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Combined Exposure to Mixture of Chemicals. An Impossible Challenge?

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1. Introduction

The harmful effects of the human activities on health and the environment are known for a very long time but the public awareness is recent and dates from second half of the 20th century. Living organisms are almost constantly exposed to many stressors. Among them, chemical pollutants play a major role. A wide range of chemical substances act as pollutants, ranging from simple inorganic ions to complex organic molecules. Some metals such as cadmium, mercury, lead provoke adverse effects of human health when they are present at high level of exposure. Radioactive isotopes may be harmful to organisms, depending on the dose and type of radiation. Numerous organic compounds are also known to be noxious: hydrocarbons (i.e. benzene, polycyclic aromatic hydrocarbons, PAHs), polychlorinated phenols (PCPs) polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated biphenyls (PBBs), organochlorine pesticides (OCs), organophosphorus pesticides (OPs), carbamates, pyrethroid insecticides, phenoxy herbicides, rodenticides, organometallic compounds and so on (Walker et al., 2001). Some of these chemicals are of concern because of their human toxicity. Other chemicals cause damages to non-human biota but are not believed to be harmful to humans. Finally, some other pollutants are not directly toxic to humans or other biota at current environmental concentrations, but are capable to modify environmental features causing major environmental damage (i.e. chlorinated fluorinated carbons, CFCs, known to drastically disturb the chemistry of the stratosphere). In this chapter, only the pollutants harmful to living organisms are considered, keeping in mind that non chemical stressors may act at the same time on biota.

Any substance can have adverse effects on cell biology and/or on whole organism, but this depend on dose and chemical speciation. Toxicity and ecotoxicity are defined as the capacity to cause injury to a living organism (human or not) defined with reference to the quantity of substance absorbed, the way in which the substance is taken up and distributed in time (single or repeated doses), the type and severity of injury, the time needed to produce the injury, the nature of the organism(s) affected, and other relevant conditions (Duffus et al., 2009).

Any chemical of concern has to be taken up by an organism before it can produce an effect. Once absorbed, the potentially toxic substance will be distributed throughout the organism and the absorption of the toxicant will result in a toxic effect and a response defined as the percentage of the exposed population showing the defined toxic effect. To quantitatively

describe toxicological effects of a given substance, one has to define a reference value that characterizes safe exposure. Very often, median dose lethal to 50% (LD_{50}) of a test population was used as a reference, whereas an increasing number of toxicologists and ecotoxicologists favour now the benchmark concentration (BMC) or dose (BMD). The benchmark concentration (or dose) is the statistical lower confidence limit on the concentration (or dose) that produces a defined response (called the benchmark response or BMR, usually 5 or 10 %) for an adverse effect compared to the background, defined as 0%.

After pollutant uptake, subsequent elimination and clearance of the substance from the organism will occur due to various biological and biochemical processes. The biological half-life is the parameter used to describe the progressive reduction in the pollutant internal concentration. Similarly, in environmental compartments such as air, water, soil, sediment, the pollutant concentration may decrease or not depending on various ecological processes and chemical properties of the pollutant. Persistence is the key concept which describes the ability of a substance to stay in a given environmental compartment. The way in which the substance is distributed in time (single or repeated doses) is also a key factor modulating toxicity of chemicals. Consequently, one has to distinguish between acute, subchronic or chronic toxicity, which may be very different for a given chemical. Therefore, LD_{50}, BMD, biological half-life, persistence, and ways of exposure are very important issues in risk assessment for toxicant effects on humans or non-human biota.

Toxicological and ecotoxicological studies have produced a considerable corpus of knowledge which has been used to draw rules and regulations for managing chemicals of concern. However, most toxicological and ecotoxicological studies focus on exposure and effects of single compounds, whereas in real world, organisms are submitted to many pollutants often acting at low doses and at the same time. The chemical substances do not act independently. The living organisms are permanently exposed to multiple substances acting in a concomitant way. It is therefore crucial for scientists and policy-makers involved in the field of (eco)toxicology to develop, use and refine efficient methods for risk assessment of combined exposures to various toxicants and chemical mixtures. Up to now, most of the methods for the management of chemical compounds are based on single-substance risk evaluations. When risk assessment of multiple chemicals are required, single-substance toxicity data are used to derive mixture toxicity using a limited number of methods and models. The objective of the present chapter is to give a brief overview of the methods currently available to assess combined exposure toxicity. We will first give some basic concepts and terminology, and we will review the state-of-the-art of the current available tools and methodologies. Then, we will use the case-study of wood preservative toxicity to illustrate some of the difficulties and gaps of the current methodologies.

2. The general framework of chemical risk assessment. Basic concepts and terminology.

2.1 The four stages of the environmental risk assessment of chemicals.

Numerous biological, physical, and chemical *stressors* are harmful to humans, biota, and ecosystems. These agents are perceived like threats and cause concerns within the human society. They may exert *adverse effects* at different biological levels: they disturb molecular and cell biology, but also the physiology of whole organism. *Responses* occur at population, community, and/or ecosystem levels. Various means have been implemented to face these environmental and health problems.

Chemical risk assessment may pursue various objectives. One may try to reduce human exposure to chemicals of concern. An other frequent goal is the reduction of health effects. Risk assessment may also be devoted to the mitigation of ecological impacts, or to the protection of vulnerable populations. Evaluation can be done before (*a priori* assessment) or after (*a posteriori* assessment) exposure to toxicants.

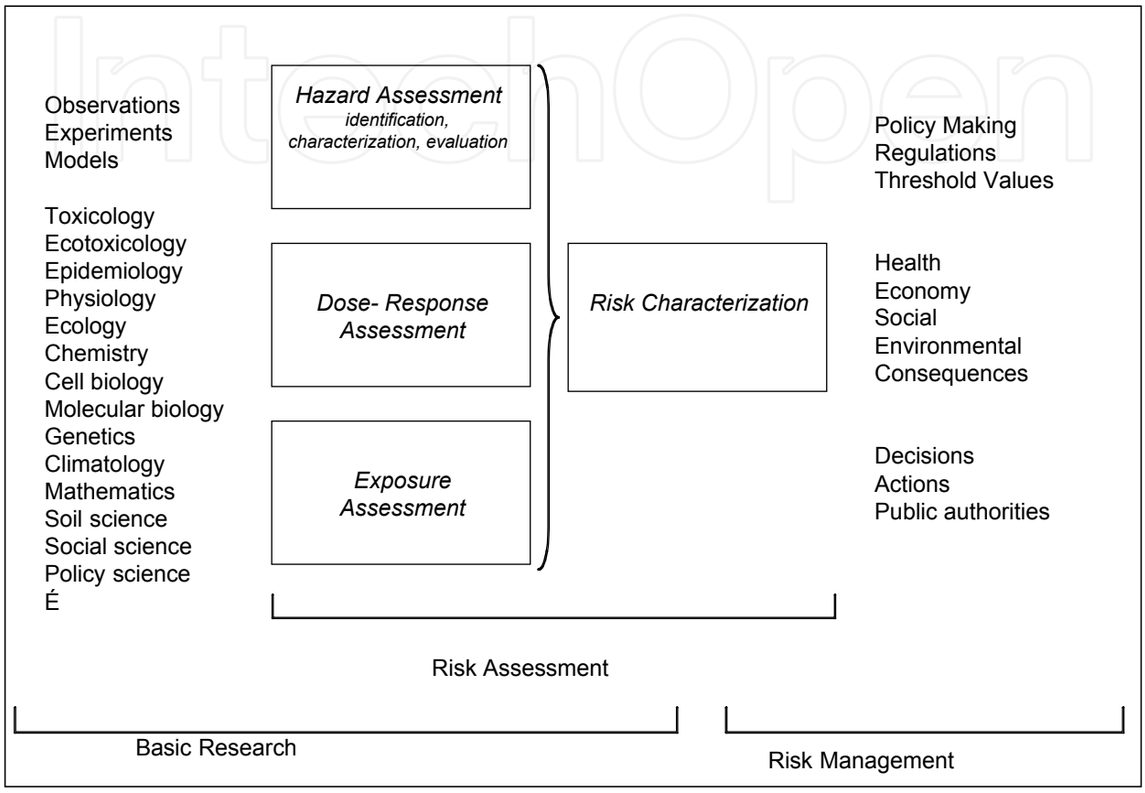

Fig. 1. The four stages of the environmental risk assessment of chemicals.

