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Toxicological Evaluation of Complex Mixtures: Prediction and interactions – A Review

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Contents

List of abbreviations and acronyms

ACGIH	American Conference of Government Industrial Hygienists
LCA	Life Cycle Analysis
ADEME	Environment and Energy Management Agency
ADI	Acceptable Daily Intake
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
AFSSA	French Food Safety Agency (currently ANSES)
AFSSET	French Agency for Environmental and Occupational Health Safety (currently ANSES)
AL	Acceptable level
ASTEE	Scientific and Technical Association for Water and the Environment
ATSDR	Agency for Toxic Substances and Disease Registry
B (a) P	Benzo (a) pyrene
BEEP	Scale of Potential Ecotoxic Effects
BINWOE	BINary Weight of Evidence
BMD	Benchmark Dose
BRGM	Geological and Mining Research Office (see : Bureau de Recherches Géologiques et Minières)
CA	Addition of concentrations (doses)
CE	European Commission
CIRC	International Agency for Research on Cancer (see IARC)
CNRS	Scientific Research National Center
COT	Committee on Toxicity of Chemicals
CR	Cancer risk
CRI	Cumulative Risk Index (= IRA)
CSHPF	Superior Council of Public Hygiene of France
DGAL	General Direction of Food
ADI	Acceptable daily intake (see ADI)
LD50	Lethal Dose 50%
LOAEL	Minimum dose with observed adverse effect
DR	Dose Ratio
NOAEL	Dose with no observed adverse effect
ECB	European Chemicals Bureau
ECETOC	European Center for Ecotoxicology and Toxicology of Chemicals
EChA	European Chemicals Agency
EDA	Effect Directed Analysis
EFSA	European Food Safety authority
EIFAC	European Inland Fisheries Advisory Commission
EMEA	European Medicines Evaluation Agency
EQRS	Quantitative Assessment of Health Risks
ERC	Excess Collective Risk
ERE	Ecosystem risk assessment
ERI	Excess of Individual Risk
ERS	Health risk assessment
ERU	Excess of Unit Risk
FAO	Food and Agriculture Organization of the United Nations
FS	Safety factor
GC	Gas chromatography
PAH	Polycyclic Aromatic Hydrocarbons
HCN	Health Council of Netherlands
HI	Hazard Index
HII	Hazard Index interaction
HPLC	High Pressure liquid Chromotography
HQ	Hazard Quotient
HRS	High-resolution mass spectrometry
IA	Independence of action
IARC	French International Agency for Research on Cancer (see CIRC in French)
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ICPE	Installation Classified for the Protection of the Environment
ILSI	International Life Sciences Institute
INERIS	French National Institute for Industrial Environment and Risks
INRS	French National Institute for Research and Security
INVS	French Institute for Public Health Surveillance (see Institut de Veille Sanitaire in French)
IPCS	International Program on Chemical Safety

IR	Risk Index
IRIS	Integrated Risk Information System, US-EPA toxicology database
IRSST	The Institut de recherche Robert-Sauvé en santé et en sécurité du travail-QUEBEC
ISPED	Institute of Public Health, Epidemiology and Development
ISS	Integral Search System
I-TEF	International Toxic Equivalent Factor
I-TEQ	International Toxic Equivalent Quantity
JECFA	Joint FAO / WHO Expert Committee on Food Additives
Kow	Octanol / water partition coefficient
LOAEL	Lowest Observed Adverse Effect Level
MEDD	Ministry of Ecology and Sustainable Development (now Ministry of Ecology, Sustainable Development, Transport and Housing)
MoA	Mode of action
MOE	Margin of exposure
MRL	Minimum Risk Level
MTM	Multi Test Macroinvertebrates
NCRP	National Council on Radiation Protection and Measurements
NIOSH	National Institute for Occupational Safety and Health
NMA	Maximum acceptable level of the substance
NOAEL	No Observed Adverse Effect Level
NOEC	No Observed Effect Concentration
NRC	National Research Council
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
OEHHA	Office of Environmental Health Hazard Assessment
WHO	World Health Organization
OSHA	Occupational Safety and Health Administration
NATO /CCMS	Committee on the Challenges of the Modern Society of the North Atlantic Council
PBPK / PD	Physiologically Based Pharmacokinetic and Pharmaco-dynamic
PCB	Polychlorinated biphenyls
PCB (- dl)	Polychlorinatedbiphenyl (Dioxin-like)
PCDD	Polychlorinated dibenzodioxins (or dioxins)
PCDD / Fs	Polychlorinated dioxins, furans
PCDF	Polychlorinated dibenzofurans (or furans)
PEC	Predicted Environmental Concentration (expected concentration of the substance in the environment)
PlCT	Pollution lnduced Community Tolerance
PNEC	Predicted No-Effect Concentration
PODI	Point starting
POP	Persistent organic pollutants
PTWI	Provisional tolerable weekly intake
QSAR	Quantitative structure-activity relationship
REACH	Registration, evaluation, authorization and restriction of chemicals
RECORD	Association for Cooperative Research on Waste and the Environment
REL	Recommended Exposure Limits
RfC	Reference concentration
RfD	Reference Dose
RP	Reference Point
IRISC	Integrated Risk Information System of Closure
RIVM	Dutch Institute for Environmental Health
RPF	Relative Potency Factor
RSD	Waste Health Network
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products
SFSE	French Society of Health and Environment
SFSP	French Society of Public Health
TCDD	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin
TDI	Tolerable Daily Intake
TEF	Toxic Equivalency Factors
TEQ	Toxic Equivalent Quantity
TGD	Technical Guidance Document
IT	Toxicity Index
TIE	Toxicity Identification Evaluation
TLV	Toxic Level Value
TPH	Total Petroleum Hydrocarbons
TTC	Threshold of Toxicological Concern
TTD	Target-Organ Toxicity Dose
UF	Uncertainty factor
US-EPA	United States Environmental Protection Agency
UT	Toxic Unit
OELs	Occupational exposure limit values
VME	Average Exposure Value
VTR	Toxicological Reference Value

WEA Whole Effluent Assessment
WET Whole Effluent Toxicity
WoE Weight of Evidence
WR Radiation weighting factor

Abstract

The evaluation of chemical mixtures is a complex subject and follows several approaches.

To strengthen the scientific basis of the toxicology of chemicals mixtures, studies have been carried out to determine the biological concepts and basic formulas of mathematics for the extrapolation of low doses.

The extrapolation of these doses should be considered as a key issue in the assessment of potential health risks from exposure to chemical mixtures in the atmosphere, by-products of drinking water disinfection, or in recombinant additives ..., etc.

Clearly, the intervention of biologists, biomathematicians and bioengineers in toxicology mixtures is essential for the development of this. Studies on complex mixtures use multidisciplinary knowledge.

The risk of complex mixtures remains a challenge. Before the results of the toxicity test can be used to adjust the risk assessment calculations, it is important to assess the chemical composition and to understand the mechanism of chemical interactions observed in animals chronically exposed to low doses of chemical mixtures.

The current development of exposure biomarkers allows the assessment of the internal dose of exposure to toxic substances, integrating all the media and pathways of contact, thus allowing a precise assessment of the risk to human health.

Finally, it is time to initiate research projects related to this theme, and more particularly to the development of toxicological and eco-toxicological tests, to better study interactions at low doses. This will not only improve scientific knowledge, but also provide essential skills to increase safety against exposure to complex mixtures.

A battery of tests seems essential to evaluate the toxic potential of the mixtures, and to better understand the different possible interactions between the substitutes.

However, the toxicological and eco-toxicological and risk assessment models appear to be limited by, on the one hand, the non-specificity of the mechanisms of action but at stake, and on the other hand, their lack of representativeness of in vivo effects

It would therefore be interesting and desirable for these tests to be better understood, in order to define and interpret the mechanisms of action of the mixtures.

This bibliographic review aimed to provide some answers to the central question which is: the nature of the possible interactions between contaminants that can influence their toxicities.

Keywords: mixtures; additive effect; synergy; potentialization; cumulative risk; interaction; exposure; pesticides; hydrocarbons

General Introduction

1. General context

Humans are constantly exposed to chemicals, especially in the workplace, and to the effects of mixtures of pollutants and extremely complex chemical contaminants [Burgess, 1995] which may differ for each mixture depending on the chemical composition. However, knowledge on the toxicity of mixtures of chemical agents is almost non-existent since they come under recent sciences (Toxicology and Ecotoxicology).

Admittedly, risk assessment methods associated with mixtures have been developed in recent years, which despite their imperfections, have made it possible to make significant advances in the protection of exposed persons. However, the latter mainly focus on the study of the effects of chemical compounds alone. Yet living organisms are rarely exposed to a single toxic substance and molecule [WHO, 2009; Kortenkamp et al. 2009].

This is why the subject of multiple exposures and their effects is today of capital importance both for the population in general, as well as for the scientific community and regulatory authorities as evidenced by the positions taken by the WHO (2009) or the European Union on the toxicity of mixtures. Thus, improving the knowledge in question appears to be a major subject of interest and research [Hansen et al., 1998; Carpenter et al., 1998].

2. Objectives

The objective of this bibliograohic review, which is not exhaustive, is to study the different mixtures of chemical substances, and to identify the possible interactions between the constituents of the mixture and their effects.

Indeed, most workers are exposed to multiple contaminants. The health criteria for these exposures usually do not consider the possibility of interactions between these agents. However, it is probable that a combined exposure to several substances implies an interaction between these substances.

Exposure to mixtures of chemical substances in the workplace raises many questions regarding the possibilities of interaction between them.

Thus, this bibliographic review takes on all its importance since the mixture based on chemical substances raises more and more concerns and questions in the industrial environment by the various toxicologists and risk assessors linked to acute and chronic exposure to mixtures.

However, the most majority of toxicological studies focus on substances considered independently of one another. Studies involving several substances (generally 2 to 4) are limited in number, and do not represent actual exposures (other compositions of the mixture, different chronology of simultaneous or consecutive exposures, etc.). Complexity being considered the main reason why mixtures have not been well studied.

Finally, this bibliographic review aimed to provide some answers to the central question which is: the nature of the possible interactions between contaminants and their consequences on the toxicity associated with a mixture.

To this end, a state of the art of the bibliography research available on the subject is presented in the first chapter, then the different approaches to the toxicological evaluation of mixtures of chemical substances are explained, and finally the most relevant approaches used in this field are highlighted.

3. Problem of mixtures

The term “chemical mixture” is understood to mean all the substances (identified or not), regardless of their sources or their temporal or spatial proximity, which may jointly contribute to toxicity in the study population [US-EPA, 2000].

In some cases, the mixtures are very complex, made up of compounds that are formed simultaneously as a by-product of a source or a process (emissions from combustion plants or diesel fumes). In other cases, the mixtures of compounds are commercial products (gasoline, chemical solvents, pesticide formulations, etc.) and possibly emitted into the environment.

There is another category of mixtures made up of compounds (without chemical or commercial link) placed in the same place for treatment or storage (example of waste storage). Multi-chemical exposures are very common, such as those linked to air and soil pollution by human industrial and agricultural activities, to food additives, to the contamination of water and beverages by substances formed during water disinfection, etc.

The health criteria relating to these exposures usually do not consider the possibility of interactions between these contaminants, which can lead to a modification of the toxicity. However, this factor should be given high priority [NIOSH., 1996].

The constituents of the mixtures can interact with each other at the level of absorption, distribution, metabolism, or excretion. But, unfortunately, no current toxicological analysis approach allows considering these toxicological interactions, as there is no special strategy and standard protocol available to determine the toxic and genotoxic effects of complex mixtures.

This is, in large part, due to the fact that there are no tools to predict the magnitude of toxicokinetic interactions in complex mixtures of chemicals or to consider toxicokinetic interactions in the risk analysis. complex mixtures of pollutants. The combined effects are scientifically difficult to apprehend.

The evaluation of a mixture does not depend on its individual ingredients, but rather on a knowledge of their combined toxicity when used in different proportions. This can be explained by the synergistic or antagonistic effect [Bliss, 1939].

Six decades after Bliss's publication in 1939, few studies have looked at the interactive effects within chemical mixtures. Studies by Yang [1994] have shown that 95% of toxicology resources are devoted to studies of simple pure chemicals.

However, mixtures of chemicals present in the environment are most often complex and consist of parent compounds, transformation or reaction products, residues, and potentially inert materials.

The REACH regulation required the registration of more than 30,000 chemical substances in use today, and put in place a process to complete the missing information on the dangers of these substances and to identify appropriate risk management measures. Indeed, exposure to combinations of pollutants sometimes has unintended consequences, resulting in a toxic response that is significantly higher or lower than the simple sum of the responses induced by the components of the mixture taken individually. The toxic effects of the mixtures can be acute and chronic, carcinogenic, genotoxic or others.

Such effects, which are the consequence of what are called "toxicological interactions", can be beneficial (a chemical product confers protection against the toxic effects of another product) or dangerous (the toxicity associated with a product. is increased in the presence of another product).

The development of new scientific concepts has recently appeared to define complex exposures that better reflect reality. Thus, "the exposome" was proposed in 2005 bythe molecular epidemiologist and director of the International Agency for Research in Cancer in Lyon, Christopher Paul Wild (Wild 2005), “who defends the need for a rebalancing of cancer research - which according to him then granted a quasi-privilege exclusive to genes - in the direction of these famous environmental factors” [Guchet, 2017].

This new paradigm takes into consideration all the sources of pollution or exposure likely to contribute to the deterioration of the health of individuals, i.e. all the routes of exposure to a pollutant and, when possible, the interactions between pollutants.

Thus, this study will be structured in two main parts. The first devoted to the literature review on the different types of interactions of complex mixtures and the assessment of the toxicological risk of complex mixtures. Subsequently, in a second part, the mechanisms of action of complex mixtures and the risk analysis of complex mixtures will be discussed, according to the literature, focusing on their genotoxicity and reprotoxicity and ends with recommendations regarding risk assessment of exposure to complex mixtures.

1.- TOXICOLOGY OF MIXTURES: state of the art

In the absence of an exhaustive list of the effects that each of the constituents can cause, and the lack of knowledge of the dose or the concentration at which the response takes place, it is very always difficult to know the overall effect of a mixture. Indeed, by definition, the concentration of the constituents of complex mixtures is variable and the constituents are sometimes unknown.

Before getting to the heart of the matter, it would be interesting to give general definitions of the terms used in this bibliographic review.

1.1. Definition of mixtures

- **Simple mixture**: it is a mixture containing "two or more identifiable components but the toxicity of the mixture can be, in adequate proportion, characterized by a combination of the toxicities of the components and their interactions".
- **Complex mixture**: mixture made up of a number of components such that any assessment of its toxicity from the toxicity of its components is too uncertain to be used.

The chemical composition can vary over time or depending on different conditions under which the mixture is produced. The components may be simultaneously formed as a by-product of a process, produced intentionally, or may coexist as a result of disposal practices. The risk assessments of complex mixtures are preferably based on the toxicity and exposure data of the mixture. Gasoline is an example of a complex mixture [US-EPA 2000; ATSDR, 2004; Monosson, 2005].

- **Similar mixtures**: mixtures which are slightly different but for which identical characteristics are expected (their fate in the environment, transport, physiological processes, and toxicity). These mixtures can consist of the same components in almost the same proportions with only a few different compounds. Similar mixtures have the same biological activity or are believed to have the same type of biological activity due to their composition. They act by the same mechanism of action or have the same critical effect, such as combustion fumes from diesel engines.

1.2. The different types of interactions

In studies published in the mid-twentieth century on the combined actions of chemical mixtures, the scientific community underlines the existence of two distinct mechanisms: interaction and non-interaction.

In terms of risk analysis, the non-interaction corresponds to the additivity - method used in the case of relatively simple mixtures comprising at most a dozen compounds -.

The concept of interaction includes all other cases where the effects of a chemical mixture is different from the first two types of action. This results in either a higher effect (ie synergism, supra-additivity) or a lower effect (ie antagonism, infra-additivity) compared to that expected on the basis of single additivity (Casse et al. 1998). A simplified diagram is presented (Figure 1).

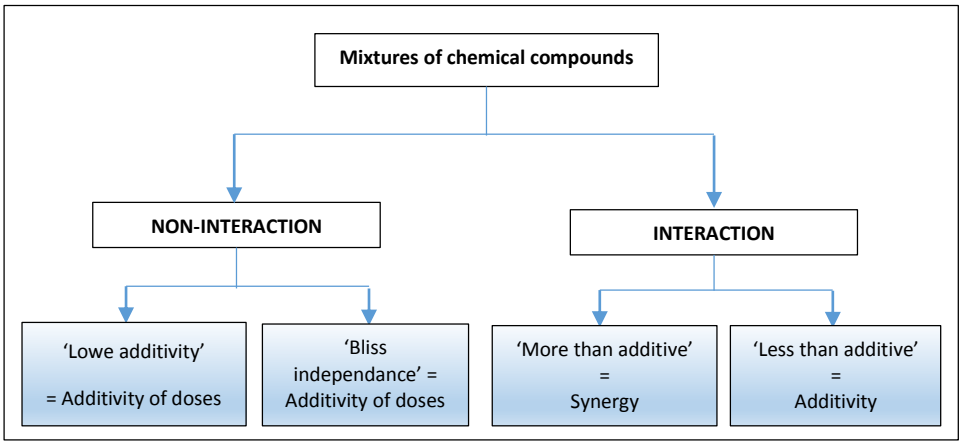


Figure 1: *Summary diagram of the main types of combined actions used for mixtures of chemical compounds*

- **additivity**: taken "by default" for the evaluation and the prediction of the effects of the mixtures

  - when data on the interaction are not available - ;

- **supra-additivity**: when an interaction produces an effect greater than the sum of the individual substances;

- **infra-additivity**: for which the interaction leads to an effect lower than this sum [adapted according to the R-425 report on the impact of toxicological interactions on the management of situations of exposure to multiple contaminants].

Tables 1 and 2 summarize the different terms used to describe the toxicological interactions, frequently described by: antagonism, additivity, potentiation, and synergy [Hertzber., 2000], as well as the combined actions of the components of the mixtures [Cot, 2002 and Seed et al., 1995].

Interaction		Model	Observed effects
Infra-additivity	Antagonism	0 + 3 = 2	Decrease
		-2 + 3 = 1	
Additivity	Addition	1 + 2 = 3	Lack of interaction
Supra-additivity	Potential	0 + 3 = 7	Increase
	Synergy	1 + 2 = 7	

Table 1: *Possible interactions between chemicals*

Concept	Term used in this report	Synonym (s)	Effects observed
Non-interaction	Dose addition	Simple similar action Additivity Concentration addition Simple joint action Summation Loewe additivity	Chemicals have the same effect on the body and differ only in potency: hence the combined effect of two agents can be estimated from the total dose both agents together
	Independent action	Simple dissimilar action Simple independent action Independent joint action Effect / response addition Bliss independence	Chemicals have differing effects on the body and hence the combined effect of two agents is equal to the separate effects of each agent given alone
Interaction	Synergism	Increase Potentiation Supra-additivity	The combined effect of two agents is greater than would be seen if no interaction had occured
	Antagonism	Depotentiation Sub-additivity Inhibition Infra-additivity Negative synergy Masking	The combined effect of two agents is less than shoud be seen if no interaction had occured

NB: Carbon tetrachloride and ethanol (ethyl alcohol), for example, are both toxic to the liver, but combined they cause much greater damage than the sum of their hepatotoxic effects.

