evolutionary context: lessons from the analysis of biological responses of oligochaete annelids to metal exposure. Environ. Pollut. 179, 343–350.
Peakall, D.B., 1994. The role of biomarkers in environmental assessment (1). Introduction. Ecotoxicology 3 (3), 157–160.
Reinecke, A., Reinecke, S., 1996. The influence of heavy metals on the growth and reproduction of the compost worm *Eisenia fetida* (*Oligochaeta*). Pedobiologia 40 (5), 439–448.
Rial-Otero, R., et al., 2004. Parameters affecting extraction of selected fungicides from vineyard soils. J. Agric. Food Chem. 52 (24), 7227–7234.
Rider, C.V., LeBlanc, G.A., 2005. An integrated addition and interaction model for assessing toxicity of chemical mixtures. Toxicol. Sci. 87 (2), 520–528.
Rizhiya, E., et al., 2007. Earthworm activity as a determinant for N2O emission from crop residue. Soil Biol. Biochem. 39 (8), 2058–2069.
Sanchez-Hernandez, J., 2006. Earthworm biomarkers in ecological risk assessment. Reviews of Environmental Contamination and Toxicology. Springerpp. 85–126.
Schnug, L., Leinaas, H.P., Jensen, J., 2014. Synergistic sub-lethal effects of a biocide mixture on the springtail *Folsomia fimetaria*. Environ. Pollut. 186, 158–164.
Schreck, E., et al., 2012. Ecological and physiological effects of soil management practices on earthworm communities in French vineyards. Eur. J. Soil Biol. 52, 8–15.
Song, Y., et al., 2009. DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia foetida*) induced by atrazine. Soil Biol. Biochem. 41 (5), 905–909.
Spurgeon, D.J., Hopkin, S.P., 1996. The effects of metal contamination on earthworm populations around a smelting works: quantifying species effects. Appl. Soil Ecol. 4 (2), 147–160.
Spurgeon, D., Hopkin, S., Jones, D., 1994. Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny): assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. Environ. Pollut. 84 (2), 123–130.
Spurgeon, D.J., Weeks, J.M., Van Gestel, C.A., 2003. A summary of eleven years progress in earthworm ecotoxicology: the 7th international symposium on earthworm ecology· Cardiff· Wales· 2002. Pedobiologia 47 (5–6), 588–606.
Stenersen, J., 1979. Action of pesticides on earthworms. Part I: the toxicity of cholinesterase-inhibiting insecticides to earthworms as evaluated by laboratory tests. Pestic. Sci. 10 (1), 66–74.
Teuschler, L.K., 2007. Deciding which chemical mixtures risk assessment methods work best for what mixtures. Toxicol. Appl. Pharmacol. 223 (2), 139–147.
UEP Agency, 2002. Interim Reregistration Eligibility Decision for Chlorpyrifos. US Environmental Protection Agency, Washington, DC.
Uwizeyimana, H., Wang, M., Chen, W., 2017. Evaluation of combined noxious effects of siduron and cadmium on the earthworm *Eisenia fetida*. Environ. Sci. Pollut. Res. 24 (6), 5349–5359.
Van Gestel, C., Van Brummelen, T., 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Ecotoxicology 5 (4), 217–225.
Van Gestel, C., Weeks, J.M., 2004. Recommendations of the 3rd international workshop on earthworm ecotoxicology, Aarhus, Denmark, August 2001. Ecotoxicol. Environ. Saf. 57 (1), 100–105.
Velki, M., Hackenberger, B.K., 2013. Biomarker responses in earthworm *Eisenia andrei* exposed to pirimiphos-methyl and deltamethrin using different toxicity tests. Chemosphere 90 (3), 1216–1226.
Wang, J.H., et al., 2012a. The combined stress effects of atrazine and cadmium on the earthworm *Eisenia fetida*. Environ. Toxicol. Chem. 31 (9), 2035–2040.
Wang, J.-h., et al., 2012b. Biochemical responses of earthworm (*Eisenia foetida*) to the pesticides chlorpyrifos and fenvalerate. Toxicol. Mech. Methods 22 (3), 236–241.
Wang, Y., et al., 2015. Ternary toxicological interactions of insecticides, herbicides, and a heavy metal on the earthworm *Eisenia fetida*. J. Hazard. Mater. 284, 233–240.
Warne, M.S.J., Hawker, D.W., 1995. The number of components in a mixture determines whether synergistic and antagonistic or additive toxicity predominate: the funnel hypothesis. Ecotoxicol. Environ. Saf. 31 (1), 23–28.
Xiao, N.-W., et al., 2006. Biomarkers responses of the earthworm *Eisenia fetida* to acetochlor exposure in OECD soil. Chemosphere 65 (6), 907–912.
Xu, J., et al., 2006. Toxicity effect of combined contamination of two herbicides to earthworm. J. Agro-Environ. Sci. 5, 018.
Yang, G., et al., 2015. Joint toxicity of chlorpyrifos, atrazine, and cadmium at lethal concentrations to the earthworm *Eisenia fetida*. Environ. Sci. Pollut. Res. Int. 22 (12), 9307.
Žaltauskaitė, J., Sodienė, I., 2010. Effects of total cadmium and lead concentrations in soil on the growth, reproduction and survival of earthworm *Eisenia fetida*. Ekologija 56 (1–2), 10–16.
Zang, Y., et al., 2000. Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*. Environ. Pollut. 108 (2), 271–278.
Zhang, Z.S., et al., 2009. Bioaccumulation of total and methyl mercury in three earthworm species (*Drawida* sp., *Allolobophora* sp., and *Limnodrilus* sp.). Bull. Environ. Contam. Toxicol. 83 (6), 937–942.
Zhou, C.-F., et al., 2013. Subacute toxicity of copper and glyphosate and their interaction to earthworm (*Eisenia fetida*). Environ. Pollut. 180, 71–77.