Whatever the objective, the Environmental Risk Assessment (ERA, Fig. 1) is usually carried out in four stages. Hazard is a set of inherent properties of a substance, a mixture of substances, or a process that make it capable of causing adverse effects to living organisms or the environment. Hazard is a source of danger: during *hazard assessment*, the dangerosity of a chemical is evaluated independently of the probability of occurrence of the damage. During this stage, the potential causes of damage are exhaustively reviewed and clearly identified. The substances of concern and the adverse effects that they may produce are identified and a list is made. All the relevant informations relating to toxicity are gathered in the form of material safety data sheets (MSDS). Then, the hazard characterization consists of the qualitative and quantitative description of the hazard associated with the agent of concern. During the second stage, the relationship between exposure to a hazard (dose) and the resultant adverse effects (responses) are comprehensively described and *dose-response assessment* is produced. Dose-response assessment always involves extrapolation of results from an experimental or observation group to an entire population. This stage necessarily includes a part of uncertainty, which has to be clearly stated before managing decisions. The third stage is devoted to *exposure assessment*: a complete description of exposure is

performed. Exposed populations, levels and pathways of exposure are studied. Then, all these data are integrated during the *risk assessment* stage which aims to produce a quantitative description of the probabilities of the damage. Risk assessment provides quantitative estimation, including uncertainties, of the severity and probability of occurrence of known and potential adverse effects of a substance in a given population.
The environmental risk assessment is based on multidisciplinary approaches involving observations, experiments, and models from various fields of science. Once an ERA is available, *policy makers* have to define regulations, which often result in threshold values. *Public authorities* are in charge of the *risk management* involving relevant decisions and actions. Such procedure is used worldwide, but one has to keep in mind that it has been implemented for single-chemical toxicity. It fails to be fully efficient to predict risks linked to combined exposure to multiple chemicals. Although some potential environmental hazards involve significant exposure to only a single chemical, most instances of environmental contaminations involve concurrent or sequential exposures to several compounds that may induce similar or dissimilar effects over exposure periods ranging from short-term to lifetime (U.S. EPA, 2000).

2.2 Basic concepts and terminology.

A quick survey of the scientific literature may convince anybody that there is a very rich terminology in the field of mixture toxicity, but this terminology remains sometimes unclear and sometimes contradictory. In the following paragraphs, we try to summarize the main concepts and definitions.
A very high number of terms are applied to toxic substances in the scientific literature. We will consider that a *contaminant* is any substance detected in a place where it is not normally found. *Pollutant* is any chemical found in the environment causing adverse effects or harms to living organisms, or disturbances to the ecosystem structure and function. Toxicologists often refer to *toxicant* for any substance that is capable to provoke injuries to living organisms as a result of physicochemical interactions under circumstances which are thought likeky to happen. *Poison* is nearly a synonym of toxicant, but is usually applied to substances deliberately used to impair the health of the organism or to kill it. *Drug* is any substance that, when absorbed into a living organism, may alter its functions. *Biocides* are substances intended to kill living organisms. *Pesticides* are specific biocides intended to kill pests.
Following the U.S. Environmental Protection Agency (1986, 1987), a *mixture* will be defined as any combination of two or more chemical substances, regardless of source or of spatial or temporal proximity, that can influence the risk of chemical toxicity in the target population. Mixtures may be highly complex originating for a single source or process as by-products (diesel exhaust, municipal incinerator, etc.). In other instances, chemical mixtures are man-made commercial products (e.g. pesticide formulations, PCBs, gazoline). In some other cases, environmental releases, waste disposals, or storages of various chemical compounds cause combined exposures. Multichemical exposures are ubiquitous, including air, water, soil and food contaminations from various sources.
Scientific literature contains many definitions, about chemical mixtures and mixture toxicity. Therefore, key concepts must be clearly listed and defined before describing toxicity of chemical mixtures. Table 1 gives a summary of the most commonly used definitions. A *chemical mixture* corresponds to any set of multiple chemical substances regardless of their sources that may jointly cause toxicity in the target population. The *components* of the

mixture may or may not be identifiable. *Similar components* are components with the same or similar biological activities. Literature often refers to this chemical mixture as whole mixture or mixture of concern. A mixture can be simple or complex. A *simple mixture* is considered as any mixture that toxicity can be adequately characterized with the help of the combination of the single toxicities and interactions of its components. Usually, such simple mixture contains a small number of identifiable single chemicals. Unfortunately, real world case studies most often involve complex mixtures. One has to consider as a *complex mixture* any mixture containing so many components that it is not possible to properly characterize its toxicity from data based on components' toxicities and interactions. Risk assessment of complex mixture are based on toxicity and exposure data on the whole mixture. Mixtures that displayed similar characteristics for transport, fate, physiological processes, and toxicity are known as *similar mixtures*. Very often, they only differs by a small number of features. Moreover, similar mixtures frequently contain groups of components that are similar in chemical structure and biological activity and also originate together from the same kind of sources (e.g. diesel exhaust, municipal incinerator). Such similar components belong to the same *chemical class*.

component	single chemical that may enter in the composition of a chemical mixture
similar component	single chemicals with the same or similar biological activities.
chemical mixture	any set of multiple chemical substances regardless of their sources that may jointly cause toxicity in the target population.
simple mixture	any mixture containing two or more identifiable single chemicals, but few enough that the mixture toxicity can be adequately characterized.
complex mixture	any mixture containing so many components that any estimation of its toxicity contains too much uncertainties and error to be useful.
similar mixtures	mixtures that are expected to have comparable characteristics for toxicity.
chemical class	any group of components displaying similar chemical structure and biological activity.

Table 1. Definitions and key concepts widely used in assessment of mixture toxicity. Components and mixtures (U.S. EPA, 1987)

The concept of toxicological similarity is based on data dealing with the biological activities of chemicals. In this matter, the literature frequently refers to the *mode of action* as a series of events and processes starting with interaction of an agent with a cell, causing disturbances and damages (Table 2). The sequence of events has to be supported by experimental evidences and a clear link must be identified between the adverse effect and the chemical. The reference to the *mechanism of action* implies a more detailed understanding and a deeper description of the cascade of events. One has to clearly distinguish between aggregate and cumulative exposures. *Aggregate exposure* refers to single chemical toxicity. It is the whole exposure to a single chemical whatever the *exposure pathways* (food, water, air, residential uses, occupational) and the *exposure routes* (oral, dermal, inhalatory, external). The associated risk is the *aggregate risk. Cumulative exposure* and corresponding *cumulative risk* refer to multiple chemicals, whatever the pathways and routes. One has to emphasize that temporality of exposure/effects plays a key role in aggregate and cumulative exposure assessments.

mode of action	series of events or processes resulting in an adverse effect.
mechanism of action	detailed description and understanding of the molecular events explaining biological activity
aggregate exposure	demographic, spatial and temporal characteristics of exposure to a single chemical through all relevant pathways and routes.
aggregate risk	risk associated with aggregate exposure.
cumulative exposure	aggregate exposure to multiple chemicals.
cumulative risk	risk associated with cumulative exposure
exposure pathway	any physical way that contributes to a physical interaction between chemicals and living organisms
exposure route	any process that permits the entry of a chemical into an organism or the interaction between the toxicant and the organism

Table 2. Definitions and key concepts widely used in assessment of mixture toxicity. Biological action, exposures, risks. (U.S. EPA, 1987 & WHO ICPS, 2009)

Several other concepts may be remembered, because of their importance in predicting toxicity and assessing risks. It is now well established that *speciation*, the occurrence of an element in different forms, is crucial to understanding its toxicity (Duffus et al., 2009). The *chemical species* include isotopic composition, electronic or oxidation state, and/or complex or molecular structure. *Bioaccessibility* is the potential for a substance to come in contact with a living organism. For instance a substance trapped inside a particule is not bioaccessible, whereas a part of the substance adsorbed on the surface of a particule are accessible. *Bioavaibility* describes the potential for a substance to be taken up by a living organism. Bioavaibility depends on both physicochemical properties and biological capabilities.