Table 2: *Terms used to describe the combined actions of the components of mixtures (COT, 2002 and Seed et al., 1995)*

1.2.1. Antagonism

The phenomenon of antagonism occurs when the interaction between two components of the mixture leads to a total toxic response less than the sum

of the individual responses (Figure 2). Antagonism is the basis of many medical treatments and antidotes for poisoning.

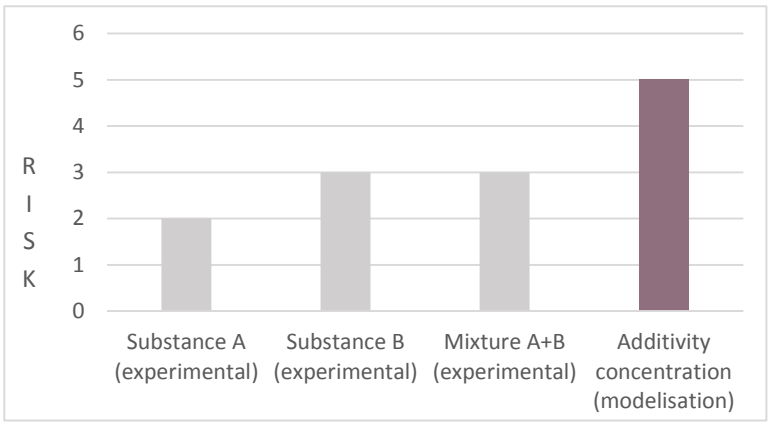


Figure 2: *Antagonism model*

For example, ethyl alcohol (ethanol) can counteract the toxic effects of methyl alcohol (methanol) by preventing it from coming into contact with the enzyme responsible for the oxidation of methanol. Another typical example of this class of interactions is the protection conferred by selenium against mercury toxicity.

1.2.2. Additivity

Additivity occurs when the combined effect of two or more chemicals is equal to the sum of the effects of each chemical taken individually (no direct interaction) (Figure 3).

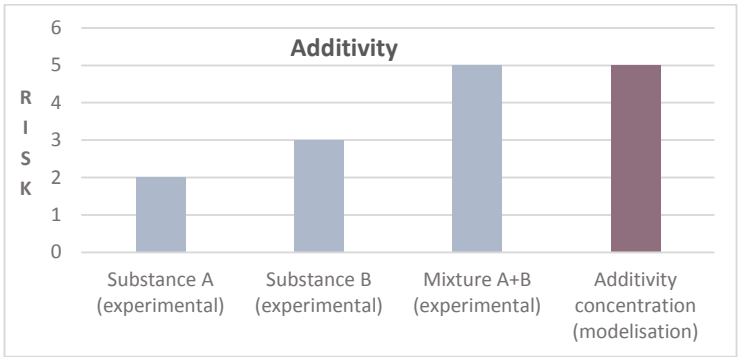


Figure 3: *Additivity model*

The risk analysis of mixtures is generally based on the additivity of doses (concentrations) (a) and additivity of responses (b) models.

(a) **Additivity of doses**: this notion was developed in 1926 by Loewe and Muischnek, and assumes that each substance intervenes in the toxicity of the mixture in a manner proportional to its effectiveness and its dose and that the substances act in the same way (from a point of mechanistic view), so that the same response can be obtained by replacing one compound, totally or in part, by another, but with different amplitude potentials [Calabrese, 1991].

It is believed that each compound in a mixture may contribute to the overall effect observed by acting in proportion to its concentration, and that dose additivity means that the combined response of the substances in the mixture is greater than that of each substance taken. individually [Kortenkamp et al., 1999; US EPA, 1986].

However, this notion of dose additivity does not take into account the possibility of toxicological interactions whereas, from a biological point of view, several interactions could take place [Haddad et al. 1999]. They can be of the toxicokinetic type if the substances follow the same metabolic pathway (alteration ofabsorption, elimination, biotransformation, and distribution), or toxicodynamic type (alteration of the intensity of toxic effects) if the molecular target is identical [Fournier et al. 2014].

(b) **Additivity of responses**: according to this approach, the response of a mixture is estimated by the fraction of the responses of the individual substances that compose it. An example of an additivity response as well as an example presented by cadmium and mercury are illustrated in Figure 4. This second model is based on the principle that the substances in a mixture act independently of each other [Calabrese, 1991].

Scientists who have worked on toxicological and ecotoxic interactions favor the concentration additivity model since it overestimates the effects of the mixture, unlike the response additivity model which certainly gives more precise estimates, but often slightly lower than the actual toxicity of the mixture [RECORD, 2011].

The additivity of concentrations is recommended in the European Union, the United States and by international organizations because of its simplicity [Reffstrup 2010; Zeman 2008; Faust and Scholze 2004].

Finally, it should be noted that the use of the two additivity models for the risk analysis of substances mixed with these two models is limited due, on the one hand, to the absence of reference values or standards for one to several substances constituting certain mixtures, and on the other hand, the deterministic aspect of these models. This is because they do not consider the variability or uncertainty associated with parameters, including reference and exposure values. Therefore, it seems essential to launch studies on mixtures of at least 4 compounds in order to be able to set a recognized threshold beyond which synergies or antagonisms could be concluded.

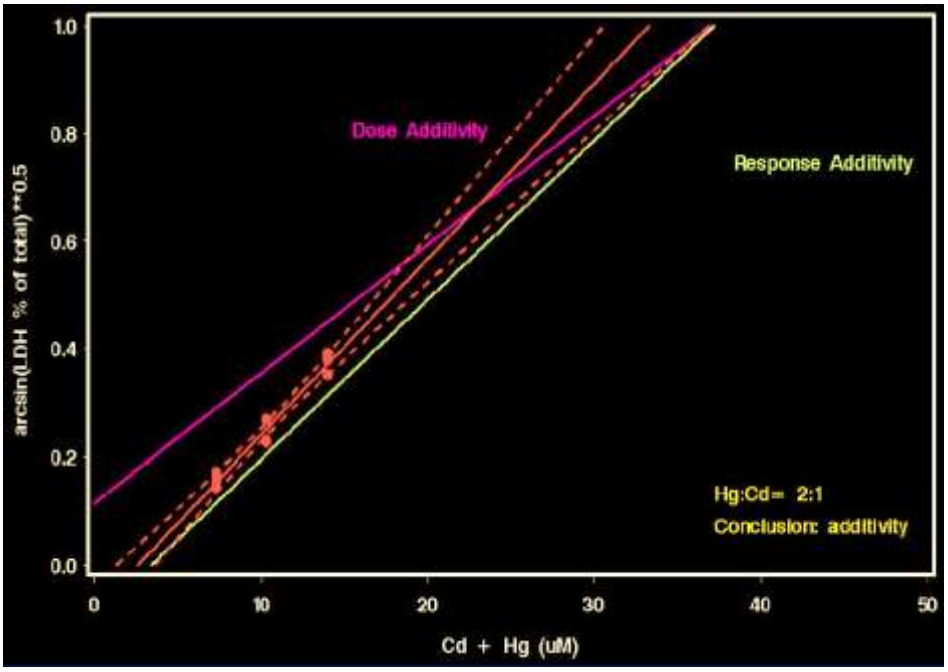


Figure 4: *Additive response curve between Mercury and Cadmium (Hg: Cd = 2: 1)*

1.2.3. Potentiation

When a substance causes an increase in the toxicity of another substance, without itself producing the toxic effect considered (Figure 5), we will speak of potentiation [R-425: Impact of toxicological interactions on the management of hazardous situations. exposure to multiple contaminants]. In other words, this phenomenon occurs when a substance which usually does not have a toxic effect or has negligible toxicity is combined with a chemical, which has the effect of making the latter much more toxic.

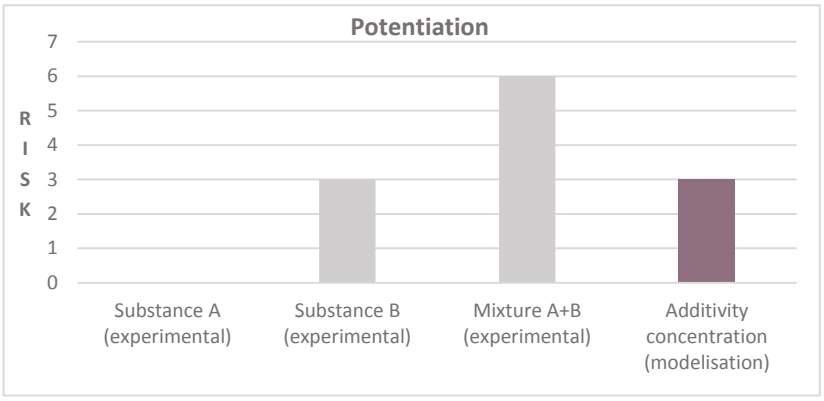


Figure 5: *Potentiation model*

The work that has focused on ketone-haloalkane interactions is a good example. Also, isopropanol has no effect on the liver, but may increase the hepatotoxicity of carbon tetrachloride. Likewise, the work on ketones-haloalkanes is a good example.

1.2.4. Synergy

It is the most studied type of interaction in toxicology by research laboratories. It designates the interaction between at least two "compounds" their combined effect is greater than the sum of their own effects (this definition, used by the US-EPA 2000, relates to the influence on the toxicity observed without consider the real modes of interaction).

Synergistic responses are observed when all the substances in a mixture induce toxicity, but the combination of their responses is greater than the sum of the responses of each component of the mixture taken individually (Figure 6).

The increased toxicity seen on combined exposure to haloacetic acids and haloalkanes is a good example, as are the effects of carbon tetrachloride and ethanol on the liver (both toxic to the liver, but in combination they cause serious damage), or the effect of exposure to asbestos and cigarette smoke on the lung.

Inhalation of radon decay products and routine smoking have synergistic effects on the incidence of lung cancer [Morisson et al., 1998]. The incidence of lung cancer caused by occupational exposure to asbestos is much higher in smokers than in non-smokers.

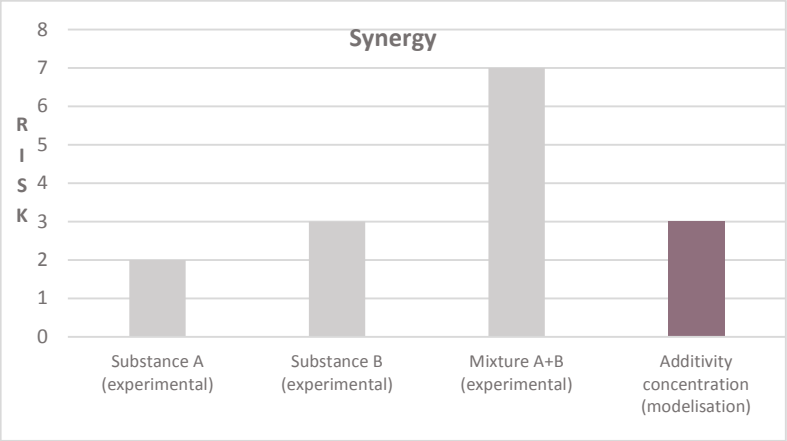


Figure 6: *Synergy model*

Obviously, smoking, considered alone, represents exposure to a very complex mixture. Environmental tobacco smoke (ETF) contains over 4000 chemical compounds including over 50 carcinogens and mutagens. These include polycyclic aromatic hydrocarbons (PAHs), aromatic amines and other compounds. But this does not change the fact that the addition of a second environmental factor, radon or asbestos, has the consequence of increasing the risk.

In toxicology, when chemicals have synergistic effects, the potential risks they present are reassessed taking into account their synergistic characteristics.

Remark: It is possible to increase the toxicity of certain insecticides several times, in particular pyrethrin (from chrysanthemums) and synthetic pyrethrins (pyrethroids) by adding compounds that are not insecticides. These synergistic products are sesamin, sesamolin, piperonyl butoxide, MGK-264 (bicycloheptene dicarboximide) and sesamex.

Piperonyl butoxide (PBO) is probably the most widely used synthetic pyrethrin synergist [Winckel et al. 2006]. The insecticidal activity of pyrethrins increases tenfold when one part of piperonyl butoxide is added to nine parts of pyrethrin. However, this molecule has harmful effects on human endocrine functions and on the environment. All the more so, the PBO has a greater persistence than the active substance. Residues are detected at levels which are abnormally high, mainly in cereal products, and even in certain organic products (highlighted in the Casdar projectSECURBIO which made it possible to identify the contaminant residues with which organic products could be confronted: pesticides, GMOs and mycotoxins).

Piperonyl butoxide (CAS: 51-03-6) is classified and CAT2 "Potential for endocrine disruption. In vitro data indicating potential for endocrine disruption in intact organisms. Also includes in-vivo effects that may, or may not, be ED-mediated. May include structural analyzes and metabolic considerations" for its "overall dangerousness". Therefore, organic farming products containing PBO have been banned [Annex VIII of Regulation (EC) No 889/2008, additives and auxiliaries which can be used for the preparation of foodstuffs]. This decision took effect on March 31, 2018.

On the other hand, barbiturates have amplified effects on the central nervous system (CNS) and cause CNS depression when consumed together with general anesthetics, alcohol (acute consumption), narcotic pain relievers and pain relievers. 'other sleeping pills / sedatives [examples adapted from Klaassen, 2001].

The experimental study carried out by Mumtaz in 2005 elucidates the interactions within mixtures. In fact, renal cortical cells were exposed to 0.4 µM of mercury chloride, or 0.20 µM of cadmium chloride, or else Hg: Cd according to the 2/1, 5: 1, 10: 1 rations.

The cytotoxicity was measured by the release of LDH (Lactate Dehydrogenase) for 24 hours and the exposure of controls not treated with cadmium mercury chloride. The results are shown in Figures 7 and 8.

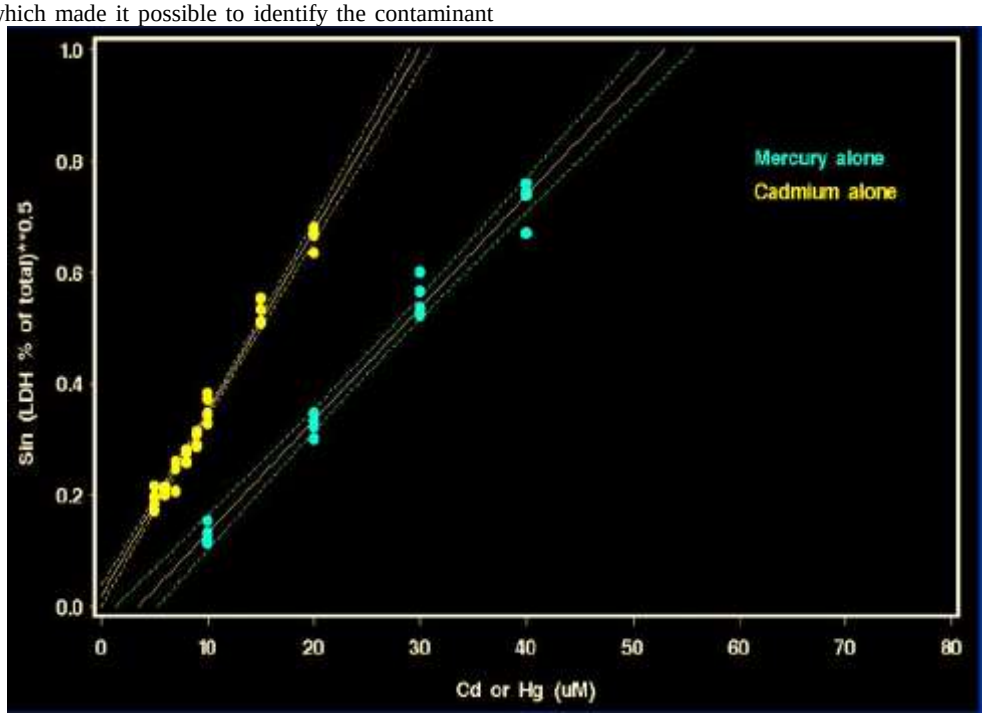


Figure 7: *Curves representative of Cadmium and Mercury in the total absence of interaction*

The curves illustrate the additivity responses between cadmium chloride and mercury chloride at a ration: Hg: Cd = 2: 1, and an absence of additivity (synergistic response) when the Hg: Cd ration is of the order of 10: 1.

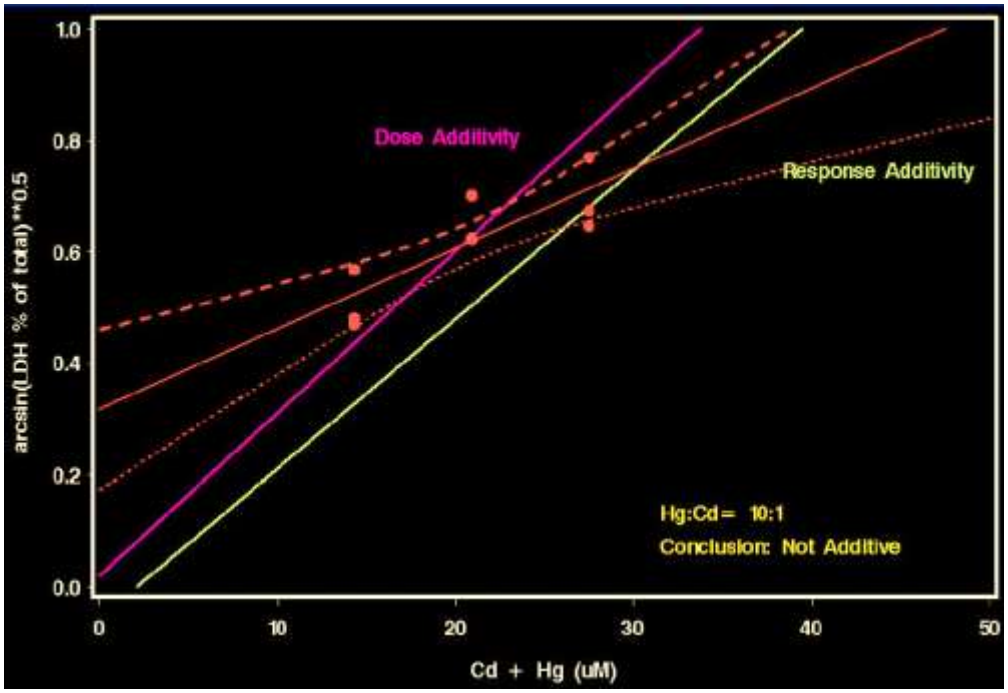


Figure 8: *Curves representing the absence of an additive response between Mercury and Cadmium (Hg: Cd = 12: 1) - Synergy type interaction*

The French National Institute for Industrial Environment and Risks (INERIS) has listed other types of interactions, in this case inhibition (in the case where the substance has no effect on a certain target organ but which, in the presence of another toxic substance, makes it less toxic; and Masking (when the components produce opposite or competing effects and the effects produced, by their combination, are less important than those suggested by toxic effects of components).