3. Non interactive chemicals. Additivity.

Very early, Plackett & Hewlett (1952) have identified the four possible types of joint action for mixtures (Table 3).

Types	Similar joint action	Dissimilar joint action
Non interactive	Simple similar action (concentration addition)	Independent joint action (response addition)
Interactive	Complex similar action	Dependent joint action.

Table 3. The four possible types of joint action for mixtures (Plackett & Hewlett, 1952).

The four types essentially refer to binary mixtures. In real world, chemical mixtures often contain numerous substances. Moreover, interactions are thought at the molecular level in terms of mode of action. Other interactions between chemicals may occur at other biological levels. Nevertheless, these authors have clearly distinguished two key points of joint action: (i) the similarity or dissimilarity of the modes of action and (ii) the dependence or independence of chemical actions. Indeed, mixture components exert their toxicity independently or not. They may also have toxicological interactions or not. These properties have been used to define different ways of assessing mixture toxicity.

Revisiting the concepts from Plackett & Hewlett, Ashford (1981) has distinguished six possible combination mechanisms for the joint action of mixtures or drugs (Table 4). The

author considers that the different subsystems (i.e. nervous, cardiovascular, endocrine subsystems…) have to be studied independently. Thus, it becomes possible to estimate the respective contributions of the different subsystems to the response of the whole organism. He has also proposed to take into account possible interactions between chemical substances occurring into the different subsystems. The model also identifies the possibilities of partial interactions between chemicals. Such partial interactions better correspond to the real physiology of the organisms.

Correspondence between compounds in the mixture	None	Some	All
Common sites of action (similarity)	Dissimilar (and noninteractive	Partially similar	Fully similar
Common subsystems (dependence)	Independent (and noninteractive)	Partially dependent	Fully dependent

Table 4. The six possible combination mechanisms for the joint action of toxicants (Ashford, 1981)

A key concept in understanding mixture risk assessment is *toxicologic similarity*. In this case, one assumes a similar mode of action across mixture components. Sometimes, the mode of action is not the same and components act only on the same target organ.
In contrast, *independence of action* is defined as mixture components that cause different kinds of toxicity, or effects in different target organs. The term *additivity* is used when the toxicity of the combination of chemicals can be estimated directly for the sum of the exposure levels (*dose additivity*) ot of the responses (*response additivity*).

3.1 Dose additivity or concentration addition.

When the components of a chemical mixture have the same mode of action, the mixture toxicity is assessed by the sum of the dose of the components (Loewe & Muischnek, 1926). The dose additivity or concentration addition (CA) concept is devoted to similarly acting toxicants. Sprague (1970) proposed a derived concept: the toxic unit approach (TU). In this hypothesis any component can be replaced by another if they display the same action mechanism as long as the corresponding relative toxic potency allows to obtain a similar toxic unit.
This method has been refined and is currently used to assess the toxicity of several chemical classes (US EPA, 2000). One considers that each component of the mixture behaves as a concentration or dilution of every other chemical in the mixture. The response of the combination is the response expected from the equivalent dose of an index chemical. This index chemical is selected as the basis for standardization of toxicity of components in a mixture. The index chemical must have a clearly defined dose-response relationship. The equivalent dose is the sum of component doses scaled by their toxic potency relative to the index chemical.

$$C_m = \sum_{k=1}^{n} C_k \times RPF_k \tag{1}$$

where
C_m is the mixture concentration expressed as an equivalent of the index chemical,
C_1 is the concentration of the index chemical,
C_k is the concentration of the k component,
RPF_k is the relative potency factor relative to the index chemical ($RPF_1 = 1$).
PCDDs and PCDFs commonly called dioxins, are by-products of combustion processes. PCBs were manufactured in the past for a variety of industrial uses, as electric insulators, dielectric fluids and hydraulic fluids. Most countries banned the manufacture and use of PCBs in the 1970s. Improper handling of PCBs is responsible of a continuing source of environmental contamination. Dioxins, furannes, and co-planar polychlorobiphenyls (PCBs) are Persistent Organic Pollutants (POPs), which are known to have the same mechanism of action since they are Aryl hydrocarbon Receptor (AhR) ligands. AhR is a cytosolic transcription factor that is normally inactive, bound to several chaperones. Toxicity results of the activation of AhR signaling pathways.
2,3,7,8-tetrachlorodibenzodioxin (TCDD) is the most potent congener of this group and is considered one of the most potent toxicants and carcinogens known to date. Since PCDDs, PCDFs, and PCBs occur as complex mixtures in food, this chemical class of compounds poses some risks for humans. Consequently, methods have been developed to assess cumulative risk related to dioxins and dioxin-like compounds thanks to the World Health Organization.

$$TEQ = \sum_{i=1}^{n} Ci \times TEFi \tag{2}$$

where
TEQ, toxic equivalency quantity is expressed in toxic equivalents of the 2,3,7,8-TCDD, i.e. the index chemical,
C_1 is the concentration of the 2,3,7,8-TCDD,
C_i is the concentration of the i component,
TEF_i is the toxic equivalency factor, that is the relative potency factor relative to the index chemical ($TEF_1 = 1$).
TEFs values are estimates derived from experimental data (see for instance Van den Berg, 1998). TEFs have been recently reevaluated (Van den Berg, 2006) and uncertainties were assumed to be within 1 order of magnitude. The underlying principle of effect additivity has been confirmed by recent data.
When the chemical components have the same mode of action, but the mechanism of action is not accurately known, it is not possible to use the RPF or TEQ methods with a high level of confidence. In such cases, an alternative method has been proposed. The hazard quotient is the ratio of the potential exposure to the substance to the level at which no adverse effects are expected. The hazard quotient is based on the estimation of exposure and its comparison with a reference level supposed to be acceptable.

$$HQ_i = \frac{E_i}{RfD_i} \tag{3}$$

where
HQ_i is hazard quotient for the substance i,

E_i is the exposure to the substance i,
RfD_i is the reference dose (acceptable level) for the substance i.
This hazard index method is a simple addition method: the hazard index is the sum of hazard quotients for substances that affect the same target organ.

$$HI = \sum_{i=1}^{n} HQ_i \tag{4}$$

where HI is the hazard index for the chemical mixture.
A more simple additive method has also been used. The *point of departure index* (PDI) consists in the addition of the *no observed adverse effect level*s (NOAEL) or benchmark doses (BMD). All these methods require additivity of the doses or concentrations.
The *margin of exposure* (MOE) method is rather close to the HI and PDI methods. It is based on the estimation of the ratio of the no-observed adverse effect level to the estimated exposure dose. Similarly, margins of exposure of components of a mixture may be summed.
Basic concepts supporting dose additivity or response additivity are briefly summarized in Table 5. Unfortunately, none of these methods takes into account possible interactions between the components of the mixture.

index chemical	The chemical selected as the basis for standardization of toxicity of components in a mixture. The index chemical must have a clearly defined dose-response relationship.
dose additivity concentration addition	When each component of the mixture behaves as a concentration or dilution of every other chemical in the mixture, the response of the combination is the response expected from the equivalent dose of an index chemical. The equivalent dose is the sum of component doses scaled by their toxic potency relative to the index chemical.
response additivity independence of action	The toxic response from the combination of chemicals is equal to the conditional sum of components responses as defined by the formula for the sum of independent event probabilities.
RPF	Relative Potency Factor (see Eq. 1)
TEF	Toxic Equivalency Factor (see Eq. 2)
TEQ	Toxic Equivalency Quantity (see Eq. 2)
HQ	Hazard Quotient (see Eq. 3)
HI	Hazard Index (see Eq. 4)
PDI	The Point of departure index is the simple addition of the no observed adverse effect levels (NOAEL) or benchmark doses (BMD).
MOE	The margin of exposure is the ratio of the no-observed adverse effect level to the estimated exposure dose.