2. CONCEPTS OF INTERACTIONS IN SCIENTIFIC LITERATURE

2.1. Possible interactions in a complex mixture

The nature of the interactions cannot be defined, nor predicted with certainty [Vyshocil et al, 2001]. Indeed, the study of the combined action of chemicals in a mixture is a complex subject.

This complexity is illustrated in the work of Løkke (2010) carried out within the framework of the European program "NoMiracle" (Novel Methods for Integrated Risk Assessment of Cumulative Stressors in Europe). He studied new methods of risk assessment of cumulative stressors in Europe, and set up new tools to analyze, characterize and quantify the combined risks to health or the environment.

It should be remembered that the notions of effects associated with several chemical substances date from the end of the 19th century - beginning of the 20th century thanks to the work of Loewe and Muischneck (1926), and those of Bliss (1939) who laid the groundwork theories of toxicological interactions.

Thus, in addition to the proportion of each substance in the mixture (dose rations), the scientific literature highlights the influence of other factors on the interaction between chemical substances present in a mixture (Figure 9), and more particularly, "Environmental Stressors" identified by Lokke such: environmental exposures (drugs, atmospheric pollutants, alcohol, tobacco, etc.), and biological factors (genetic polymorphism, differences associated with age, etc.).

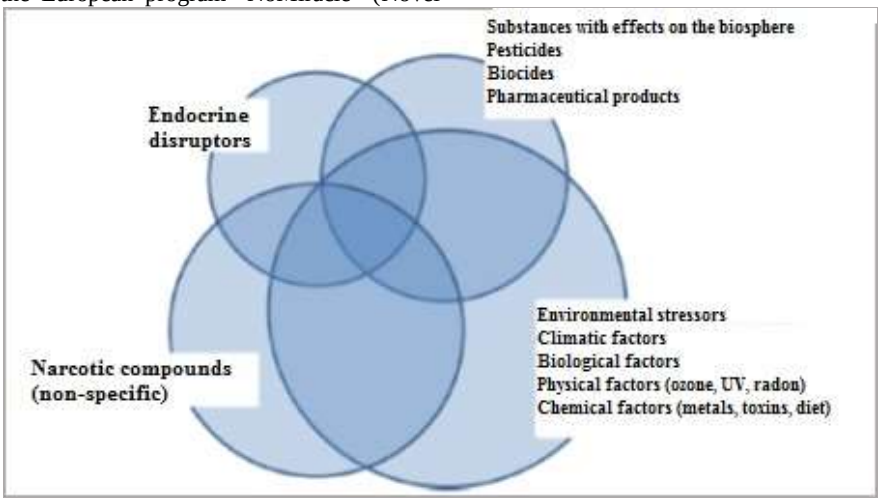


Figure 9 : *Possible interactions during multiple exposures (Løkke 2010)*

Among the main conclusions of the "NoMiracle" program:

- the importance of time to deal with the toxicity and in particular the toxicity of chemical mixtures;

- the nature of the uncertainties associated with the risk assessment (knowing that theone of the problems that arises when analyzing uncertainties is how to distinguish the relative contribution of variability - that is, heterogeneity -, and that of true uncertainty regarding the characterization of expected risks for population); and,

- the value of visualization to identify and quantify the most relevant risks.

A major finding of the project was that researchers and regulators should focus on the receptor rather than the single stressor or combination of agents.

More work needs to be done in the mechanistic understanding and interpretation of mixed effects / multiple stressors.

2.2. Interactive effects

Few studies have been done on the interactive effects of chemical mixtures after Bliss's study. Literature search has revealed that most studies focus on exposure to two chemicals or exposure to binary mixtures (Hertzberg and Teuschler 2002; Yang 1994a).

The study by Cassee (1998) emphasizes that "as a rule" mixtures should not exhibit any harmful effects on health, while Yang (1994b) suggests that even at low levels of exposure to chemical mixtures they are can cause biological effects, some of which will not be detectable by current methods.

Similarly, the study by Semences (1995) favors caution in these terms: "although some studies" support the hypothesis that adverse effects are unlikely when exposed to complex mixtures, it is "cautious. to provide for exceptions to the "" rule.

Indeed, the studies which have been carried out on chemical mixtures at concentrations close to or below the NOEC (No Observed Effect Concentration) have reported potentially dangerous biological reactions [Cavieres et al., 2002; Rajapakse et al., 2002; Welshons et al., 2003].

Over the past 15 years, research on chemical mixtures has intensified, as evidenced by review articles [Carpenter et al. 2002; Feron et al. 2002; seeds et al. 1995] and the organization of international conferences on the subject. The interest in chemical interactions is an important step in understanding the effects of the toxicology of chemical mixtures on health, as well as their environmental impact.

2.3. The toxicity of a mixture

As a general rule, the toxicity of a mixture depends on the concentration of each constituent, the duration of exposure, the sequence of administration, the frequency of exposure, and individual susceptibility, etc.

Nowadays, the toxic effects of a mixture can be predicted from the similarity of the compounds. It can be a :

- similarity in chemical structure;
- similarity in mode of action;
- similarity in induced effects.

According to the scientific consensus, as soon as chemical substances do not show any similarity between them, it can be considered that, when mixed, the risk is acceptable.

3. THE TOXIC AND ECOTOXIC EFFECTS OF MIXTURES: A COMMITTED BIBLIOGRAPHIC STUDY

3.1. Ecotoxic aspects

In 2001, ECETOC published a bibliographic study on the aquatic toxicity of mixtures, in which the authors were interested in the toxic effects of mixtures of different compounds (metals, pesticides). They compared the conclusions of studies for acute and chronic exposures, with high concentrations used in the laboratory, to other studies carried out on ecosystems or effluents and therefore using more realistic exposures. But this work exclusively concerns the prospective evaluation of chemical substances. However, the concept of risk assessment distinguishes two approaches: prospective assessment (a priori) and retrospective assessment (a posteriori) [Suter, 1993].

3.1.1. Prospective evaluation

Prospective assessment concerns the discharges, substances or materials for which we want to know the risk they represent before deciding on their use or release into the environment. This assessment is mainly based on laboratory tests and predictive exposure models. The prospective risk assessment therefore estimates foreseeable exposures and does not generally consider the response of organisms identified in the field but those of organisms used in biological tests whose ecological realism can be questioned [Forbes and Forbes, 1997].

3.1.2. Retrospective evaluation

The retrospective assessment concerns existing pollution for which we

want to know the risks for the environment. In principle, it is based primarily on measurements of in situ exposure and effects. In this context and very recently, ADEME (2005) (confronted with the evaluation of waste and derived products with a view to agricultural recovery) or AFSSA (2006) (questioning the toxicity of migrants from food packaging), present an alternative to the classic methods of evaluating mixtures by proposing to collect information on the danger of the product (mainly through bio-tests) instead of analyzes of the composition and modeling of interactions.

In summary, although the concepts of toxicological interactions between chemical compounds are not new, the interest in mixtures is relatively recent with many articles dealing with the subject [Carpenter et al. 2002; Feron, 2002; Reffstrup et al., 2010; Kortenkamp et al., 2010], and a steady increase in the number of publications over the last decade (around 600 publications per year in Pubmed since 2006, Figure 10).

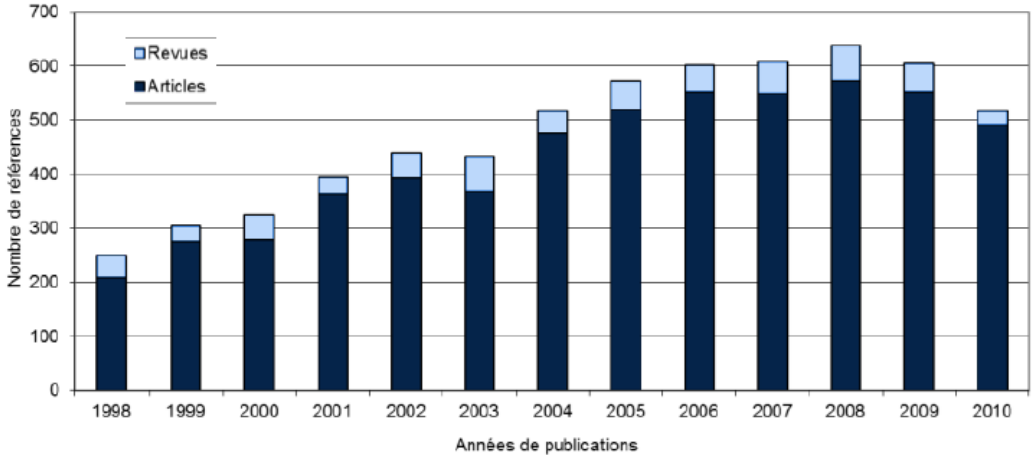


Figure 10: *Evolution of the number of citations over the last ten years for the query "mixture and toxic" in the Pubmed database (as of the date 09/09/2010)*

3.2. Case of phytosanitary products

Mixtures of chemicals used in agriculture are most often complex and consist of parent compounds (active substances and formulation aids), transformation or reaction products, residues and inert materials. There are very few data on the toxic and ecotoxic effects of these mixtures, as well as those of the co-formulants and adjuvants of pesticides.

Knowledge on the specific effects of certain formulation adjuvants, as well as on their interaction with the toxicity of the active ingredients is fragmentary and does not allow a decision to be made with certainty on the risk presented by these substances on the environment.

A realistic approach to the assessment of the risk associated with exposure to plant protection products must consider the consequences of combined exposures, as such exposures - simultaneous or sequential - could lead to effects which are quantitatively and / or qualitatively different from the effects expected in considering only the additivity of the responses generated by the products taken in isolation. Indeed, exposure to combinations of substances sometimes results in significantly greater toxicity (potentiation or synergy) or, on the contrary, lower (case of antagonism) than the simple sum of the responses induced by the components of the mixture taken. individually [Marking, 1985].

Evidence of toxic interactions in toxic mixtures should be an important aspect of the overall process of risk assessment of chemicals used in agriculture. However, this is a critical step in the evaluation procedure, since the experts most often lack reliable information on the quantitative characterization of the interactions between the molecules that go into the composition of the mixtures.

The approach currently used to assess the risk associated with exposure to mixtures of chemicals is based on the long-standing concept of additivity [Plackett & Hewlett, 1952; Sprague, 1970]. This approach may possibly be justified in the case of mixtures of substances having the same mode of action, and holds true for certain pesticides [Faust et al., 1994; Bailey et al., 1997]. However, this is not always the case, in particular for mixtures made up of molecules which have different chemical and toxicological properties.

Indeed, potentiation or synergy phenomena between active materials or between an active material and another micropollutant have often been observed [Solon & Nair, 1970; Macek, 1975; Ensenbach & Nagel, 1955; Pape-Lindtsrom & Lydy, 1997; Forget et al., 1999; Belden & Lydy, 2000; Woods et al., 2002]. The mechanisms involved are not always known, but the synergistic nature of the interaction between toxicants generally results either from an increase in the metabolic activation of one substance by another, or from the inhibition of the detoxification systems of one substance by another [Johnston et al., 1994].

Cases of antagonism have also been highlighted [Pape-Lindstrom & Lydy, 1997; Van der Geest et al., 2000; Bailey et al., 2001; Jin-Clark et al., 2002; Woods et al., 2002]. The antagonism between two substances can be of the functional type (case of substances which have opposite effects on the same physiological function), chemical (existence of chemical reactions between the substances which lead to the formation of less toxic derivatives), metabolic (modification absorption, biotransformation, distribution and / or excretion of one substance by another) or, occur at the level of biological receptors (competition for the same target; [Marking, 1985]).

However, there are very few data on the effects of mixtures of pesticides at sublethal concentrations and / or on biomarkers capable of providing information on the state of health of individuals [Bocquené et al., 1995; Forget et al., 1999; Jin-Clark et al., 2002]. Likewise, there are very few data on the effects of mixtures between active substances and adjuvants [Jumel et al., 2002].

3.3. Importance of the experimental context for the evaluation of the ecotoxicological risk

The risk assessment is generally based on the relationship between the exposure level and the maximum concentration for which no harmful effects are observed (NOAEL - No Observed Adverse Effect Level).

To characterize the risk of threshold toxic effects, the following formula is used:

$$\frac{\textbf{Exposure concentration}}{\textbf{NOAEL}}$$

<1, no adverse effects are anticipated
= 1 adverse effects can occur
> 1, adverse reactions are likely

The exposure concentration may be, for example, the Average Daily Dose (ADD) to assess a risk related to ingestion (food and water).

Two approaches are mainly studied by scientists and risk assessors: the first is to directly test the toxicity of a mixture, the second is to predict the toxicity of the mixture, based on the individual toxicity of each compound.

3.3.1. Monospecific toxicity tests

These tests form the basis of the ecotoxicological risk assessment for pesticides, although the most recent procedures involve, in some cases, the use of more complex devices such as mesocosms or microcosms. These will represent complex systems where organisms will be able to interact with each other and with the physical environment, in particular the sediments or elsewhere biodegradation processes of xenobiotics take place. In this context, organisms will be subjected to multiple routes of contamination.

Integrated systems

These systems provide relevant information for the evaluation of the fate and effects of pesticides in aquatic environments. They offer the opportunity to simultaneously identify the direct effects or "primary effects" and the indirect or "side effects" of these substances, while the monospecific toxicity tests (which simply bring together the model species such as: lymnea or stickleback and the products tested in water; the contamination of the organisms will then be done directly from the medium) carried out under simplified laboratory conditions usually only allow the evaluation of certain direct effects of the molecules tested.

More complex systems

The use of more complex systems, but nevertheless easily controllable, such as microcosms, can make it possible to take into account the phenomena which reduce (adsorption on suspended matter or on sediments for example) or increase (bioturbation, biotransformation or transformation leading to the formation of more toxic degradation products, seasonal variation in the sensitivity of organisms, etc.) bioavailability and / or toxicity of the products studied [Caquet et al., 2000]. In addition, they allow certain intra- and interspecific interactions which can influence, sometimes significantly, the nature and amplitude of the response of organisms to toxicants.

4. MIXTURE RISK ASSESSMENT APPROACHES AND METHODS

Data are limited on the toxicity of groups of chemicals or similar groups of chemicals. Where such data does not exist, evidence that different substances produce similar adverse effects on organs and / or physiological systems is brought together to create what are called assessment groups, which are used to predict outcomes. possible combined toxic effects of chemicals in the same group.

Substances in a mixture can act according to a similar "mechanism of toxicity" (ie the major steps leading to an adverse effect), and the doses can then simply be added together to predict the effects (addition of doses); or they can interact together to become more toxic (synergy) or less toxic (antagonism).

New tools, such as mathematical and biological models, are being used to predict both the organic processes of degradation and elimination of chemicals and their mechanisms of toxicity.

4.1. Assessment of the risk associated with multi-substance exposure: approaches from health agencies

The French Society of Health and Environment (SFSE) considers that the problem of mixtures is too important not to be taken into account in health risk assessments. It notes that several methods have been developed but they are still imperfect. However, these methods contribute in taking an important step in the direction of protection of exposed persones. they must therefore be used. These improvements in practices will have to evolve as research progresses in this field, by integrating new approaches.

As part of improving new approaches, the SFSE recommends:

- An iterative consideration of mixtures in health risk assessment;
- Communication, analysis and institutional recognition of the toxicological reference values for “mixtures” published in the scientific literature;
- The production of toxicological profiles for certain frequent co-exposures.

4.1.1. Approach of the Scientific Committee on Health and Environmental Risks (SCHER)

The SCHER (Scientific Committee on Health and Environmental Risks) approach is one of the relevant approaches (Figure 11). It is one of the modeling examples for mixtures of pesticides referenced in the preliminary opinion of the three non-scientific committees of the Directorate-General for Health and Consumer Protection of the EU on the "toxicity and evaluation of mixtures chemicals”.

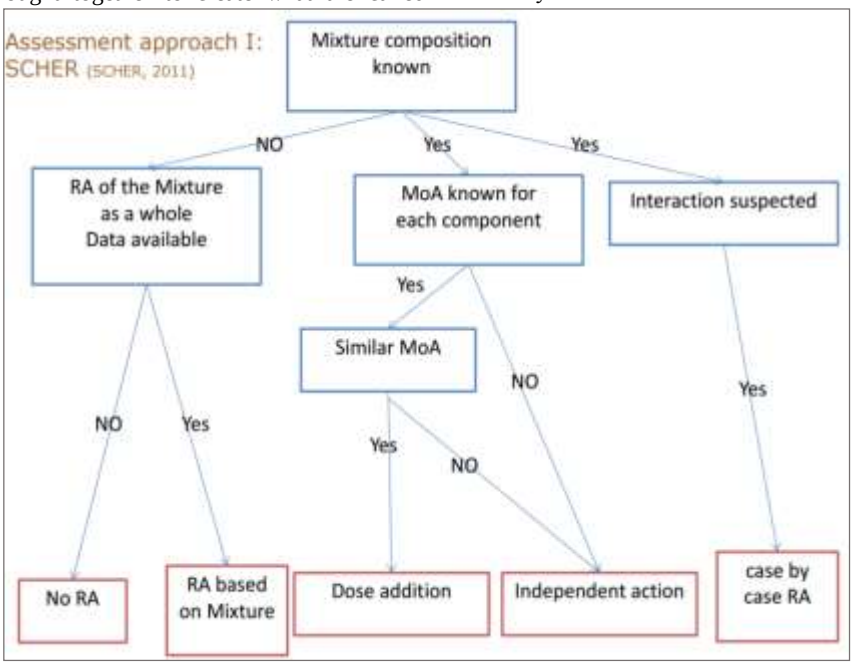


Figure 11: *Approach to assessing the risk associated with mixtures proposed by SCHER*

4.1.2. US EPA Guide

The first reference text on the risk assessment of multi-substance exposures was published in 1986 by the US EPA. The latter describes the key concepts related to exposure to mixtures of chemical compounds and their toxicity, including some specific study methods [US EPA, 1986]. In

1990, the US EPA set up a "Mix Tox" database (BD) which contains data from the literature on interactions in binary and ternary mixtures, to serve as a guide in toxicological interaction studies. However, the user of this BD is called upon to resort to toxicological judgment in the interpretation of these data within the framework of a toxicological risk analysis.

Knowledge on the consideration of chemical mixtures has been strengthened in recent years thanks to the update in 2000 of the US EPA report published in 1986. This guide describes scientific advances in terms of study methods and risk assessment, and presents three different approaches to assess the risks of mixtures depending on the nature and quality of the data available on the mixture of interest, the similar mixture, and the components of the mixture (Figure 12).