Table 5. Definitions and key concepts used for mixture toxicity assessment when components of the mixture do not interact. Additivity. Independence of action. (U.S. EPA, 2000 & WHO ICPS, 2009)

3.2 Response additivity or independence of action.

One of the first paper dealing with mixture toxicity is the one from Bliss (1939), who proposes the method known as *response additivity*. This approach is used when the mixture

components act independently on different targets. The response of the mixture is given by the sum of the responses of its components. For the noninteractive or independent types of joint action, one has to keep in mind that it is assumed that the components of the mixture do not affect the toxicity of one another.
In such a case, the toxic response from the combination of chemicals is equal to the conditional sum of components responses as defined by the formula for the sum of independent event probabilities (ATSDR, 2004). For instance, for a binary mixture, the cumulative risk may be given by Eq. 5:

$$p_m = 1 - (1-p_1) \times (1-p_2) \quad (5)$$

where
p_m is the risk related to the exposure to the mixture,
p_1 is the risk related to the exposure to component 1,
p_2 is the risk related to the exposure to component 2.

3.3 Critical overview of the CA and IA models

Two basic concepts have been generally used for predicting multiple mixture toxicity: concentration addition (CA, Loewe and Muischnek, 1926) and independent action (IA, Bliss, 1939).
It has been proved that the CA model provides highly accurate predictions of mixture toxicity when all of the components have a strictly similar mode of action, regardless of their levels and ratios in the mixture (Faust et al., 2001; Zwart and Posthuma, 2005; Junghans et al., 2006). However the CA model is not adapted to mixtures with components having dissimilar modes of action because it leads to an overestimation of the toxicity of such mixtures (Faust et al., 2003).
The independent action (IA) model is based on dissimilar actions of mixture components. In this approach, the toxicity of each component is independent and cannot be replaced by another. The basic idea of this approach is that different compounds act on different physiological systems within the exposed organisms and lead to a common toxicological endpoint. This model provides accurate predictions of the mixture toxicity when all of the components have dissimilar modes of action, regardless of their levels and ratios in the mixture (Faust et al., 2003). However the IA model is not adapted to mixtures with similar acting components because it leads to an underestimation of the overall toxicity (Faust et al., 2001; Junghans et al., 2006).
Two main difficulties still remains. First, chemical with and without the same mode of action are very often found in the same mixture. Second, components may toxicologically interact. Furthermore, interspecific differences and possible interactions at the ecological levels are not satisfactorily addressed by the available models.
Recently, Zwart and Posthuma (2005) proposed a mixed two-step approach for mixed-model (MM) calculations. The first step requires evaluation of the CA responses to each individual toxic mode of action, the second step consists in evaluating the IA effect of the different toxic modes of action. We have used such a model to assess toxicity of a mixture of wood preservatives. The experimentals, the results and the main conclusions are given in section 5 (see below). In conclusion, one has to remember that the assessment of the predicting values of the available models is still an opened question (Backhaus et al., 2003; Zwart and Posthuma, 2005; Junghans et al., 2006).

4. Interactive chemicals. Different types of toxicological interactions between chemicals

A common concern for evaluating chemical mixtures is the potential for toxicological interactions to occur from co-exposures. Usually, one considers that toxicological interactions occur when the responses observed deviate from those expected under additivity.

4.1 The Different types of toxicological interactions between chemicals.

When two or more chemicals are combined, they may interact in different ways. The most simple toxicological interactions are *synergism* and *antagonism*. Other interactions, such *potentiation, inhibition* or *masking* may also modulate possible adverse effects. Different types of toxicological interactions between chemicals are briefly summarized in Table 6.

synergism	The combined effect of several chemicals is greater than expected on the basis of the simple summation of the toxicity of each of the individual substances
potentiation	When one substance does not have a toxic effect on a system, but when added to a toxic chemical, it makes the latter more toxic
antagonism	The combined effect of several chemicals is smaller than the solitary effect of any one of those chemicals
inhibition	When one substance does not have a toxic effect on a system, but when added to a toxic chemical, it makes the latter less toxic
masking	When the compounds produce opposite or functionally competing effects at the same site or sites, so that the effects produces by the combination are less than suggested by the component toxic effects.
no influence	When one substance does not have a toxic effect on a system, and but when added to a toxic chemical, it has no influence on the toxicity of the latter chemical.

Table 6. Types of toxicological interactions (Duffus et al., 2009; US EPA, 2000; ATSDR, 2004)

The relations between additivity, similarity of the modes of action, and interactions are listed in Table 7, which gives a theoretical overview of the relations between toxicological interactions and similar or dissimilar joint actions.

	Toxicological interaction	Joint action
No interaction	Dose additivity	Simple similar action
	Response additivity	Simple dissimilar action Independent action
Interaction	Synergism	Complex similar action Effect > additivity
	Potentiation	Complex dissimilar action Effect > additivity
	Antagonism	Complex similar action Effect < additivity
	Inhibition	Complex dissimilar action Effect < additivity

Table 7. Relations between toxicological interactions and similar or dissimilar joint actions

4.2 Interactions between chemicals and newly developed methods for assessment of mixture toxicity

Besides additivity models, there are very few available methods to take into account the toxicological interactions possibly occurring between the components of a mixture (WHO IPCS, 2009). Among these methods, one has to cite qualitative *binary weight of evidence* (BINWOE) proposed from ATSDR (2007). BINWOE evaluates strength of interactions data, mechanism of action, influence of exposure duration and route, and sequence of exposure for each pair of chemicals. For instance, a method has been developed to quantitatively modify the hazard index (HI), using factors that account for interaction weight of evidence, interaction magnitude, fraction of toxic hazard of each interacting chemical pair and relative proportions of the chemicals (Teuschler, 2009; USEPA, 2000). Among the methods currently in development, one has to list Physiologically Based Pharmacokinetic (PBPK) models. Such methods have been used (Haddad et al., 2001) to compare an interaction-based HI for central nervous system effects with an additive HI for different exposure to mixtures of several hydrocarbons showing greater than additive effects at the higher total dose levels of the mixture.

The *Whole Mixture Approach* (Mumtaz et al., 1993) uses effects data from exposure to the mixture of concern. These data are treated as if the mixture behaves like a single substance. Lastly, the *Threshold of Toxicological Concern (TTC)* has been proposed for use with complex mixtures where no effects data are available (Kroes et al., 2005). This method is based on structure–activity relationships and assigns exposure thresholds for comparison with the potential exposure level.

Species differ in their sensitivity toward a single chemical as a result of differences in biological traits (De Zwart & Posthuma, 2005). At the ecosystem level, the risk of chemical exposure to a single compound may be characterized by the proportion of species from a generic species pool that is likely affected by a toxicant at a certain concentration. The *potentially affected fraction* of species (PAF) is used to quantify the risk for species assemblages. Using this concept together with the mixture toxicity models (CA and IA models), De Zwart & Posthuma (2005) have proposed a method to address the risk on direct effects on the composition of species assemblages and biodiversity. This method has still to be validated.

5. Several outcomes from a real world case-study: wood preservatives

5.1 Possible impacts of wood preservatives on aquatic organisms

Wood, especially from coniferous trees is very frequently treated with various pesticides, commonly called wood preservatives (essentially insecticides and fungicides), to prevent attacks by pathogenic agents such as xylophagous insects or lignivorous fungi. Treatments avoid alterations of the wood mechanical qualities, and consequently economic loss or lifespan reduction. Treatment occurs at different stages of the production in tree nurseries, during wood storage, or at sawmills.

Historically, sawmills were established very close to the forests in basin heads along the rivers to get easy energy from water. Consequently, the risk of contaminations of aquatic environment with wood preservative mixture is considered as very high (Gifford et al., 1996; Lyytikaïnen et al., 2001; Hingston et al., 2002, 2006). After accidental or routine releases, wood preservatives exert marked adverse effects on macroinvertebrates and fish populations, and in a more general way in aquatic communities. Moreover, one has to keep

in mind that basin heads may constitute an invaluable resource for drinking water and biodiversity.

5.2 investigations on mixture toxicity of wood preservatives

Very often, wood preservatives, as other pesticides are used as commercial solutions. These commercial solutions of wood preservatives contain mixture of several active chemicals. Therefore, in case of accidental (acute) or routine (chronic) releases in the natural environment, aquatic organisms are exposed to several chemicals at the same time (Helson & Surgeoner, 1986; Green & Abdelghani, 2004).

	mixture 0 (M0)	mixture 1 (M1)			mixture 2 (M2)		
Chemicals	Concentrations in the commercial mixture EX 2002 E.S.E.® (mM)	Concentra-tions (mM)	Pesticide ratios in mixture 1	Toxic Units (%)	Concentra-tions (mM)	Pesticide ratios in mixture 2	Toxic Units (%)
Propiconazole	3.62	3.62	45.8%	<0.01%	9888**	76.2%	23.0%
Tebuconazole	1.36	1.36	17.2%	<0.01%	2612**	20.1%	11.5%
IPBC	1.49	1.49	18.8%	0.01%	480**	3.7%	30.5%
Cypermethrin	1.44	1.44	18.2%	99.98%	0.226*	0.0017%	35.0%

Table 8. Concentrations of active substances in the commercial mixture EX 2002 E.S.E.© (M0). Pesticide ratios (%) calculated for mixture 1 (M1), and for mixture 2 (m2) based on *G. pulex* 96-h LC_{50} (*) of cypermethrin (mM) and on *G. pulex* 96-h LC_5 (**) of fungicides (mM). Toxic units ratio (%) based on respective *G. pulex* 96-h LC_{50} are indicated for mixture 1 and mixture 2.

A study was undertaken to mimick the effects of a commercial mixture containing four different pesticides with various mode of action. The results exposed thereafter have been already published in a previous paper, where experimental details can be found (Adam et al., 2009). Freshwater amphipods *Gammarus pulex* (L.) were exposed to propiconazole, tebuconazole, IPBC, and cypermethrin given separately or in mixtures. First, we assess the environmental toxicity of wood preservatives on aquatic biota starting from single chemical exposures. Then, mixture toxicities were modelled using concentration addition (dose additivity, CA), response additivity (independence of action, IA), and mixed model (MM). The modelled toxicity was compared with the measured mixture toxicity. To do that, two experiments were done, *G. pulex* were exposed to (i) a real world commercial mixture (M0, Table 8) and (ii) a laboratory-made mixture (M1, Table 8) containing exactly the same ratio of active substances than the real world commercial mixture. The only difference between these mixtures is that the commercial mixture contained unknown additives and solvents.
Acute toxicity tests were performed. *G. pulex* (L.) free from parasites were collected from an unpolluted stream (Ruisseau de la Fontaine des Ermites, France, N4712404300 E00610303200). Individuals were acclimated in freshwater to laboratory conditions at least 10 days prior to testing. Ten *G. pulex* adults (46 mm) were randomly chosen and inserted into a test chamber (a 100 mL glass container) that was maintained at 15 °C. For each acute

test concentration, six replicates were used. The mortality was observed after 24, 48, 72, and 96 h of exposure.

5.3 Rationales for the choice of the test-organism

The freshwater amphipod *Gammarus pulex* (L.) (*Crustacea, Amphipoda*) has been chosen as test-organism because of its ecological and ecotoxicological importance. This crustacean species is one of the most widespread invertebrates in European streams and it is a major component of the biomass of many streams (Welton, 1979). As a detritus feeder, *G. pulex* plays a key role in nutrient cycling in freshwater systems (Welton, 1979) and *Gammarus* species are among the most eaten prey for many fish species (Bollache et al., 2006). *G. pulex* is known to be sensitive to a wide range of pollutants and to be among the most sensitive aquatic invertebrates (Helson & Surgeoner, 1986; Mian & Mulla, 1992; Schulz & Liess, 1999; Wogram & Liess, 2001; Cold & Forbes, 2004; Van Wijngaarden et al., 2004; Bloor et al., 2005). This amphipod species can be easily grown in the laboratory and has been recommended for use in toxicity tests (McCahon & Pascoe, 1988a, b; Adam et al., 2010). Moreover, we have also investigated the impact of wood preservatives in *Gammarus pulex* L. and *Gammarus fossarum* K. (*Crustacea, Amphipoda*) populations. Results show that populations were highly impaired by treatment areas at very low pesticide contaminations. Densities and age structure of the populations were particularly modified. Results also suggested an active drift of adults from the most contaminated sites. The impact was observed throughout the year but it was higher in summer and after repeated rainfall events.

5.4 Modes of action of the tested chemicals

Propiconazole, tebuconazole, 3-iodo-2-propinyl butyl carbamate (IPBC), and cypermethrin are among the most frequently used chemicals to protect wood. Two of these pesticides, propiconazole and tebuconazole are triazole fungicides, displaying similar physiological effects: they are 14α-demethylase inhibitors and also referred to as ergosterol biosynthesis inhibitors via cytochrome P450 inhibition (Egaas et al., 1999; Iwasa et al., 2004). Tebuconazole is frequently used in agricultural areas (Berenzen et al., 2005) and propiconazole is one of the most widely distributed pesticides in the world (Kronvang et al., 2003). IPBC is a halogenated unsaturated carbamate fungicide mainly used as wood preservative (Bailey et al., 1999). Juergensen et al. (2000) hypothesized that its fungicidal property was related to the terminal iodine, whereas Jarrad et al. (2004) proposed that carbamate pesticides could act on different physiological targets by disturbing the acetylcholine esterase activity. Another commonly used pesticide in commercial mixture is cypermethrin, a pyrethroid insecticide which exerts very severe toxic effects on aquatic invertebrates. Synthetic pyrethroids are among the most widely used insecticides around the world (Hill et al., 1994; Amweg et al., 2005). Pyrethroids act by slowing the gating of the voltage-dependent sodium channels, thus leading to a sustained membrane depolarization of motor neurons (Bradbury & Coats, 1989).

5.5 Single chemical toxicity data

Dose response curves were fitted with the help of Hill's model for the single-contaminant experiment. LC_{50} were calculated by the Regtox macro (open source) running with Microsoft Excel© software. Results are given in Figures 2 & 3. Mortality response curves of the four tested substances followed sigmoid curves. LC_{50}s with their 95% confidence intervals

obtained from the Hill's model are given in Table 9. LC_{50} decreased with increasing exposure duration for the four tested pesticides.