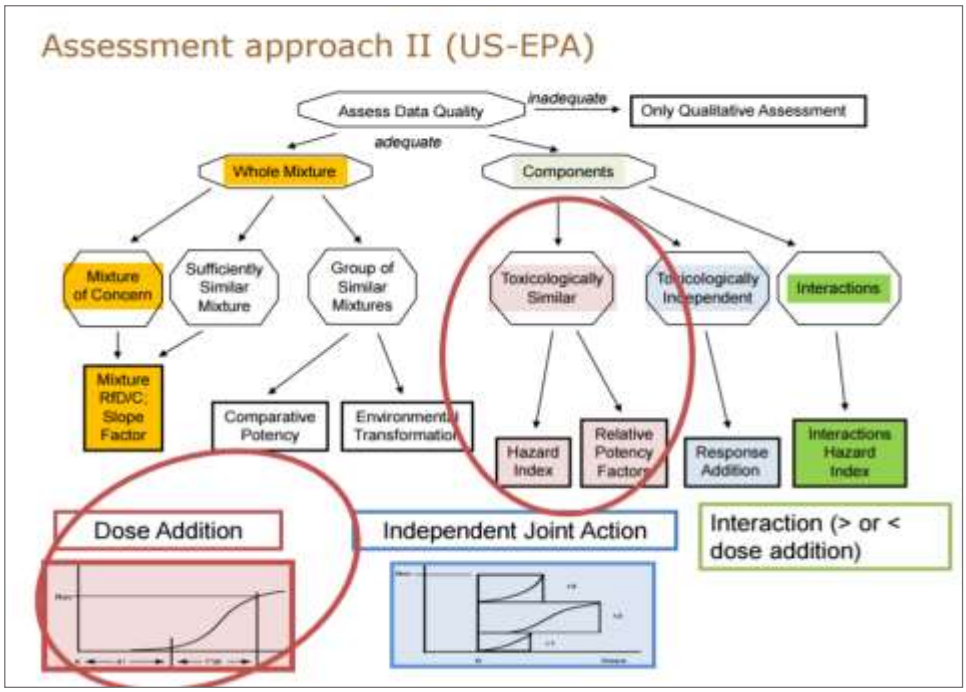


Figure 12: *Approach proposed by the US-EPA for risk assessment of mixtures*

i.- data on the mixture of interest (the mixture is considered as an entity): The approach for the mixture of interest is currently more advanced to assess carcinogenic risks, which is explained by the long experience in the use of tests mutagenicity in vitro to assess carcinogenic potential;
ii.- data on a similar mixture;

iii.- data on the components of the mixture: this approach consists in evaluating the mixture through the analysis of its compounds, it is based on the additivity of the doses for chemical substances which have the same toxicological profile (Annex 4) and on the additivity risks for substances which have independent modes of action.

This approach is adopted to assess non-carcinogenic risks.

The true toxicological mechanism of action is seldom known for a given mixture and even for most of its compounds. Therefore, judgments about the mode of action (similar or independent) of the compounds in the mixture will be uncertain.

Finally, it should be noted that the three above-mentioned approaches are based on the assumption that interactions at low exposure doses are quite low, or do not occur and are therefore neglected in the risk assessment.

Indeed, the complexity of assessing the risks associated with a mixture (study of complex exposure and toxicity data, application of scientific methods) has led scientists to recommend default methods [RECORD, 2012], while inviting the risk assessor to study the different approaches and assess the range of risk values produced to determine the toxicological mechanism of action.

Recommended methods

In cases of low exposures, lack of data on interactions, and simple mixtures (less than 12 compounds), additivity is used.

- If the compounds in a mixture have similar toxicity, then additivity of doses is recommended. In this case, the doses of the compounds are weighted by their relative potency, and they are added together. The response to the mixture will be estimated for the combined dose.

- If the toxicity of the compounds of a mixture is different, then we have recourse to the additivity of the responses. The risks are then determined for each compound taken individually. The risk of mixing will be estimated by adding up the different individual risks.

The choice of the approach to be used will be guided by certain considerations (physiological and toxicological processes, dose-response relationships for each compound, type of data available on the responses).

However, it should be noted that there is no standardized method to take into account the interactions, and even less, a biological mathematical model that could serve as a method.

Therefore, the US-EPA recommends the "addition of dose" approach for non-genotoxic toxicants having the same modes of action or acting on the same organs, and the "addition of responses" approach for evaluation. Carcinogenic risk.

4.1.2.1. Additivity of doses

This approach is used when the dose of a compound does not produce any observable effects or health concern. But if several doses are added, the effects can be seen.

A substance can be considered as an “additive dose” if it is considered to be a concentration or a dilution of each substance making up the mixture. The substances are also assumed to behave in a similar manner with respect to absorption, metabolism, distribution, elimination and toxicity.

In other words, to apply this approach, two hypotheses are put forward: on the one hand, all the compounds have the same metabolisms and pharmacokinetic and toxicological mechanisms, and on the other hand, the dose-response relationships of the compounds are similar.

Among the methods most frequently used by the US-EPA as decision-making tools, we have:

- **The Hazard Index method** (HI) which does not require extensive knowledge and is based on fairly flexible assumptions. However, it is characterized by the uncertainty of the valuation. The basic assumption of this method is that the target organ is the same for all the compounds in the mixture (non-carcinogenic toxicity).

As a general rule, the HI method is applied separately for each route of exposure, for a single specific toxic effect or toxicity for a single organ. The weighting factors of the compounds in the mixture are standardized so that their sum (which represents the indicator of risk associated with the mixture) is interpreted in relation to the benchmark value 1. Thus, exposure to the mixture is considered to be of concern if the HI is strictly greater than 1, which requires additional knowledge or compensatory measures.

- **The relative potency factors (RPF) method** and that of toxic equivalent factor (TEF). The RPF method focuses on empirical equivalence factors based on toxicity studies under well-defined exposure conditions. If the information is sufficient to show that all the toxic effects of interest share the same mode of action, then an equivalence factor is derived for each compound in the mixture relative to a reference substance. This factor will represent all toxic effects and exposure conditions.

It should be remembered that this particular case corresponds to the TEF method known for dioxins / furans, PCBs, and PAHs. The risk assessment for the mixture is carried out on the basis of the equivalent exposure and the dose-response relationship of the reference substance.

It should be noted that in each above-mentioned method, the exposure levels are weighted by a factor which represents their different toxicities (toxicologic potency) before being added together.

4.1.2.2. Additivity of responses

This approach is practiced for compounds of mixtures whose mode of action is independent (either that they act on different systems or, produce effects which do not influence each other), and whose effect can occur at low doses. for each compound, even if it is not observable in epidemiological or toxicological studies.

The risk associated with the mixture is then estimated by the sum of the risks associated with each compound acting independently. As an example, the additivity of responses is often used to assess the risks associated with mixtures of carcinogens.

Finally, the additivity of responses differs from that of doses, since it does not assume a similarity of kinetics or modes of action and / or dose-response relationships. Indeed, the risks can be combined even if the compounds of the mixture do not have the same target organs.

Let us take the example of a mixture made up of 2 compounds A and B which are independent from a toxicological point of view. The carcinogenic risk pm associated with the mixture is given by the US-EPA equation below:

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

p1 represents the risk associated with exposure to compound A and p2 to compound B.

This formula can be generalized by the following equation:

$$p_m = 1 - \Pi_{(i = 1, n)} (1- p_i)$$

Regarding mixtures composed of a few substances with low risks for each compound, the equation is simplified as follows:

$$p_m = p_1 + p_2 + \ldots + p_n$$

Except that this approach leads to an overestimation of the risks associated with mixing.

4.1.2.3. Analysis of uncertainties

The overall uncertainty surrounding the estimates of an assessment results from the actual variability of certain design parameters and / or knowledge gaps.

Thus, the adoption of the default methods proposed by the US-EPA and used to assess the risks associated with a mixture, must be accompanied by a summary of the uncertainties, in the characterization of the risks, linked to the knowledge and information available. dealing with the effects of the mixture on health, and more specifically, the identification of hazards and the dose-response relationships.

This synthesis can deal with the quality of the toxicological reference values (TRV) used for each compound (uncertainty factor, confidence level of the producer organism, etc.).

4.1.3. ATSDR approach

The scientific scene was marked by the ATSDR conference organized in 2002, thanks to the recommendations formulated below:

1. Include realistic exposure scenarios in the concentrations tested, the mixtures studied, and the exposure modes and durations must include realistic ones;
2. develop alternative, predictive and more efficient methods for the study of chemical mixtures;
3. harmonize guidelines and research on toxicokinetic and toxicodynamic aspects combined with predictive mathematical models for the study of chemical mixtures.

The ATSDR report published in 2004 [ATSDR, 2004a] provided answers to the assessment of the health risks associated with polluted sites and soils, by using the study methods published by the US EPA and, by adding improvements, namely:

- the mixture is considered as an entity, and therefore it is:
  - a mixture of known interest; or
  - a mixture similar to a known mixture;
- or again, the mixture is considered to be the sum of compounds.

The synoptics of the ATSDR approach for threshold and non-threshold substances are shown in the diagram in Annex 1.

4.1.3.1.The substances at threshold

The risk assessment strategy adopted by the ATSDR for threshold substances is formalized in figure 13.

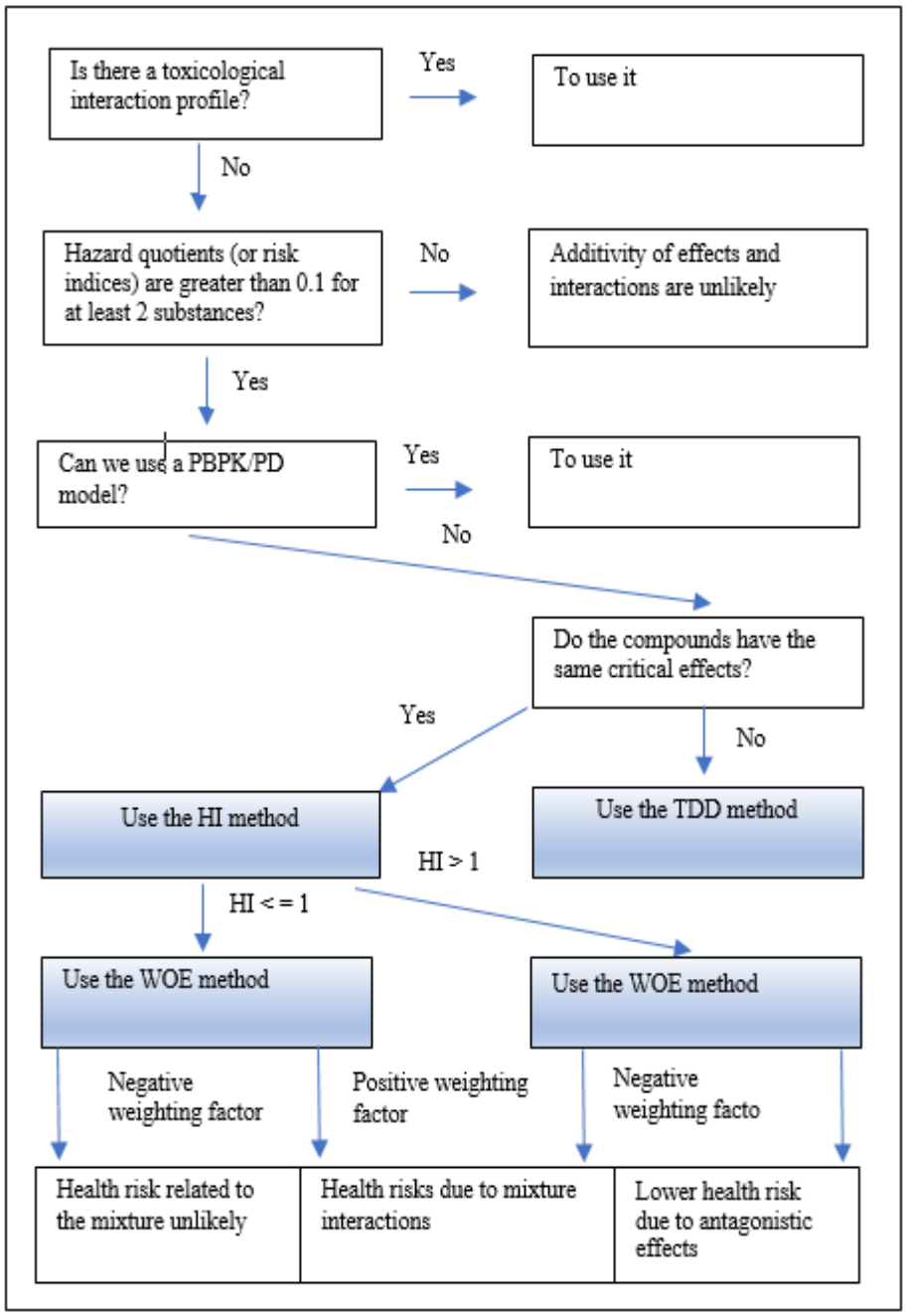


Figure 13: *ATSDR strategy for the assessment of health risks associated with mixtures of threshold substances*

- **The Hazard Index method** (HI): According to ATSDR, this approach can only be considered when at least 2 substances have a risk index greater than 0.1. The effects must relate to the same target organ and occur according to the same mechanism of action.

For substances with additive effects, the calculation of the HI is done according to the following equation:

$$HI = \Sigma_i HQi = \Sigma DJEi / VTRi$$

With:

HI: Hazard Index

HQi: Hazard Quotient or risk index of substance i

DJEi: Daily exposure dose of substance i

TRVi: Toxicological reference value of substance i.

- **The Target-organ Toxicity Dose Method** (TTD): applied for substances whose critical effects do not concern the same target organs. This method assumes that the assessor can determine a toxicological reference value (TRV) from the available studies.

It should be remembered that we are talking about a context specific to the ATSDR where only the Minimum Risk Level (MRL), relating to critical effects, are used.

In the case of health risk assessment, it is excluded to develop specific TRVs. Therefore, for a given substance, we resort to the use of other TRVs available in the reference toxicological databases.[1]than the one selected. This approach is illustrated in Table 3.

Substance A	Substance B	Substance C	Quantification of health risk
VTR retained for the ERS ⇔ kidney effect	VTR retained for the ERS ⇔ kidney effect	VTR retained for the ERS ⇔ effect on the liver	Calculation of a Risk Index (IR) for each substance + calculation of the HI for the effects on the kidney
VTR base n ° 1 ⇔ effect on the liver	VTR base n ° 1 ⇔ effect on the liver	VTR base n ° 1 ⇔ effect on the skin	TTD approach for calculating an overall IR for liver effects + TTD approach for the calculation of an overall IR for the effects on the skin
VTR base n ° 2 ⇔ nervous system effect	VTR base n ° 2 ⇔ effect on the skin	VTR base n ° 2 ⇔ blood effect	

Remark: In this example, it is considered that the effects on the same organ take place according to the same mechanism.

- **The Weight of Evidence method** (WOE): this method is applied to substances with thresholds and even without an effect threshold. It is estimated to be more precise since it makes it possible to examine whether similar effects result from potentiation (greater than additive) or from a reduction in effects (less than additive).

The WOE method proposed by Mumtaz and Durkin [Mumtaz, 1992 and 1994], proposes a weighting of the HI from the study of interactions by pair of substances (called BINWOE: binary weight of evidence).

According to the WOE method, HI is calculated according to this equation:

$$HI\ I = HI \times UF^{WOEn}$$

HI I: Adjusted Hazard Index

HI: Unadjusted Hazard Index, based on simple additivity

UF: Uncertainty factor, equal to 10 (Mumtaz, 1994)

WOEn: score describing the nature and intensity of interactions. WOEn = f (INWOE).

According to Mumtaz (1994), each BINWOE is a product of six factors belonging to the interval [-1; 1]. BINWOE = f1x f2 x f3 x f4 x f5 x f6. Annex 2 presents the factors for calculating the BINWOE score describing the nature and intensity of interactions between two substances.

This method was tested by comparing the calculated predictive score of interactions and the experimental results (study for 4 nephrotoxics) [Mumtaz, 1998]. The prediction of interactions on the target organ (the liver) has been shown to be relatively satisfactory. However, this method cannot predict the effects of the mixture on another organ. The variability of the possible concentrations of the substances composing the mixture represents a real limit.

To overcome this constraint, the US-EPA has developed an algorithm for calculating the interaction score which takes into account the differences in the proportion of the components of the mixture.

Finally, this approach seems to be interesting since it allows a quantitative evaluation of the interactions, provided that the toxicological studies are available in the scientific literature or are carried out specifically. But given the multiplicity of substances in the mixtures studied and the small number of studies carried out, this approach remains of limited scope.

- **The Toxic Equivalent Factor method** (TEF): This method is based on the use of toxic equivalence factors. It is particularly used for dioxins / furans, polychlorinated biphenyls and polycyclic aromatic hydrocarbons [INERIS, 2003].

4.1.3.2. Substances without threshold

- **Simple additivity**: the individual excess risks (ERI) are calculated for each substance and are added on condition that the ERIs are less than 0.01 and their sum is less than 0.1 [INERIS, 2006].

- **The Integral Search System method** (ISS): it makes it possible to predict the effects of a mixture of 3 or more substances from the study of binary mixtures (study of interactions by pair of substances, calculation of a weighting ratio of interactions qualifying their nature and quantifying their intensity).

This method is based on databases from the US-EPA and the National Cancer Institute which list nearly 6,000 chemical substances. In case of unavailability of toxicological data for a binary mixture, it is those of the known substances belonging to the same chemical class which will be used. Therefore, a large number of mixtures can be evaluated. However, like the WOE method, the main limitation of this tool is that the exposure levels are not taken into account.

4.1.4. Alternative assessment methods

Unable to conduct studies on all the substances and compounds of the mixtures, some scientists have proposed simplification methods to assess the risks of mixtures of known and unknown substances, in this case, the threshold of toxicological concern (TTC).

The Threshold of Toxicological Concern method (TTC): it considers that when a substance present in a mixture is below a certain exposure threshold, it is not necessary to include it in the risk assessment. And yet, the scientific literature highlights the importance of the combined effects that can occur, even if the substances in the mixture are present at levels less than or equal to the no-effect doses (NOAEL / NOAEC), or have modes of similar action.

The risks will only be truly assessed if the analysis of the composition of the mixture is exhaustive and if sufficient data are available to estimate the effects (subject of active research).

Physiologically based pharmaco-kinetic / dynamic models (PBPK / PD): These models make it possible to have an approach adapted to the exposures studied in terms of route and exposure levels, and to determine an interaction threshold. These new tools contribute to a better understanding of the toxicokinetic phase.

These models seem interesting, but they are not possible for the impact studies carried out within the framework of the health risk assessment.

The quantitative structure-activity model (QSAR): This model makes it possible to collect toxicological and ecotoxicological information on substances which are devoid of it. It is a mathematical model used to predict the physicochemical and biological properties and the fate of compounds in the environment from their chemical structure. Currently, this tool is not adequate for complex toxicological properties.

"Omics" approaches: make it possible to understand the complexity of living things as a whole, using the least restrictive methodologies possible, and to obtain a great deal of information on the cellular and / or tissue response to in vitro or in vivo exposure. They are used to highlight and identify new biomarkers (exposure [Castorina, 2003; Scherer, 2005], effect or susceptibility [Calderon, 1998]) to identify and quantify the exposures and effects associated with mixtures. [Viau, 2002], generate new knowledge on the mechanical level (modes of action), and develop new predictive toxicology tools to identify hazards.

However, these methods are now exploratory and do not seem to have a regulatory future in the short term.

4.2. Approaches adopted in the field of occupational hygiene

ACGIH, OSHA and NIOSH have adopted the HI-type approach, in this case the sum of the risk indices when the effects concern the same target organ according to the same mechanism of interaction (ACGIH, 2002; NIOSH; 2006). However, the ACGIH recommends examining the possibilities of synergy and potentiation on a case-by-case basis.

In addition, the IRSST has implemented a decision support tool for occupational physicians, toxicologists and hygienists in order to estimate the possibility of interactions of substances in a mixture in the workplace [IRSST, 2005].

This tool makes it possible to identify the effects on health, toxicokinetic data, mechanisms of action and target organs involved in the toxicity of all the chemical substances of the Regulation respecting the quality of the working environment in Quebec.

Also, the IRSST has "experience sheets" on 209 pairs of substances with an opinion on the type of interaction: supra-additivity, additivity, infra-additivity (antagonism) and "impossible to pronounce" (Appendix 3).

4.3. Risk analysis of complex mixtures

Currently, the approach used by default for the risk analysis of chemical mixtures is that of additivity. The results of this approach can be confirmed or refuted if appropriate data regarding the toxicity of the mixtures are available. Toxicokinetic interactions explain most of the deviations from additivity to the level of toxicity demonstrated by certain studies [Crishnan and Brodeur, 1991].

The additivity hypothesis of the toxic effects of substances present in a mixture is justified if these substances act on the same biological system and contribute to a common response [Goldstein et al., 1990].

In the absence of adequate data on a particular mixture, risk assessors can apply the data for each chemical, most often in an additive. According to the EPA, any information must reveal the potential for interaction (ie, synergy, antagonism). When we do not have sufficient data on the types of interactions, models of the additive response (or dose) are recommended "[EPA, 2000].

As it is difficult to determine which of the models, AR or AD, is the most appropriate for interpreting the results of toxicological studies, the analysis of the applicability of these models should be done in systematic studies, such as that of Stavenes Andersen. et al. (2009). The latter are interested in the types of interaction of substances in mixture on neurotoxic effects in vitro.

For their part, Price and Wiltshire (2009) propose the application of the probabilistic approach with the "Additivity of Doses" (AD) and "Additivity of Responses" (AR) models in order to overcome the limitations of the current approach. Their study deals more specifically with the modeling of the chronic non-carcinogenic effects of migrating substances from certain food packaging. To our knowledge, this is the first publication to introduce a probabilistic approach into the health risk analysis associated with mixtures containing components without reference values or standards.

4.3.1. The Exposome: a new concept to understand the risks of exposure

The exposome studies the exposures to which a man is subjected from his conception (intrauterine life) until his death. This disciplinary field aims to make the link between genetic factors and environmental exposures in the occurrence of diseases, such as cancer.

Three overlapping domains within the exposome have been described:

(1) a general external environment including factors such as urban environment, climatic factors, social capital, stress;

(2) a specific external environment with specific contaminants: diet, physical activity, tobacco, infections, etc.; and,

(3) an internal environment to include internal biological factors such as metabolic factors, intestinal microflora (gut microbiota), inflammation, oxidative stress.

Christopher Wild, of the International Agency for Research on Cancer, recalls that in the event of cancer prevention, it is the identification of genetic risk factors or even "genetic predispositions" which is mainly taken into account. The role of environmental contaminants in the cancerization process is largely underestimated and underestimated.

However, according to Professor Wild, chronic diseases and cancers have an environmental cause in the broad sense of the term, taking into account the different vectors of exposure (water, air, soil) but also the way of life (food, behavior, etc.).

Consequently, the concept of exposome launches new challenges and new questions towards epigenetics which shows that the environment influences the expression of genes.

A successful exposome should integrate many external and internal exposures from different sources throughout life.

4.3.2. Methods used to assess the exposure of the population to complex mixtures

The exposure of the general population to complex mixtures is assessed using different methods:

- relative power factor (RPF) as a function of a chemical index: example of chemicals which act through the inhibition of cholinesterase;
- toxic equivalence factor (TEF) or (TEQ) which makes it possible to estimate the toxic potential of the environments and the exposures linked to these compounds, in the form of a global index. We use the structural similarities between the molecules and the hypothesis of a common mechanism of action involving the Aryl hydrocarbon receptor (case of Polycyclic Aromatic Hydrocarbons).
- there are many uncertainties: variability of TEFs according to tissues, species, doses, durations and routes of exposure, ignorance of interactions between compounds. This implies that adverse effects are likely to appear, even at the background noise of exposure, and that more drastic source reduction measures must be considered;
- the risk index (HI): when the mechanisms of action are well determined (the target organ and the biological receptors involved), the methods of FPR and TEF are recommended, but in the absence of data on the mechanisms of action, HI is recommended [Hertzberg and Teuschler, 2002].

4.3.3. Toxicological evaluation of chemical mixtures

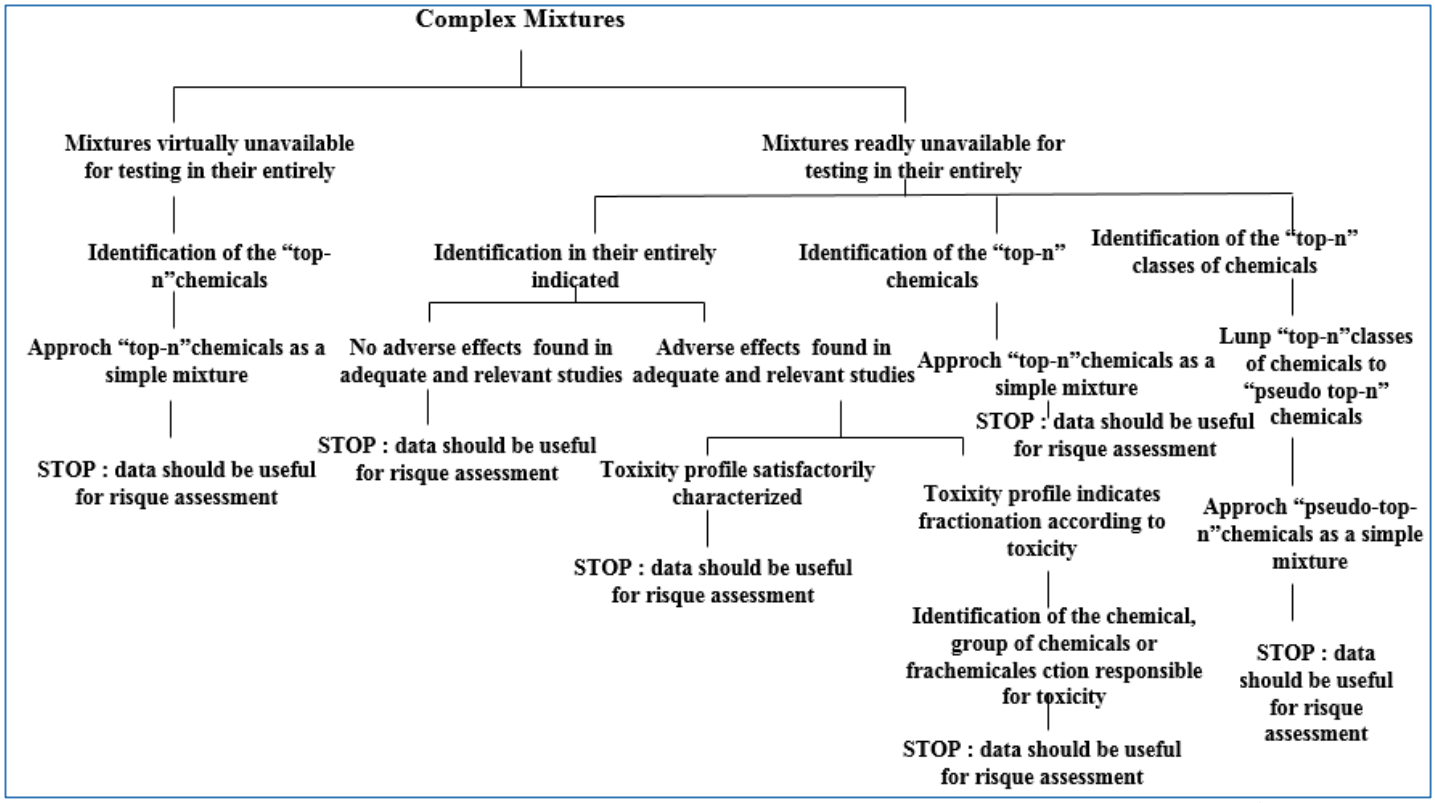
Two main types of approaches have been developed to assess the toxicity of mixtures of chemicals (Figure 14). The first, called the "bottom-up approach", focuses on the toxicity of simple mixtures [Groten et al., 2001]. It is the most used to assess the toxicity and the risk associated with exposure to mixtures of chemicals.

In the case of simple mixtures, the substances are identified and do not exceed the number of ten (10). The toxicity data are collected, initially for all the components of the mixture, then the toxicity of the mixture is estimated according to the principle of additivity: the toxicity of the

mixture is considered as the result of the toxicity of each substance in the mixture.

The second approach, called the "top-down approach", consists in directly evaluating the toxicity of a mixture as a whole in order to generate very applied data for risk evaluation [Feron et al., 1998].

This approach therefore considers complex mixtures in which all the components are not necessarily identified or quantified. With this approach, the effect of the mixture is evaluated as a whole. Indeed, the complex mixture is no longer considered as a sum of substances but as a single entity.



Source: Feron, V.J. and al., 2002. Toxicological Evaluation of Chemical Mixtures.

Figure 14: *Scheme of toxicological evaluation of complex mixtures*

These two approaches are quite different, the first being more theoretical and the second more applied. The theoretical approach concerning simple mixtures is often far from the reality of aquatic ecosystems where organisms are subjected to multiple stresses. However, because of its very simplicity, it provides new knowledge on the interactions between substances, and from a methodological point of view, it allows the development of predictive models of toxicity.

In the case of carcinogens, additivity of responses is used, while additivity of dose is applied in systemic toxicants acting by similar mechanisms [USEPA, 1986; Meek et al. 1994].

Regarding the additivity of doses, the dose of each of the components of the mixture is normalized by a protective value such as the reference dose or acceptable daily dose (danger index approach, also called the Rm approach [ACGIH, 1999]), or by the dose of a component of the mixture, usually the most toxic (toxic equivalence factor approach). These standardized doses are then added together.

A realistic approach to the risk assessment associated with exposure to xenobiotics should consider the consequences of combined exposures, as such exposures - simultaneous or sequential - could result in effects that are quantitatively and / or qualitatively different from the effects expected by considering the additivity of responses. Therefore, demonstrating the presence of toxic interactions among pollutants is an important aspect of the overall process of risk assessment of chemical mixtures. However, the "quantitative" characterization of interactions in the risk assessment of mixtures remains a challenge facing scientists and regulators.

Three approaches are possible to assess the toxicity of mixtures of chemicals:

i.-The first is to directly assess the toxicity of the mixture "as a whole" in order to generate appropriate data for the risk assessment. This approach is possible with well-defined mixtures. It would be excessively expensive and inefficient if it had to be applied to mixtures the composition of which is liable to vary over time or from place to place.

ii.-The second is an alternative approach involving the evaluation of interactions at different levels (binary, tertiary, etc.) to predict the toxicity of more complex mixtures. The results of such tests can be interpreted using statistical methods such as multivariate regression analysis. With four or five components in a mixture, this approach requires a large number of expensive and inefficient trials.

iii.- And finally, the **physiological-based modeling TCBP**[2]considered to be a potentially effective tool for the toxicological risk analysis of complex mixtures. Indeed, thanks to its mechanistic basis, it allows certain essential extrapolations to be made.

Until now, the toxicokinetics of several binary mixtures could be described by models TCBP; on the other hand, more complex mixtures have not yet been modeled due to the inability of researchers at describe the effect that would have a third Where fourth substance on the interaction at the binary level.

Tardif et al (1997) have already innovated by constructing a model for a ternary mixture (toluene, ethylbenzene and xylene). They were able to predict the interactions present between the components of this mixture

by taking into account only the binary interactions (toluene-xylene, toluene ethylbenzene and xylene-ethylbenzene). It remains to be seen whether this approach applies to more complex mixtures.

4.3.4. Work by Haddad et al. to assess the risks of mixtures

During his various works, Haddad and his colleagues (2001) were able to develop a risk assessment methodology for chemical mixtures, which takes into account the pharmacokinetic interactions between the components (Figure 15).

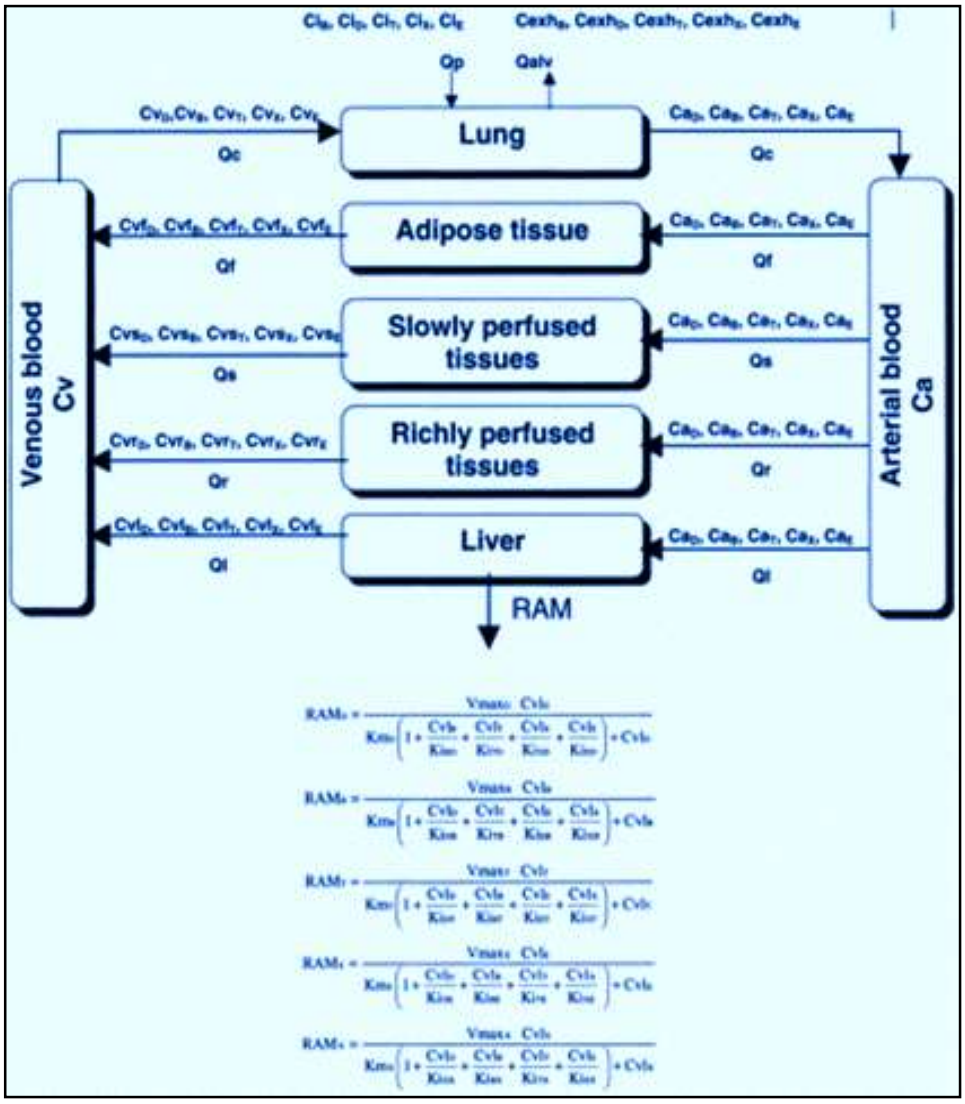


Figure 15: *PBPK modeling taking into account interactions in the risk assessment of chemical mixtures for health*

This approach is used in the event of a risk assessment forthe health related to exposure of a mixed. The primary goal is to assess therisk in itself based on an estimate, which is intended to be more precise possible, of the exposure of the target tissues to the toxic entities of the components of the mixture in question. This can be do at using the modeling of mixtures based on binary interactions [Haddad. et al., 1999 a, b] combined with the approach of BHI [Haddad. et al.,1999 a, b].

The work of Haddad et al is the first to demonstrate the possibility of addressing the topic complex of toxicokinetic interactions in the context of the analysis from mixtures in a relatively simple and mechanistic way while respecting the basic principle of toxicology: "**the response to the toxic agent is directly related to the dose received in the target tissue**'.

Recent work by researchers at the Toxicology Research Center of Canada uses data on the mechanisms to predict the existence and magnitude of toxicological interactions, at different doses, and under different exposure scenarios. The interaction mechanisms considered include the modulation by one of the components of the mixture of the absorption, distribution, metabolism, excretion or of the interaction with receptors of other components of the mixture. The impact of these modulations may not be large at low exposure levels, but in most cases remains unknown.

4.4. Main conclusions of the scientific experts in the field of evaluation

Based on the analysis of the available scientific literature, the scientific expert committees of the European Commission reached the following conclusions:

1. Under certain conditions, the chemicals will act together so that the overall level of toxicity is affected.

2. Chemicals with common modes of action will act together to produce combination effects that are greater than the effects of each component of the mixture applied individually. These effects can be described by addition of dose / concentration.

3. For chemicals with different modes of action (acting independently) the concept is not robust.

4. Interactions (including antagonism, potentiation, and synergies) generally occur at medium or high dose levels (relative to lower effect

levels). At low levels of exposure, they are unlikely to occur or are toxicologically insignificant.

5. Given the almost endless number of possible combinations of chemicals to which humans and environmental species are exposed, some form of initial filter to focus on mixtures of potential concern is needed. Several criteria for such screening are offered.

6. With regard to the assessment of chemical mixtures, there is a significant knowledge gap at present and a lack of information on exposure and the rather limited number of chemicals for which there is sufficient evidence. information on their mode of action.

Currently, there is no defined set of criteria to characterize or predict a mode of action for data-poor chemicals.

7. If no information on the mode of action is available, the dose / concentration addition method should be prioritized over the independent action approach.

To predict a possible interaction would require expert judgment and should be done on a case-by-case basis.