G. pulex exposed to propiconazole displayed 96-h LC_5 and 96-h LC_{50} which respectively occurred at 3384 and 4703 µg.L^{-1} (Fig. 2A). The main part of *G. pulex* response to propiconazole was observed during the first 24 h of exposure, then, the LC_{50} decrease was very low between 24 and 96 h of exposure (Table 9). As for propiconazole, tebuconazole lethality on *G. pulex* displayed a threshold concentration: 96-h LC_5 and 96-h LC_{50} occurred respectively at 804 and 1643 µg.L^{-1} (Fig. 2B). Again, as for propiconazole, the main part of tebuconazole lethality on *G. pulex* is expressed in the first 24 h of exposure (Table 9).

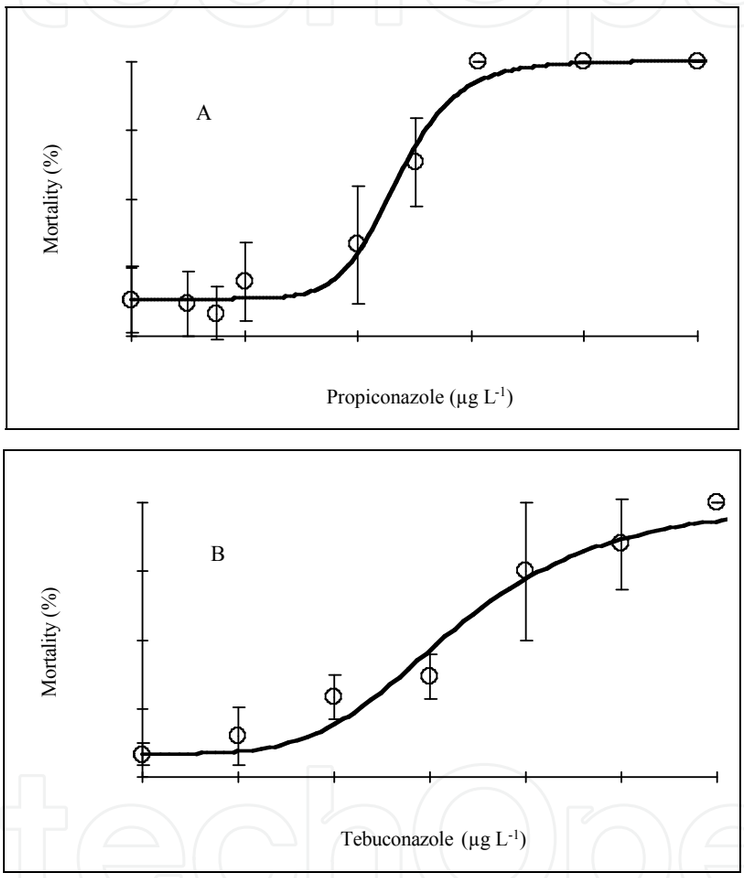

Fig. 2. Dose-response curves obtained by Hill's model after 96 h of exposure of *G. pulex* to triazole fungicides: propiconazole (A) and tebuconazole (B). Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in µg L^{-1}. Plots represent the mean (+/-95% CI) of 6 replicates.

Lethality provoked by IPBC on *G. pulex* was observed at very low concentrations. IPBC 96-h LC_5 and 96-h LC_{50} occurred respectively at 135 and 604 µg.L^{-1} (Fig. 3A). Contrary to triazole fungicides, the lethality caused by IPBC on *G. pulex* was low in the first hours of exposure, but strongly increased between 24 and 48 h of exposure. The IPBC LC_{50} decrease was higher than 90 % between 24 and 96 h of exposure (Fig. 3A). As for IPBC, mortality caused by cypermethrin on *G. pulex* was observed at the lowest concentrations. Cypermethrin 96-h LC_5 and 96-h LC_{50} occurred respectively at 0.03 and 0.09 µg.L^{-1} (Fig. 3B). As for triazole

fungicides, and contrary to IPBC, the lethality caused by cypermethrin on *G. pulex* appeared mainly during the first 24 h of exposure (Table 9).

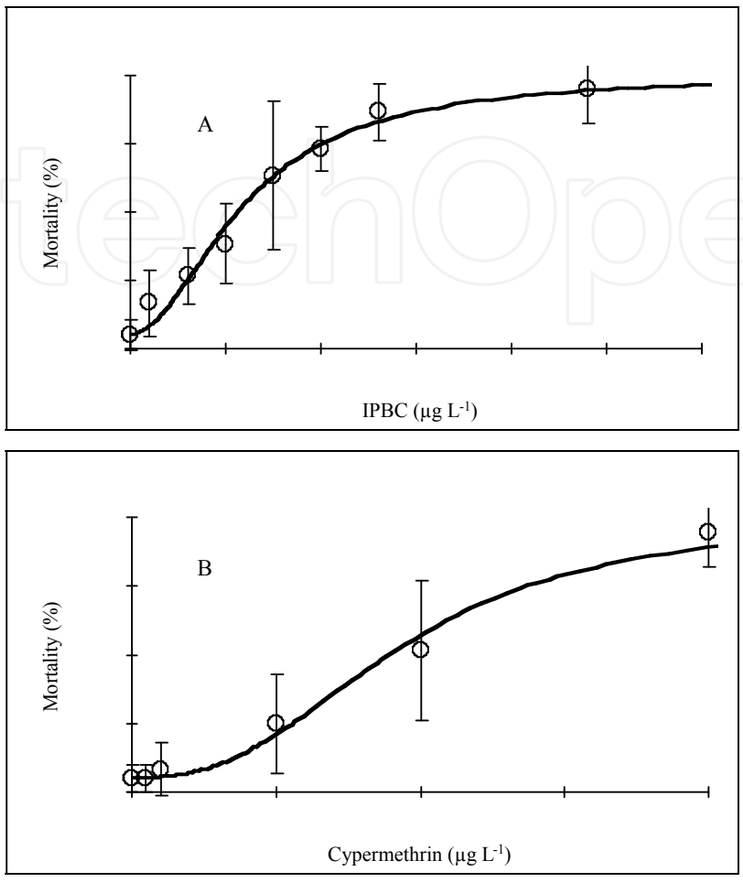

Fig. 3. Dose-response curves obtained by Hill's model after 96 h of exposure of *G. pulex* to a carbamate fungicide IPBC (A), and a pyrethroid insecticide cypermethrin (B). Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in µg L^{-1}. Plots represent the mean (+/-95% CI) of 6 replicates.

Chemicals	24h		48h		72h		96h	
	LC_{50} µg L^{-1}	r	LC_{50} µg L^{-1}	r	LC50 µg L^{-1}	r	LC_{50} µg L^{-1}	r
Propiconazole	5156-5507	0.9988	5037-5331	0.9981	4844-5177	0.9971	4439-4919	0.9947
Tebuconazole	2196-2444	0.9817	1823-2172	0.9848	1541-1903	0.9838	1471-1745	0.9905
IPBC	8438-13712	0.9906	929-1294	0.978	605-742	0.9967	517-661	0.9954
Cypermethrin	0.116-0.135	0.9996	0.098-0.116	0.998	0.084-0.103	0.9933	0.082-0.101	0.9957

Table 9. LC_{50} (95% IC) for propiconazole, tebuconazole, IPBC, and cypermethrin obtained after 24, 48, 72, and 96 h of *G. pulex* exposure, and Pearson's correlation coefficient (r) between observed mortality data and predicted lethality values obtained by Hill's model.

When given independently at environmentally realistic concentrations, propiconazole and tebuconazole (triazoles fungicides) were not toxic for *G. pulex*, 3-iodo-2-propinyl butyl

carbamate (IPBC, fungicide) was moderately toxic, and cypermethrin (pyrethroid insecticide) was extremely toxic. 96-h LC_{50} were respectively 4703, 1643, 604, and 0.09 µg L^{-1}.

5.6 Estimates of the mixture toxicity with the available models

The tested mixtures contain chemicals having similar and dissimilar toxic modes of action. Consequently, such mixtures are not expected to display dose additivity (CA) or response additivity (IA). Assessment of toxicities with these CA and IA models is expected to differ from those measured experimentaly on the whole mixtures (M0, M1). A mixed-model (MM) with a two-step approach according to Zwart & Posthuma (2005) was used to produce estimates of the mixture toxicity based on of single chemical toxicity data. During the first step, concentration addition is used to evaluate the CA responses of triazole fungicides according to Faust et al. (2003) who demonstrated the following relationship:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \tag{6}$$

where
ECx_{mix} is the effect concentration (x%) of the mixture,
the individual concentrations c_i are added up to c_{mix},
p_i is the constant proportion of the chemical i in the mixture, i.e. $p_i = c_i / c_{mix}$.
The second step consists in evaluating the IA responses of the different toxic modes of action for triazole fungicides, IPBC and cypermethrin.
The independent action model takes into account the relative relationships between response probabilities in test organisms. The dose relationships can be calculated by multiplying the probabilities of nonresponse (Bliss, 1939):

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \tag{7}$$

where
$E(c_{mix})$ is the overall effect (scaled from 0 to 1) of a mixture of n components at the total concentration c_{mix} ($c_{mix} = c_1 + \ldots + c_n$),
$E(c_i)$ is the effect of the compound i if applied singly at the concentration c_i that corresponds to its concentration in the mixture.
The tested mixtures contain chemicals having similar and dissimilar toxic modes of action. Such mixtures are expected to have an intermediate toxicity between CA and IA toxicity predictions.
We have tested a mixed-model (MM) with a two-step approach, as proposed by Zwart and Posthuma (2005): the first step requires evaluation of the CA responses to each individual toxic mode of action, the second step consists in evaluating the IA effect of the different toxic modes of action for triazole fungicides, IPBC and cypermethrin. Dose-response curves predicted by the three mixture toxicity models (Fig. 4) were superimposed and no significant difference occurred between cypermethrin (Fig. 3B) and the M1 dose-response curves (Fig. 4). The lethal effect of M0 (commercial mixture EX 2002 E.S.E.® from Dyrup©) was significantly higher (Wilcoxon test, p = 0.044) than those observed with M1 which did not contain any commercial additives (Fig. 4).

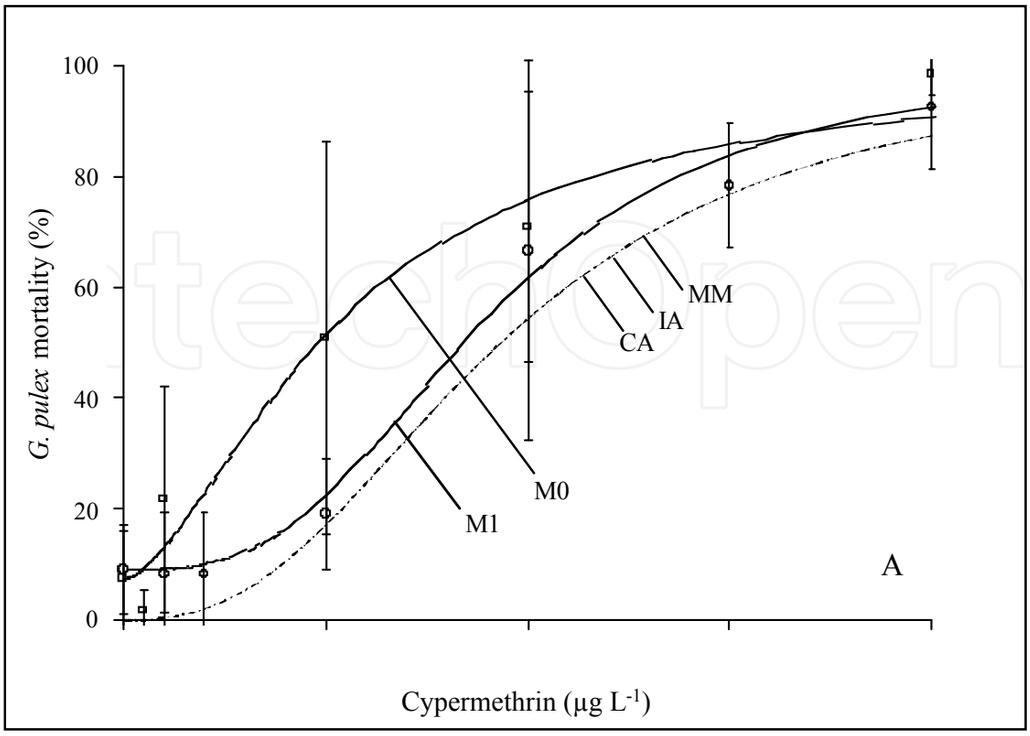

Fig. 4. Dose-response curves obtained for *G. pulex* exposed to M0 or M1 (A) after 96 h of exposure. Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in terms of cypermethrin concentration (µg.L^{-1}) in mixtures. White plots give the mean percentages (+/-Standard Deviation) of 6 replicates. Circle plots correspond to M1. Square plots correspond to M0, commercial solution EX 2002 E.S.E.© (A). Solid lines are the dose-response curves obtained by Hill's model. Predicted dose-response curves calculated by CA, IA models and MM are represented with dotted lines.

CA, IA models and MM have proved to be equally relevant for predicting mixture toxicity of M1. The three predicted dose-response curves were superimposed, and we could not discriminate a better one in this case. The lethality of this mixture for *G. pulex* was mainly caused by cypermethrin. The lethality of fungicides was too low to be observed at the tested concentrations. No synergism or antagonism has been detected between pesticides at the concentration ratio tested in M1. In conclusion, when amphipods were submitted to a mixture mimicking the composition of a commercial solution (18.2% of cypermethrin, 45.8% propiconazole, 17.2% tebuconazole, 18.8% IPBC), the overall toxicity was equal to that of the most toxic component, namely cypermethrin. But, when organisms were submitted to the real commercial mixture containing pesticides, solvents and additives, the toxic effects were markedly higher.

Another mixture (M2) used the same ingredients as M1, but with ratio of pesticides determined on the basis of 96-h LC_{50} for cypermethrin and 96-h LC_5 for the three other components. Cypermethrin represented only 0.002% of the total amount of active substances concentrations in M2, but it still represented 35.0% of the overall mixture toxicity expressed in terms of Toxic Units (Sprague, 1970). Fungicides concentrations in M2 were higher than in M1 (Tab. 9). With M2, the dose-response curves predicted by CA, IA models, and MM were different. Moreover, measured M2 toxicity was higher than toxicities predicted by the

CA, IA, and MM mixture models (Fig. 5). This indicated a synergism occurring between the four pesticides at this ratio-level. M2 toxicity was about 2.5, 17 and 18 fold stronger than predicted by respectively CA, IA models and MM as regards its 96-h LC_{50} (Fig. 5).