On the basis of these conclusions, decision support trees to assess the risk of mixtures are proposed. By way of example, Figure. 16 describes the risk assessment process associated with mixtures of a chemical nature.

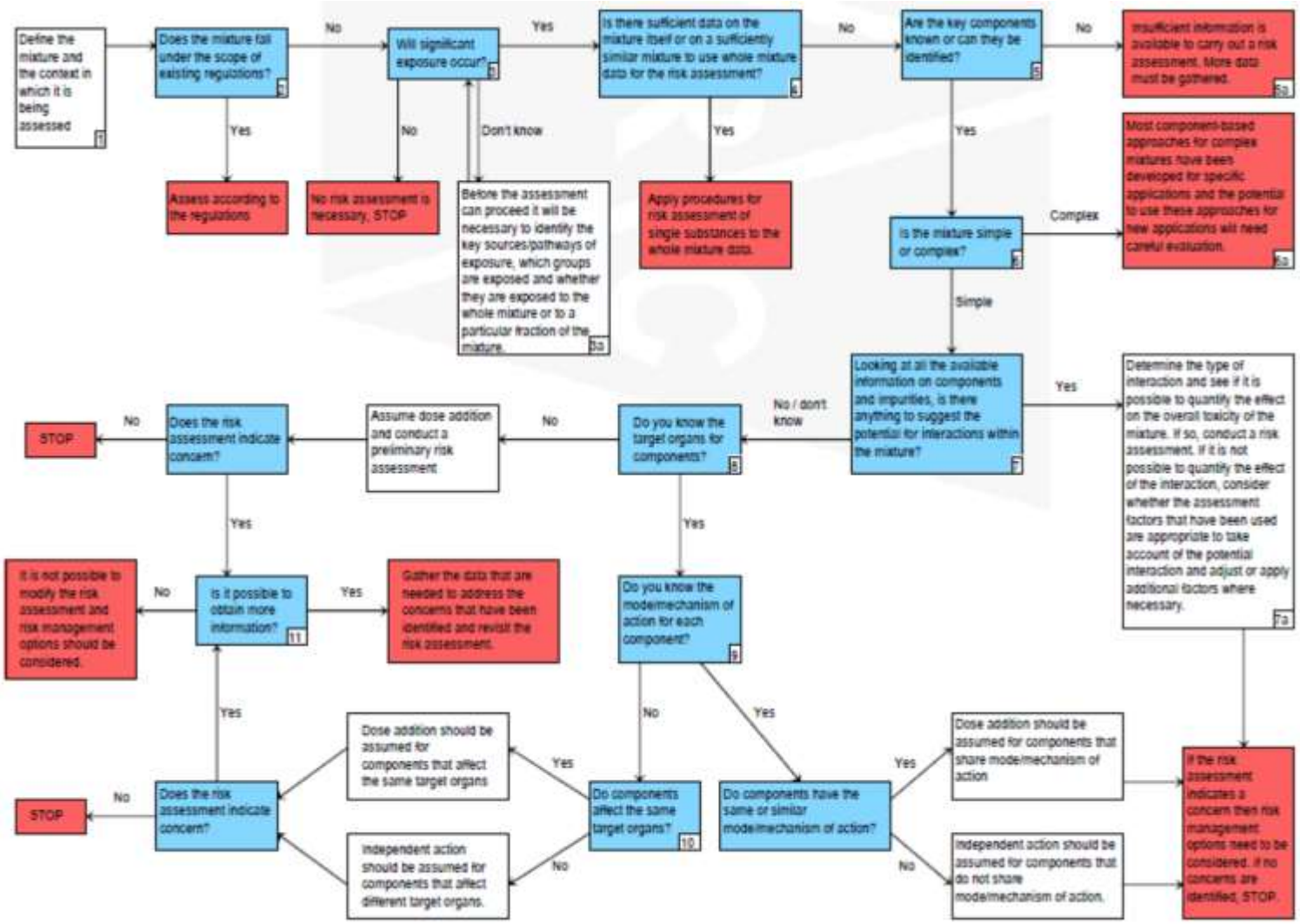


Figure 16: *Decision tree for risk assessment of chemical mixtures*

Source: IGHRC, 2009

II.- MECHANISMS OF COMPLEX MIXTURES AND THEIR EFFECTS ON HEALTH / REPROTOXICITY AND GENOTOXICITY

1.- EXPERIMENTAL EVALUATION OF THE TOXICITY OF BINARY MIXTURES OF INDUSTRIAL SOLVENTS

Knowledge of the toxic mechanism of a substance improves the possibilities of prevention and allows the design of chemicals that are better tolerated. It often avoids overexposure and guarantees a better understanding of fundamental biological mechanisms.

Certain chemical, physical or biological agents would be capable of modifying the frequency of hereditary diseases in humans or, would be at the origin of the appearance of cancers. However, many chemicals that cause cancer have mutagenic activity.

This is because the initiation stage of carcinogenesis involves DNA damage in somatic cells. If this lesion is not corrected it can fix itself irreversibly (this is called a mutation) and tests that determine mutagenic activity could also identify chemicals that are capable of causing cancer.

Thus, a large part of the in vitro and in vivo tests which are described in the guidelines, and which form the basis of genetic toxicology tests, are used to study both mutagenic activity (demonstration of the ability to damage l (deoxyribonucleic acid or DNA) and the possible carcinogenic activity of the chemicals.

1.1. Toxicological interactions in binary solvent mixtures: studies by McDermott et al.

Mc & Dermott et al. [2008] evaluated in vitro toxicological interactions occurring in binary mixtures of solvents. These authors chose two hydrophobic solvents: toluene and n-hexane, and a relatively hydrophilic solvent: methyl ethyl ketone (MEK).

T-Jurkat cells were exposed for five days to three different concentrations of these solvents, one of which corresponded to the LOAEL (minimum level inducing adverse effects).

Toxicity is measured according to 3 criteria: (i) damage to the cell membrane (concentration of the enzyme lactate dehydrogenase), (ii) disturbance of the concentration of calcium ions (Ca2 +) and, (iii) change in status glutathione redox.

Using the dose-response relationships for each solvent, the authors found that all the combinations of toluene and n-hexane induce supraadditive interactions for the three toxicity criteria. The same is true for MEC / n-hexane and MEC / toluene concerning the effect on lactate dehydrogenase and glutathione. Only the combinations involving the ECM resulted in an infra-additive effect on the Ca^{2+} concentration.

The study by Mc Dermott et al. shows that “mixed exposures to certain organic solvents in the workplace can generate supra-additivity at the level of oxidative stress and biochemical changes, and probably at the level of the immune system. "[Krishnan, 2008].

Furthermore, it should be noted that McDermott et al. demonstrated in vivo the existence of supra-additive interactions for most binary mixtures, in particular toluene, n-hexane and methyl ethyl ketone. These interact with each other at the metabolic level [Krishnan and Brodeur, 1991].

As for the results on in vitro synergy reported by McDermott et al., They must be interpreted through pharmacokinetic models, while taking into account the metabolic interactions. In fact, the pharmacokinetic model will make it possible to establish the atmospheric concentration of the solvents used in the in vitro studies. This in vitro - in vivo extrapolation will provide a better understanding of the risks for the organism exposed to the combinations. Subsequently, the threshold for these interactions can be assessed in relation to the exposure limit values of these solvents.

1.2. Association of bladder cancer with chemical exposure in the workplace: a study by Richardson et al.

Richardson et al. [2007] studied the association of the incidence of bladder cancer with exposure to chemicals in the workplace. In this study, cumulative exposures to 12,456 different industrial chemicals were estimated using participants' employment history information and an occupation-exposure matrix (developed in the United States). The study population consists of males aged 20 and over at the time of diagnosis (between 1983 and 1990) selected from the patient list for the province of British Columbia in Canada (British Columbia Cancer Registry).

Exposure to different substances in the workplace is assessed using data from the National Occupational Exposure Survey (United States) which is based on the occupation-exposure matrix. These data present the probability of exposure to a specific substance for a trade, or for a specific position in a given industrial sector. Logistic regression analysis reveals a significant association with exposure to petroleum products or additives, lubricating oils, paints, and soaps or detergents.

A principal component analysis indicates that five components (representing 29 chemicals such as: heptane, hexane, methyl-tert-buthylether, propenoic acid, sulfonic acid) account for more than 75% of the variance total.

The exposures comprising the first and second major components are mainly due to jobs in the slaughter industry and at petroleum service stations. The third main component corresponds to exhibitions in the fields of car construction and repair. A considerable proportion of the exposures in the fourth major component is attributable to the occupation of truck driver. Members of the 4th and 5th major components also had exhibits at petroleum service stations. Out of the seven chemical agents for which data are available for evaluation, a significant dose-response relationship and a statistically high risk were observed for 4 agents: mineral oils, benz (a) anthracene,

The case-control study by Richardson et al. reveals a significant positive association between the risk of developing bladder cancer and exposure to 29 chemical agents associated with particular occupations.

Mineral oils, benz (a) anthracene, 4-chloro-ortho-toluidine and diesel engine exhaust are the 4 chemical agents that explain an increase of at least 50% in bladder cancer observed in the population of workers studied. A better knowledge of the exposure dose and the mechanism of action of these substances will make it possible to re-evaluate the exposure levels without significant risk.

In general, the interpretation of the data leading to the risk assessment is not carried out in the same way depending on whether the constituents of the mixture have an identical mode of action or not. Thus, it is on the basis of a common mode of action (genotoxicity) leading to carcinogenicity that the effect of PAH mixtures is estimated**.**

When the information shows that all the toxic effects of interest sharing the same mode of action are sufficient, an equivalence factor is derived for each compound in the mixture relative to a reference substance. It represents all toxic effects and all exposure concentrations [INERIS, 2006]. This particular case corresponds to the method of toxic equivalence factors (TEF). The risk assessment associated with the mixture is then carried out on the basis of the equivalent exposure and the dose response relationship of the reference substance.

1.3. Genotoxicity of polycyclic aromatic hydrocarbons alone and in mixture: work of TOXALIM

The work carried out by the TOXALIM team within the framework of the HYDROMEL project [polycyclic aromatic hydrocarbons (PAHs) alone and in mixture], was carried out with the objective of improving the predictability of the effects of mixtures and, to study the modes of action as well as the mechanisms of interaction between certain compounds with respect to certain tissue targets or biological functions (CIME).

These studies led to the proposal of a new list of toxic equivalence factors (TEFs) for PAHs based on a recently developed in vitro genotoxicity test (γ-H2AX), compared to benzo (a) pyrene, the best known PAH [Audebert et al., 2012]. Work is continuing to determine the levels of uncertainty associated with interactions between PAHs when the latter are mixed.

Prior knowledge of the mode of action of substances may also be at the origin of the deliberate choice to test a mixture of compounds whose mode of action (or target) is different, but which will produce an effect of the same nature. The CIME project strategy consists of evaluating, on different targets, the effect of a ternary mixture of endocrine disruptors, each of whose constituents has a specific mechanism of action relating to de-masculinization or feminization (Figure 17), but which are all capable of producing the same final biological effect.

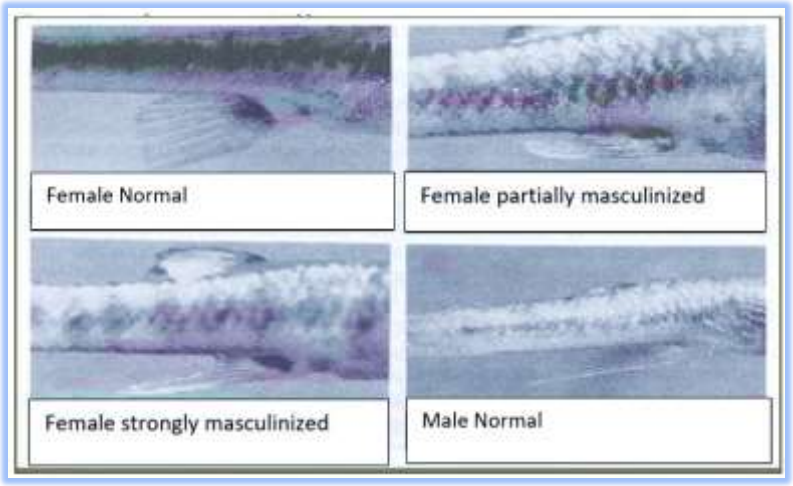


Figure 17: *Effects of effluent from a paper mill (complex mixture) on the development of gonopods in the female Heterandia formosa*

1.4. Some notable results of studies and research programs

The EXPOMATPEST (Impact of a maternal exposure to a pesticide mixture on immunity, haematopoiesis and central nervous system in offsprings. Goal: Improving knowledge on mechanisms (cellular and molecular changes) involved in pesticide mixtures toxicity. Pesticides tested: of Chlorpyrifos, Endosulfan and Atrazine alone.) research program has shown that pesticides alone (atrazine, chlorpyrifos, endosulfan), at doses at which they are supposed to have no harmful effect on health, disrupt hematopoiesis, immunity as well as the expression of certain liver genes linked to stress and cellular toxicity. These effects have been observed after pre- and postnatal exposure, some of which have been observed in young pups from weaning.

At the hematopoietic level, the mixture does not exert an effect superior to that of pesticides alone. On the other hand, on short-term synaptic plasticity, it exerts a greater effect than that of the pesticides individually considered in certain 14-week-old animals.

For the hepatic component, the mixture overall reproduces the modulations induced by pesticides alone with regard to the expression of stress and toxicity genes. In general, the impact of the mixture can hardly be predicted from the impact of pesticides alone since, depending on the parameter studied the mixture may or may not exert an effect greater than or equal to that of the pesticides taken individually. Tests carried out in vitro on the same mixture using murine or human hepatocytes in primary culture indicate that the effects observed on the mixture are mainly explained by the effects of the most active pesticide (endosulfan or chlorpyrifos depending on the tests) [Rouimi et al., 2012].

From the serum samples collected within the framework of the EXPOMATPEST project, a metabolomics analysis was carried out ("Exposure to individual pesticides or in combination: evaluation of biomarkers", EPICEE, supported by ANSES). For both sexes, dietary exposure to pesticides alone is associated with a metabolic footprint distinct from that observed in unexposed animals. In addition, in males and females exposure to the mixture is characterized by metabolic changes different from those observed in individuals exposed to pesticides alone. It should also be noted that metabolic disturbances between the different animal groups appear from weaning.

These results show that it is possible to develop the metabolomic approach to characterize the plasma biomarkers of dietary exposure to low doses of pesticides alone or in mixture [Merhi et al., 2010].

During the work preceding the CIME project, it was observed that the deleterious effects caused by the genistein-vinchlozolin mixture were generally more pronounced than those obtained during exposure with the molecules alone [Eustache et al., 2009; Kouidhi et al., 2012].

In the case of CIME, aimed at studying the genistein-vinchlozolin-bisphenol A mixture, the effects obtained with bisphenol A alone are, in most cases, superior to those obtained with mixtures containing bisphenol A and are close to the effects obtained with the genistein-vinchlozoline mixture at equimolar doses. In general, continuous exposure to mixtures at low doses, including to the ternary mixture, does not have the same effects on the physiological parameters measured according to the generation considered, and some increase over the generation (not exposed), reflecting the probable establishment of epigenetic mechanisms which remain to be elucidated.

In addition, the PERICLES program (exposure to mixtures of active substances and possible combined effects on human cells) addressed the problem of pesticide cocktails to which consumers are most likely to be exposed, starting from exposure. of the French population, in order to understand the nature of the cocktail effects in vitro on cells of human origin [Crépet et al., 2012]. The main cocktails and vector foods were determined by a statistical classification method developed for this purpose.

7 cocktails containing 2 to 6 pesticides were identified and in vitro toxicological tests, evaluating cell viability, genotoxicity, activation of xeno-hormonal receptors and other biomarkers were carried out on cell

lines representative of different groups humans (liver, kidney, intestine, brain, colon) [Cravedi et al, 2012].

Thus, the study carried out made it possible to demonstrate more or less significant cytotoxic effects depending on the cocktails and cell lines. A cocktail was found to be genotoxic on a hepatic line (HepG2), with a greater effect than that resulting from the sum of the effects of the constituents of the mixture [Graillot et al., 2012]. Other modes of action, such as the transactivation of the PXR receptor in liver cells, have shown infra-additive effects of the mixtures tested [Rouimi et al., 2012].

The results show a great variability in the sensitivity of the cell models and of the tests carried out. In fact, these cocktails produce additive effects but also supra or infra-additive effects, which makes it difficult to predict the response of the cocktails from the results of the responses and the doses of the pesticides alone.

Conclusion

These various scientific projects and the network of partners that has been set up have made it possible to deal with research questions on exposure to mixtures, interactions between constituents of a mixture, the importance of the exposure window, relevant biological systems, modeling of observed effects.

They also required the development of new tools at the biological, analytical or statistical level. Thus, was developed an in cellulo assay in 96-well culture plates based on the phosphorylation of histone H2AX intended to evaluate the genotoxicity of the mixtures and to compare it with that of the constituents examined individually. This test was applied to mixtures of hydrocarbons or pesticides. Other work is underway, in collaboration with INERIS, with a view to developing models capable of organizing these data and interpreting them.

In terms of analytical chemistry, analyzes of urinary pesticide metabolites seem to have a bright future. The interest of this semi-targeted approach comes from the fact that the data acquisition is carried out in a global way (full HRMS) and that it is therefore possible a posteriori to search for pesticides or their metabolites not initially selected.

The rapid development of metabolomics and the prospects that this approach opens up in terms of identifying biomarkers capable of revealing cell and tissue dysfunctions early and without a priori have led INRA to develop this type of study, in particular. to apply it to mixtures.

The data acquired over the past three years and those currently being analyzed suggest that the assumption regularly made in risk assessment, which consists of not considering the interactions between the components of a mixture, does not reflect reality. Knowledge of these interactions is currently fragmentary, making it difficult to take them into account. This is a real field of research to be explored.

2. QUANTITATIVE AND MOLECULAR MECHANISMS OF COMPLEX MIXTURES (SYNERGY, ANTAGONISM)

The study of chemical mixtures is limited for a certain number of reasons, and more particularly, for the complexity and heaviness of its implementation, and the difficulty of interpretation. It is much easier to study a single compound in an animal study and get the response information to a given dose.

An almost infinite number of combinations of the mixtures are possible, and often we do not know what is most important and What recommended dose range should be investigated. Few studies have been devoted to the interactions of mixtures, or even, of two chemicals.

In everyday life, we are exposed to multiple substances, and to the biological effects of different chemicals. However, homogenizing statistics on how to deal with complex mixtures is a new science which is under development [El Masri et al., 1997].

The approach currently used to assess the toxicity associated with exposure to mixtures of chemicals is based on the long-standing concept of additivity [Plackett and Hewlett, 1952; Sprague, 1970]. This approach may possibly be justified in the case of mixtures of substances having the same mode of action [Plackett and Hewlett, 1952; Sprague, 1970]. However, this is not always the case, in particular for mixtures made up of molecules which have very different chemical and toxicological properties.

Complex mixtures can act according to two different types of mechanisms: quantitative and molecular interaction.

2.1 Quantitative mechanisms

When several substances simultaneously access the living cellular environment, they can interact in such a way that the results do not constitute an extrapolation of the effects that these same substances would cause when acting individually.

The molecular mechanisms of the interaction are manifested by an acceleration, an inhibition or an absence of effect at the level of each of the successive phases of the penetration, the diffusion, the access to the cellular targets, the fixation on the molecular receptors, the metabolic transformation, and excretion.

The result is expressed by an increase in the effects observed (synergy or potentiation), a decrease (antagonism), or neutrality with or without additivity, if the factors all have an activity on the organism.

2.1.1. Interaction between substances that do not produce the same effect

Xi, applied alone, produces the effect (ei) at the dose Dei. Put in the presence of one of the Y or Z effectors, it produces the same effect (ei) at the D'ei dose [Bounias, 1999].
Such an observation implies the implementation of series of measurements of the effect E for various doses of Xi, in the presence of one of the effectors, either at fixed doses or at doses proportional to those of Xi.

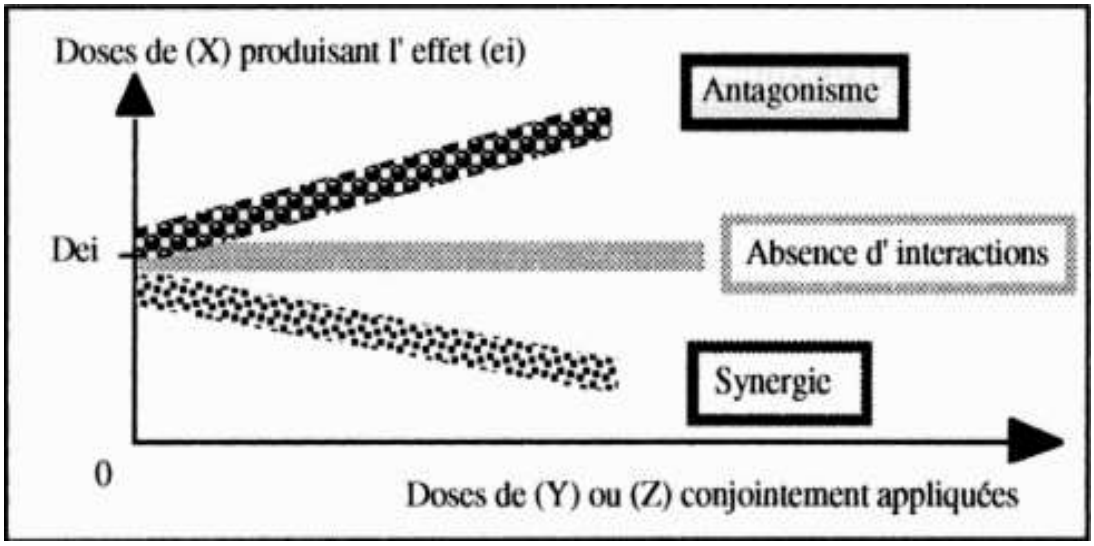


Figure 18: *Loewe curves, in case the interacting substances do not produce the same effect*

From the curves giving the responses as a function of the doses (Figure 18), an adequate modeling then makes it possible to deduce precisely what would be the dose of Xi which would produce the effect (ei) taken on the corresponding coordinate axis, in the presence of of one of the effectors. Repeating the operation for different doses of one of the effectors administered at the same time as X [Loewe et al., 1926].

2.1.2. Interaction between substances producing competing effects

In the presence of an Xai antagonist, substance Xi produces effect (ei) at doses D'ei higher than Dei. While in the presence of a synergist Xsi, we obtain D'ei less than Dei.

In the case of the absence of interaction, if the substances Xi and Xii act on the same targets, the system amounts to adding doses of the same product Xo. However, this does not result in the linearity of dose / effect responses, which must always be considered specifically. Such measurements then lead to particular representations of the interaction phenomena, which are all presentation variants of the same phenomenon.

The Loewe curves (Figure 19) still express the observable phenomena, but in a different way illustrated. The Diei dose is the dose of the interactive factor Xii producing the same effect (ei) as that produced by the Xei dose of Xi.

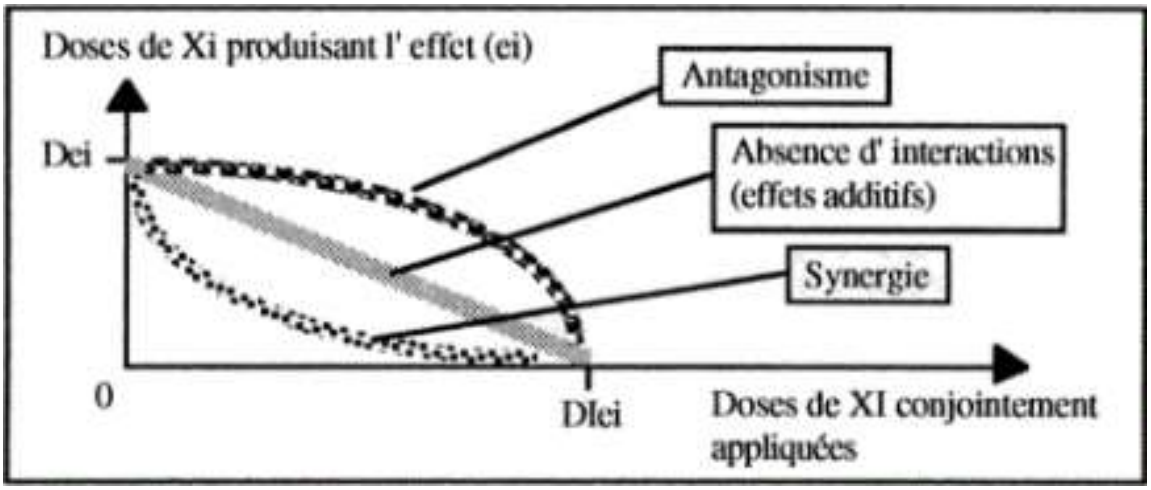


Figure 19: *Loewe curves, in the case where the interacting substance produces the same type of effect*

The concentration addition model is the most commonly used model to predict the effect of mixing. It is also commonly applied for the risk assessment of mixtures of substances. This model is also called the dose additivity model or the Loewe additivity model. The concentrations of each toxic component of the mixture are added to predict the toxicity of the mixture. Both substances are believed to have the same mechanism of toxic action [Berenbaum, 1985; Dreschner and Boedecker, 1995; Feron and Groten, 2002].

2.2. Molecular mechanisms of interaction: the case of pesticides

Any alteration of a physiological function results in a modification of the response of an organism to toxicants. Table 4 summarizes the various cases that may arise.

Let us take the case of pesticides, in fact, the fight against pests comes up against the problem of acquiring resistance, in particular, by selecting strains of parasites or predators with more developed and better adapted detoxification systems. This is why the pesticide industry has reinforced the effectiveness of its preparations by adding to the active ingredient inhibitors of the molecular detoxification system, such as inhibitors of cytochrome P hydroxylases.450.

This class of enzyme gives aromatic molecules a water solubility which makes them more easily abhorrent. Their inhibition thus increases the persistence of the toxicant in the organism.

Altered organs, tissues or function	Results	Interaction type	
		intentional	**unintentional**
Skin tissue	penetration		
Lung tissue	increased	synergy	increased toxicity
Digestive tract	scaled down	antagonism	reduced toxicity
Liver			
Metabolism	stimulated	antagonism	resistance
detoxifying	inhibited	synergy	aggravated intoxication
Kidneys			
excretion	blocking	synergy	aggravated intoxication
	stimulation	antagonism	detoxification
Neurotransmissi(	inhibited	according to the altered functions *	

Table 4: *Examples of physiological mechanisms leading to quantitative interactions*

(*) An alteration of the physiological system can result as well from an infection as from a psychic shock: the pathogenic germs, or the causes of the stress behave in this case, like factors of interaction whose role, although major, can go unnoticed during toxicological analyzes limited to the study of the effects of a substance acting in isolation in a control organism free of germs or preserved from sufficient stress [Benhadou et al., 1997].

Other inhibitors such as carbamates can synergize the action of pyrethroids by inhibiting the functioning of esterases which hydrolyze their molecules. AMPcyclase inhibitors synergize the action of formamidine pesticides - disrupting metabolic regulation - by stimulating cAMP production.

In general, a synergistic effect results from any interaction with the synthesis, the cellular translocation, the functioning of the active site, the molecular stability, and the degradation of any enzyme involved in the transformation of a toxic molecule.

2.2.1. The Fomesafen

Another example of mixtures altering physiological functions is represented by Fomesafen; it is a molecule of the diphenyl-ethers family used relatively specifically for post-emergence weed control of protein crops (soybeans and beans in particular).

These types of herbicides are inhibitors of protoporphyrinogen oxidase, the last enzyme in the heme biosynthetic pathway (the reaction is catalyzed either by a ferro-chelatase for hemoglobin and cytochromes P450, or by an Mg-chelatase for chlorophyll).

In animals, it has been shown that fomesafen and other diphenyl ethers (eg oxadiazon) act on the synthesis of hemes and can be the cause (in the event of prolonged exposure) of the appearance of porphyrias and abnormalities of hepatocytes in rodents. In addition, the disruption of the heme and hemoprotein biosynthetic pathway has a side effect, the proliferation of peroxisomes thereby affecting peroxidation phenomena (lipids, hydrogen) [INERIS report, 2006].

2.2.2. The diquat

In animal cells, diquat - a non-selective herbicide from the bipyridyl group, used for weeding many crops - can be transformed into a free radical, interacting with water to form the superoxide radical O-2, which is not very toxic in itself. same but, which in turn can generate the hydroxyl radical OH- very harmful. Once the cascade is initiated, the radicals formed will react with a number of constituents and cell structures, causing lipoperoxidation of membranes, denaturation of certain proteins and DNA damage.

2.2.3. Agral 90

It is a wetting agent based on polyethoxylated nonylphenols, used in an extemporaneous mixture with many commercial preparations of pesticides.

Highly polyethoxylated nonylphenols are of very low toxicity to living organisms, but their toxicity increases as biodegradation processes reduce the length of the ethoxylated chain and increase that of the hydrophobic chain. The mono-and di-ethoxylated derivatives of nonylphenol are notably known for their reprotoxicity, linked to their endocrine disrupting effect [INERIS report, 2006].

It should be noted that the mixture formed by the herbicide (Fomesafen or diquat) and Agral 90 groups together two types of substances which are likely to be found in aquatic environments and to accumulate in some of their compartments. Arrived in these environments, the two types of molecules can act independently on the organisms, but it is not excluded that the interactions between them modify their modes of action and their respective toxicities.

2.3. Methodology of health risk assessment

The National Research Council (1983) defines risk assessment as "an activity which consists in evaluating the toxic properties of a chemical and the conditions of human exposure to this product, with a view to ascertaining the reality of a chemical. human exposure and to characterize the nature of the effects which may result therefrom".

Several methods have been developed in terms of health risk assessment; they are still imperfect, but nevertheless, contribute to the protection of the exposed persons. In this regard, theFrench Society of Health and Environment (SFSE) recommends:

- an iterative consideration of mixtures in health risk assessment;

- communication, analysis and institutional recognition of the toxicological reference values for “mixtures” published in the scientific literature; and,

- the production of toxicological profiles for certain frequent co-exposures.

The general approach to the assessment of health risks is based on four axes: identification of hazards, definition of dose-response relationships, assessment of human exposure and finally, characterization of health risks.

As for chemical mixtures, there are two evaluation approaches: either the mixture is evaluated as a whole (approach based on the mixture), or it is evaluated from available data on the compounds that constitute it (substance-by-substance approach).

Several approaches have been developed to take into account mixtures in the assessment of health risks, the sum of the hazard quotients[3] (QD) or excess individual risks[4] (ERI), relative toxicity factors (relative potency factors or RPF) through PODI (point of departure index) [Sarigiannis and Hansen, 2012]. These approaches are based on the additivity of doses or effects after grouping substances by toxicological target (same organs, e.g. kidney; or same effects, e.g. cancer), which is one of the default hypotheses recommended for evaluation of mixtures. This additivity hypothesis is therefore freed from more complex processes including certain types of interaction (antagonism, potentiation), which are considered a priori as unlikely [Kortenkamp et al., 2009] during low dose exposures. The limiting factor is, as always in health risk assessment, the availability of data,

For the choice of the level of approach, a compromise is often to be found between the number of substances to be considered and the number of substances for which we have homogeneous information allowing their effects to be aggregated. An iterative approach is therefore recommended.[5]. In this regard, when the additivity hypothesis is posed, the sum of the QDs or the sum of the ERIs is an operational default practice frequently used in first level risk assessment. It has the advantage of simplicity in an iterative approach logic. In this case, the first step is to identify and group together all the substances likely to expose the same population and having a common effect - generally reduced during this first step to its carcinogenic action or on the same target organ - by taking account of all the potential effects and not of the only critical effect from which the TRV[6] (Reference Toxicological Value) is constructed. The second is to add the QDs, or ERIs, of the substances grouped together. The approach is therefore a priori protective, which does not pose a decision problem as long as the sum of QD is less than 1 or the sum of the ERIs does not exceed the acceptable value. Otherwise, however, a more in-depth approach is required.

Regarding certain groups of substances, such as dioxins, furans and PCB-dl or PAHs, a more complex approach focusing on the relative toxicity of the substances with respect to each other is used since the toxic equivalence factors (TEFs) based on a common mechanism of action (binding to the arylhydrocarbon receptor) are recommended by "recognized" expert bodies (WHO, INERIS). However, this approach requires more in-depth knowledge of the mechanism of action of substances.

Thus, it seems that bringing to the attention of health risk assessment practitioners approaches of this type (such as the toxic equivalence factor or similar approaches [Fournier et al., 2014] published in the literature. example: phthalates [Hannas et al., 2011], various anti-androgens [Kortenkamp and Faust, 2010], fungicides [Jensen et al., 2013] for reprotoxic effects, or even the genotoxicity of PAHs [Audebert et al., 2012]), would be a first step to broaden the spectrum of families for which mixtures could be taken into account, like what is done for molecules which bind to the arylhydrocarbon receptor (PAH, dioxins, furans and PCB-dl).

An inventory of toxicological reference values (TRVs) that can be used for whole mixtures (e.g., diesel particles) could also be made available. Institutional recognition by publication in the databases of national or international expertise agencies would be a plus for taking into account routine health risk assessments, when possible. This would also respond to the wish of expert bodies to integrate these approaches and to the expectations of the population informed about the "cocktail" effects.

In addition, it could be useful to gather information, like the ATSDR (Interaction Profiles for Toxic Substances), on the additivity of risks and to propose approaches for certain co-exposures. This could be done for exposure situations for which taking into account the mixing aspect makes it possible to improve prevention. Aldehydes in indoor environments, chlorination by-products of drinking water are examples of frequent co-exposures, while examples of situations where concentrations may be high including service station environments (aromatic pollutants), laundry (solvents), polluted soils (metals, hydrocarbons, solvents, etc.).

The Risk Assessment Framework for Combined Exposures to Multiple Chemicals [Meek et al., 2011] of the World Health Organization (WHO) and the International Program on Chemical Safety (IPCS) discusses the impact on human health from combined exposures to multiple chemicals. The objective of this framework is to develop a "fit for purpose assessment" that uses only the necessary resources. This framework imposes the default assumption that substances act by adding dose, and it describes a phased approach, shown in Figure 20.

The WHO and IPCS framework can be applied taking into account the methods available and the level of improvement possible based on the data available to conduct each of the hazard and exposure assessments and perform the subsequent risk characterization.

[3] .- Hazard Quotient (QD) = Exposure / TRV (Toxicological Reference Value).

[4] .- Excess of Individual Risk (ERI) = Exposure × TRV.

[5] .- Of course, if preventive actions appear necessary without taking mixtures into account, taking into account the mixing aspect is generally unnecessary

[6].- The term toxicological reference value (TRV) is the generic name of the values which make it possible to calculate the amplitude of the risks to human health linked to exposure to a given substance for a given duration and route of exposure . They are constructed from toxicological or epidemiological studies establishing quantified relationships between exposure levels and effects. Science gaps and the protection of human health are integrated into their development.

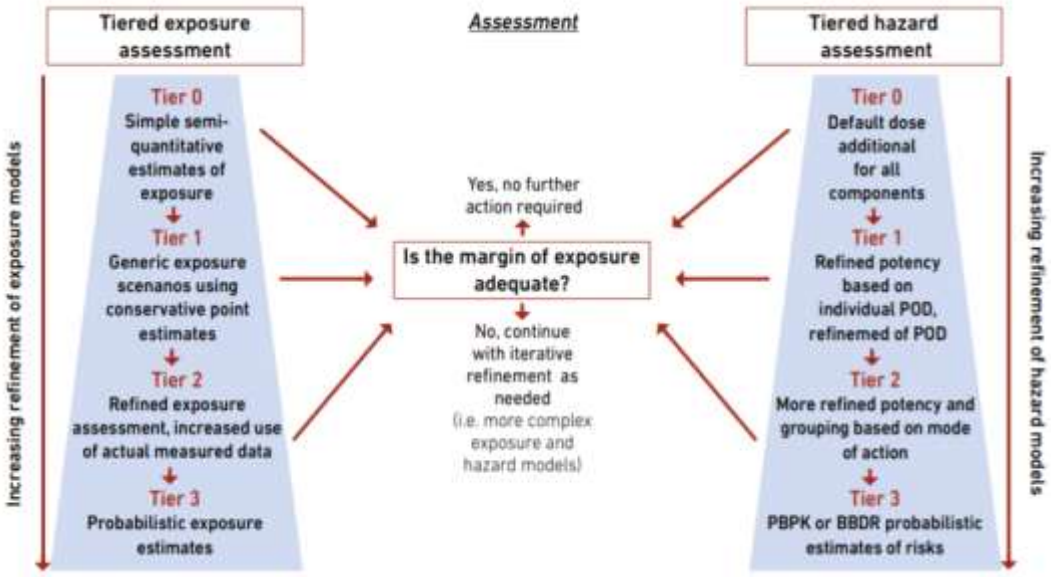


Figure 20: *Diagram of the WHO and IPCS framework for risk assessment of combined exposures to chemical mixtures (source: Meek et al, 2011) Extracted from: A screening tool for assessment of health risks from combined exposure to multiple chemicals in indoor air in public settings for children: methodological approach. Copenhagen: WHO Regional Office for Europe; 2021.*

Finally, in terms of management, these approaches based on additivity make it possible to identify the substance (s) in the mixture that contribute the most to the health risk (Table 5). However, the limits and uncertainties of these models (linked to the modes of action, the number of constituent substances, the ratios, the times and frequencies of exposure, etc.) lead us to favor the methods of the mixture of interest for get rid of possible supra or infra-additive interactions (appendix 5 describes the interactions that could increase the overall toxicity of chemical compounds in a mixture and how to deal with them in a risk assessment).