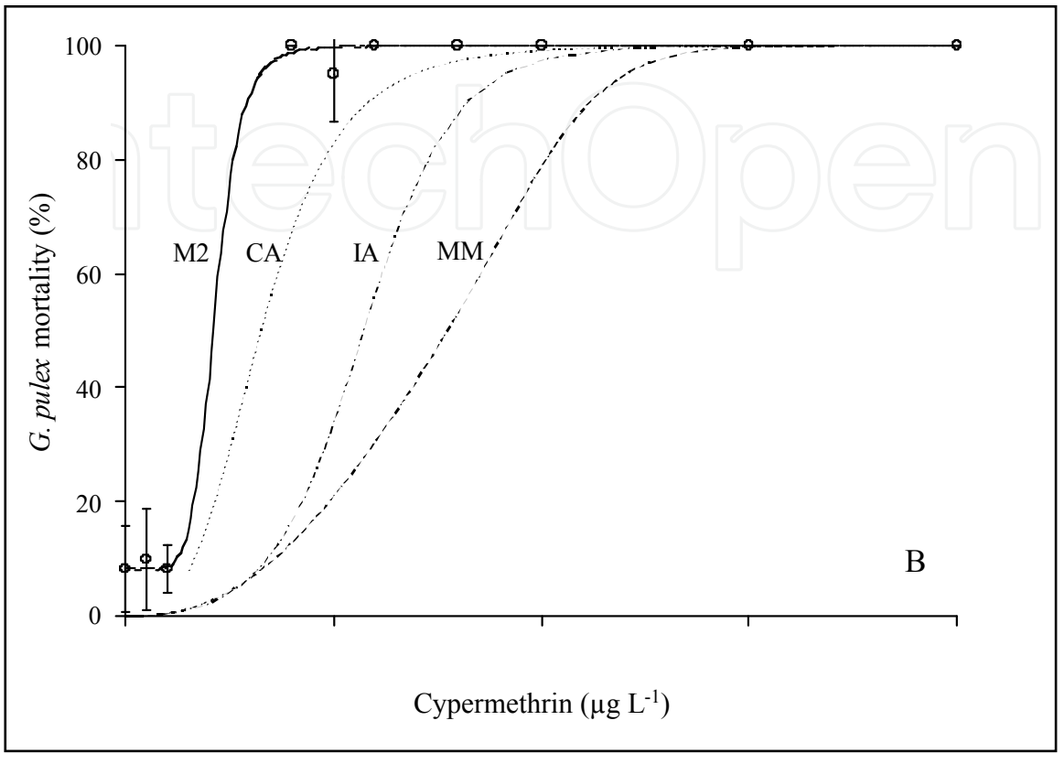

Fig. 5. Dose-response curves obtained for *G. pulex* exposed to M2 (B) after 96 h of exposure. Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in terms of cypermethrin concentration (µg.L^{-1}) in mixtures. White plots give the mean percentages (+/-Standard Deviation) of 6 replicates. Solid lines are the dose-response curves obtained by Hill's model. Predicted dose-response curves calculated by CA, IA models and MM are represented with dotted lines.

M2 was designed according to cypermethrin 96-h LC_{50} and fungicides 96-h LC_5. IPBC, cypermethrin and both triazole fungicides acting on three different physiological targets (Coats et al., 1989; Levine et al., 1999; Jackson et al., 2000; Juergensen et al., 2000), we could presume that the MM was the most relevant approach currently available for predicting toxicity of this mixture (Zwart and Posthuma, 2005). The observed M2 mixture toxicity reached up to 18 times the predicted one. This result suggests that a relatively high synergism would occur between active substances in M2. This third mixture with only 0.002% cypermethrin showed lethality 2.5 to 18 fold higher than those predicted by the commonly used models.

5.6 Lessons learnt from the case-study of wood preservative mixture toxicity

The present results (Fig. 4 & 5) show that interactions between active substances would depend on the ratio between chemicals displaying acute toxicity. Consequently, in real world, relevant environmental risk assessment of chemical mixture has also to take into

account changes that may occur in the natural environment. Indeed, pesticide environmental concentrations are known to change at different rates because of differences in degradation rates and transfer properties. The initial pesticide ratio of the commercial solution was likely to be modified between the treatment area and the contaminated aquatic environment. Furthermore, aquatic biota is typically exposed to brief pulses of pesticides in their natural environment (Liess et al., 1999). Then, aquatic organisms are expected to be exposed to fluctuating ratios of pesticides displaying different toxic interactions. Thus, relevant risk assessment should also consider possible patterns of pesticides ratio exposure.
Acute toxicity tests with M0, the corresponding commercial wood preservative mixture, have revealed a higher toxicity on *G. pulex* than observed with M1. In the present study, additives present in M0 commercial solution were likely to modify interactions between active substances and their toxicity expression (Stratton, 1985; Krogh et al., 2003). Additives could also act because they are themselves toxic, or they facilitate pesticides intake, or they reduce activity of detoxification mechanisms (Holloway and Western, 2003; Green and Abdelghani, 2004; Paul et al., 2005). Whatever the mechanism operating, the commercial solution containing additives displayed a higher toxicity than a mixture differing only by the absence of these additives. Therefore, when the composition of the mixture is not known with accuracy, available mixture toxicity models failed to predict ecotoxicity effects even if the accurate contents in active compounds are known. In the present case, toxicity predicted by mixture models was markedly underestimated. Consequently, ecotoxicological risk assessment of wood preservative mixture on aquatic systems have to be based on reliable data obtained by testing the overall commercial mixtures and cannot be calculated from single component toxicity data. CA, IA models, and MM were of limited interest for the environmental risk assessment of wood preservative mixtures especially because the use of additives in the commercial mixtures prevents from predicting toxicity. The present results give evidence that toxicity assessment of wood preservative mixtures should be necessarily based on toxicity experiments performed with real commercial solutions and not be derived from single chemical toxicity data. Furthermore, the present data strongly suggest that the environmental impacts of wood preservative mixtures might be frequently underestimated.

6. Concluding remarks

During the last ten years, mixture toxicology has undergone a remarkable and productive development (University of London, 2009). Because of resource and time limitations, direct toxicological information will never be available on all the possible mixtures to which humans or living organisms are exposed. Single chemical risk assessment has proven to be efficient at its own scale, but fails for the multiple combination of pollutants and various stressors existing in real life. The current methods available to assess mixture toxicity from single chemical toxicity data suffer from severe limitations, except in cases where additivity stands. In other cases, there does not exist any turn-key solution. The responses to health and environmental concerns cannot be only given by laboratory-based approaches and paradigms. The temporality of the exposures and related effects is insufficiently taken into account. Efforts should be made to better estimate exposures. This implies that models of exposure have still to be developed. The effects of low dose are probably insufficiently taken into account. The sensitivities of the various species must be apprehended better. Statiscally based methods may usefully supplement mechanistic approaches. Uncertainties have to be better estimated and taken into account. Biomarkers of effects, environmental monitoring,

biomonitoring, surveillance and population surveys are essential to an accurate exposure assessment. In this context, progress is still to be made to better understand the mechanisms and modes of action of toxicants. The potential of the omic-technics must be investigated. Taking into account interactions between chemicals and between chemicals and the environment remains a very difficult, but compulsory and exciting challenge.

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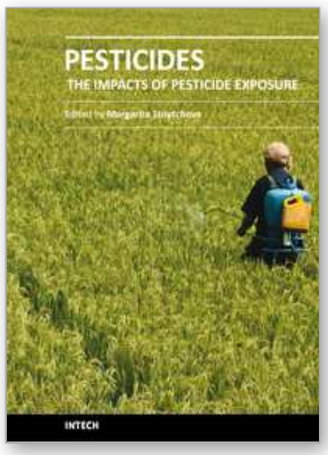

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Pesticides are supposed to complete their intended function without â€œany unreasonable risk to man or the environmentâ€. Pesticides approval and registration are performed â€œtaking into account the economic, social and environmental costs and benefits of the use of any pesticideâ€. The present book documents the various adverse impacts of pesticides usage: pollution, dietary intake and health effects such as birth defects, neurological disorders, cancer and hormone disruption. Risk assessment methods and the involvement of molecular modeling to the knowledge of pesticides are highlighted, too. The volume summarizes the expertise of leading specialists from all over the world.

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