In the absence of data on the mixture, the Hazard Index (HI) is used instead to assess the health risks.

Procedure	Acronym	Required data	Validity of application	Hypotheses	Advantages	Disadvantages
Hazard Index	HI	The maximum acceptable level for each compound (e.g., RfD or ADI reference dose). Exposure data	The "dose-response" data for the compounds are adequate, as are the exposure data at low levels. HI is also used for compounds acting on the same target organ	Simple similar action - toxicological similarity	Transparent, understandable, relates directly to the actual exhibition; wide use and easy understanding of acceptable risk	Rfd (or ADI) is not a suitable starting point. The method involves an uncertainty factor (UF). If the UFS is not the same for all the compounds in the mixture it will affect the result.
Toxic equivalency factor	TEF	Toxicity data for compounds, dose-response data for reference compound, exposure data	Data rarely available. A value of TEF is applied to all end points; the method limited to mixtures of compounds with weak chemical classes of similarity	Simple similar action - toxicological similarity across criteria	Transparent, understandable, relates directly to actual exposure and toxicity data	In some cases, a complicated method to use. Requirement of dose-response data for the index compound
Margin of exposure	MO and	Starting point: NOEL or BMD10, exposure data	The "dose response" data for the compounds are adequate, as are the exposure data.	Single similar action - toxicological similarity	Directly relates to direct exposure and toxicity data not based on regulation regulated by a parameter like ADI	No criteria to define the size of an acceptable margin of exposure
Target-organ Toxicity Dose	TTD	NOAEL or LOAEL	VTR for critical effect One or more TTDs for other adverse effects	Substances that do not have the same mode of action or the same target organ	Takes into account all the effects of substances	Need for toxicological studies available for each substance to identify the NOAEL or LOAEL for each target organ
Point of Departure	PODI	NOEL or BMD10, exposure data	The "dose response" data for the compounds are adequate, as are the exposure data.	Single similar action - toxicological similarity	Refers to direct exposure and toxicity data not based on an endpoint such as ADI	No criteria to define the scale so that the PODI is acceptable
Procedure	**Acronym**	**Required data**	**Validity of application**	**Hypotheses**	**Advantages**	**Disadvantages**
Cumulative risk index	SHOUT	NOEL or BMD10, or maximum acceptable level for each compound (Rfd, ADI). Exposure data	The "dose response" data for the compounds are adequate, as are the exposure data.	Single similar action - toxicological similarity	Combines MOEs for chemicals with different UFs	Rfd (or ADI) is not a suitable POD. Method less understandable and transparent than that of the HI. Complex calculations
Relative potency factor	RPF	Toxicity data for compounds, dose-response data for reference compound, exposure data	Concentrations measured relative to the concentration of the reference substance. Applicable for one effect and one route of exposure	Simple similar action - toxicological similarity across criteria	Requires less precision than for the TEF approach	Limited to a single effect and a single route of exposure
Hazard index interaction	Hii	Maximum acceptable level for each compound, a number of factors, exposure data	Few valid data: data limited to interactions	Binary interactions are the most important. The importance of the interactions depends on the proportions of the compounds	Is supposed to represent interactions (binary)	The determination of BINWOE is complex. Compensation factors are not supported by experimental data. No systematic method for choosing UFs

Table 5: *Advantages and disadvantages of dose additivity methods (Adapted from Reffstrup et al., 2010)*

3. GENOTOXICITY AND REPROTOXICITY OF COMPLEX MIXTURES

Chemical pollutants are classified according to their structures or their probable modes of action. Indeed, both classifications are interesting. As for the chemical structure, the classification is carried out as follows:

- polycyclic aromatic hydrocarbons (benzo (a) pyrene);
- organochlorines and organobromines (pesticides, dioxins, PCBs, polybrominates);
  - aromatic amines;
  - organophosphates (sarin, chlopyrifos);
  - nitrosamines;
  - fibers: asbestos;
  - heavy metals;
  - others (toxins like aflatoxin);
  - mixtures: tobacco, fine particles, tars.

These compounds are also classified according to their main mode of action, namely:

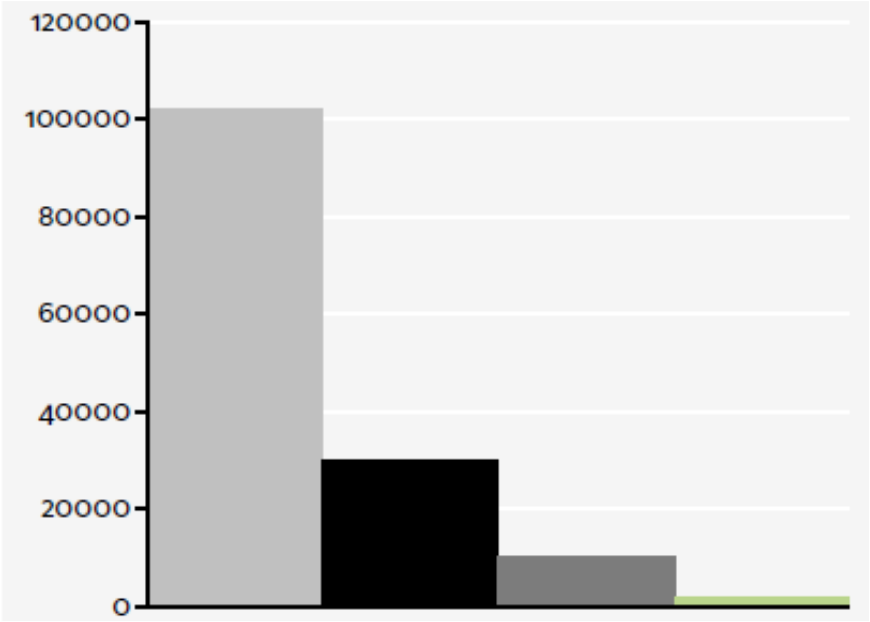
- **Direct genotoxics**: physical agents, benzo (a) pyrene, aflatoxin;
- **Non-genotoxic** : own cell signaling (dioxin, AhR receptor for dioxin and polycyclic aromatic hydrocarbons, pesticides, PXR receptor capable of binding drugs and pesticides); endocrine disruptors (activation or inhibition of cell signaling, estrogen mimetics, organochlorine pesticides); enzyme disruptors (organophosphates); cellular stresses (oxidative stress, asbestos, dioxin, etc.);
- **Indirect genotoxics**: deposition toxicity (particles, asbestos, inflammation); toxicity from multiple origins (mixtures or not).

To quantify genotoxicity, several methods are possible, including the comet test (COMET test) developed for our model organism [Erbes et al., 1997]. This method makes it possible to visualize breaks in DNA strands: after isolation of the nucleus, the genetic material is subjected to electrophoresis. The DNA nucleus remains dense in the case of an unaltered cell, whereas in the case of a single or double strand break, a comet will be visible, the tail of the comet being more or less important depending on the damage to the genetic material. Note that the mutation is considered to be the real hub of carcinogenesis since the discovery of critical cancer genes whose mutation is a sign of the malignant cell state among the thousands of other mutations usually present in the transformed cell.

Studies of genotoxicity and reprotoxicity of samples taken from the environment are often made difficult by the fact that they are usually mixtures of chemicals. Significant research to determine the combined effects of mixtures of chemicals has been initiated with a view to determining derive general principles that can be used for the toxicological evaluation of complex mixtures. One example is the studies carried out by Carpenter, Kirby and Müller,

3.1. Studies conducted by Carpenter et al. in 2011 on the genotoxicity and reprotoxicity of complex mixtures

Pimentel et al [1995] report that around 80,000 chemicals are used today, almost 10% of which are identified as carcinogenic, and that the use of chemicals tripled from 1941 to 1995 (Figure 21).



Chemicals on the EU market 1981-97

Chemicals included in REACH

Chemicals produced in volumes of more than 10 tonnes per year

Chemicals known to be CMR (Carcinogens, Mutagens, Toxic for Reproduction), PBT (Persistent, Bioaccumulative, Toxic) or vPvB (Very Persistent, Very Bioaccumulative)

In reality, few chemicals have only a single cell target. Most act at multiple locations, on different cell types, or in some cases, at multiple targets in the same cell type.

There can be quite different actions on the kidney, liver, and brain, depending on the presence of genes, receptors, and cellular regulators in specific types of cells. When targets that regulate other organs and cells are affected (eg, the thyroid or pancreatic beta cells), the impact of the chemical agent is much greater.

One study suggests that "as a rule", mixtures below the NOAEL[7] should not present any health concern (interactive effects have been reported at the level of LOAEL[8]s) [Cassee et al., 1998], while another researcher suggests that even low-level exposure to chemical mixtures can cause subtle biological effects, some of which may not be discernible by current methods [Yang, 1994; Monosson, 2005].

Another review found that although a few studies "support the hypothesis that adverse effects are unlikely when the components of the mixture are present well below their different thresholds," it would be more prudent to "provide exceptions to the rule" [Seed et al., 1995]. Indeed, over the past 15 years, several studies of chemical mixtures (in the case of pesticides) at concentrations close to or below NOEC or NOAEL have underlined the harmfulness of these products [Cavieres et al., 2002; Rajapakse et al., 2002; Welshons et al., 2003].

The great contemporary concern with chemical mixtures arises from the possibility of synergistic interactions of compounds, more than from their additivity.

Epidemiological studies conducted by Erren have shown that smoking and exposure to asbestos exert synergistic effects on the incidence of lung cancer [Erren et al., 1999].

Smoking is involved in around 30% of all cancers, and mainly tumors of the lungs, but also of the pancreas, bladder, kidneys, oral cavity and esophagus.

Most cancers are linked to our way of life. Indeed, food includes a large number of chemical genotoxic agents including nitrosamines, polycyclic aromatic hydrocarbons, and heterocyclic amines (HA) which can be reproduced during reactions involving a mixture of amino acids, carbohydrates, and creatine or creatinine. The latter were found to be extremely genotoxic during mutagenesis tests.

According to the Carex survey in France, 5 million employees in France are potentially exposed to 139 carcinogenic substances or mixtures listed, at that time (1990-1993) by the International Cancer Research Center in Lyon, in groups 1 (carcinogens in humans) and 2A (probable carcinogens in humans). Currently the number of agents and groups of carcinogenic agents (groups 1 and 2A) stands at 152 (September 2003) [Picot., 2004].

Apart from asbestos, the example of which is widely discussed, we regret the virtual inexistence of reliable data relating to other products, except for some work by INERIS on HPA and benzene.

Nowadays, chemical pollution is suspected to cause one in two cancers. Among the substances produced and marketed, many of them are PCBs, PVCs, phthalates or brominated flame retardants which are carcinogenic, mutagenic and reprotoxic (CMR) substances. The reprotoxicity of these substances may partly explain the sterility of 15% of European couples.

3.2. Studies conducted by Kirby et al on the toxicity of simple and complex mixtures

In Kirby's studies, a series of model compounds and simple mixtures including polycyclic aromatic hydrocarbons (PAHs), pentachlorophenol (PCP), and halogenated aliphatic hydrocarbons (HAHs) were analyzed. Mixture toxicity was investigated using microbial genotoxicity assays and cytotoxicity assays with renal and neural cells.

The majority of binary mixtures described additive responses, except for a limited number of samples where binary mixtures induced inhibitory effects. As an example, benzo (a) pyrene (BAP) alone induced renal death of 30% of cells, while an equimolar dose of chrysene and BAP produced cell death of only 1.6%.

The results of examinations of the binary mixtures, carried out by Kirby, indicate that the results did not deviate significantly from the additivity. The toxicity of complex mixtures could not be examined. This could be due to the chemical interaction or, simply due to the presence of unidentified chemicals, such as alkyl PAHs or PAHs which are not included in the standard risk assessment process. And therefore, the risk associated with complex mixtures exists.

Before the results of the toxicity test can be used to adjust the risk assessment calculations, it is important to fully appreciate the chemical composition, and to understand the mechanism of the chemical interactions observed in animals chronically exposed to it. low doses of chemical mixtures. Tables 6 and 7 illustrate the example of genotoxicity of mixtures of PAH, BAP Benzo (a) pyrene, Anthracene, Chrysene.

	BAP	CHRY	ANTH	BAP + CHRY
Genotoxic[at]	++	+	-	++
Renalb	++	+	-	-
Neuralb	+	-	-	+

Table 6: *Examples of genotoxicity of mixtures: In vitro effect of PAHs*

	BAP	CHRY	PCP	BAP + PCP
Genotoxic[at]	++	+	++	++
Renalb	++	+	-	-
Neuralb	+	-	-	-

[at] Genotoxicity assays: (-), less than or equal to twice the solvent control;

(+),> twice solvent control; (++),> four times solvent control.

[b] Neural and renal cytotoxicity assays: (-), no significant difference

($p < 0.05$) between test sample and control; (+), significant difference

($p < 0.05$) between test sample and control; (++), significant difference

($p < 0.05$) between two positive test samples.

Table 7: *In vitro effects of BAPs and PCPs*

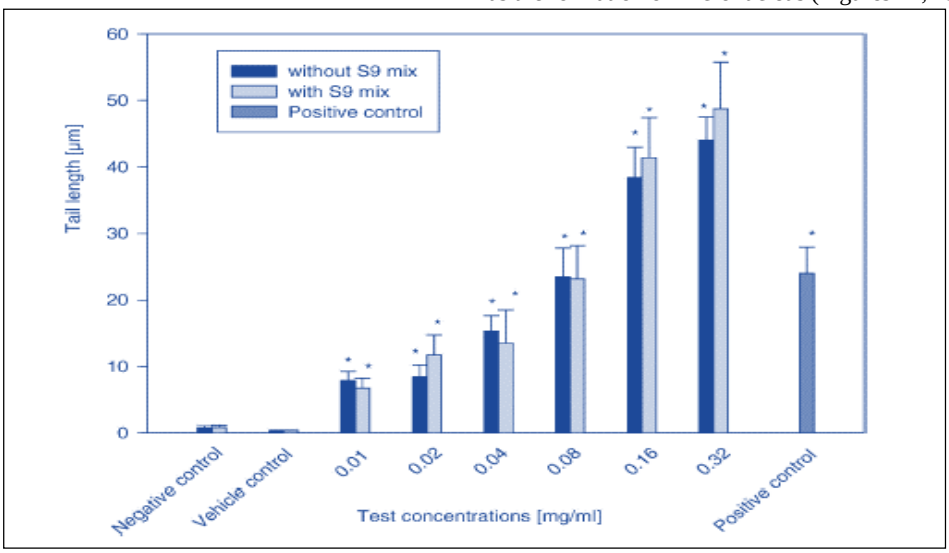
[7]. NOAEL: No Observed Adverse Effect Level.

[8]. LOAEL: Lowest Observed Adverse Effect Level.

3.3. Examples adapted from the article by Müller et al., 2002

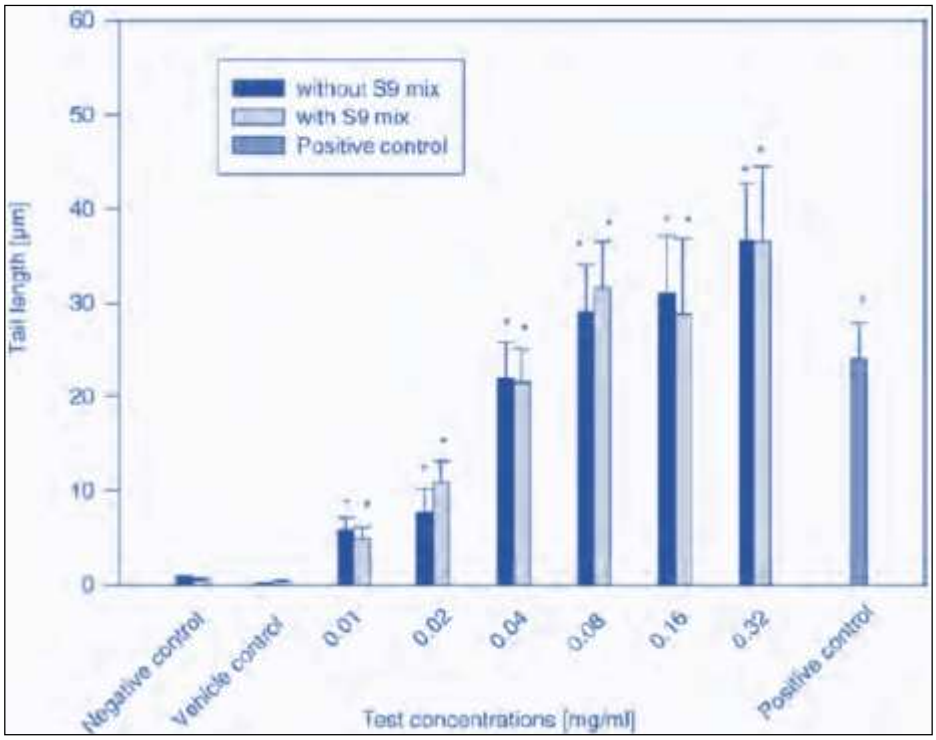
In order to test the genotoxicity of hydrocarbons produced as waste in industries, in vitro tests (comet analysis and micronucleus test) were carried out. The genotoxic potential of this mixture of hydrocarbons was examined on various types of human cells (lymphocytes and normal bronchoepithelial cells) and rat hepatocytes.

The results showed that the complex mixture of perhalogenated hydrocarbons acts as a direct genotoxic and causes DNA cleavages as well as the formation of micronucleus (Figures 22, 23 and 24).



Source : Müller et al. 2002.

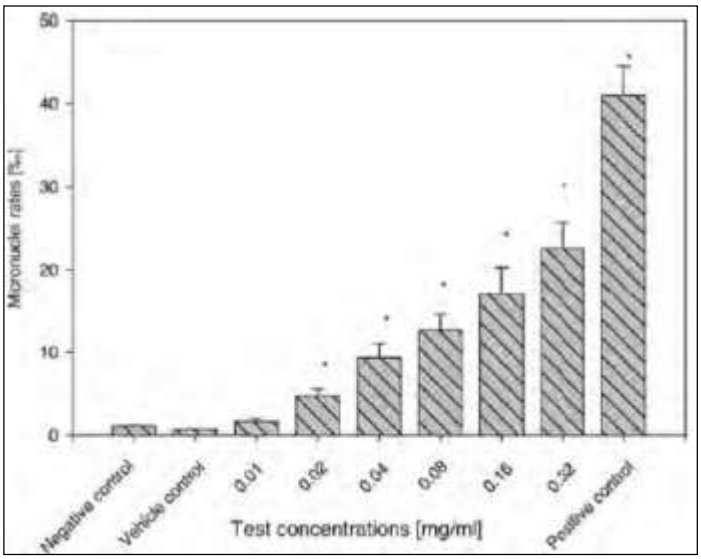
Figure 22: *DNA damage in human lymphocyte cells after treatment with a complex mixture compared to the negative control (Comet test)*



Source: Müller et al. 2002.

Figure 23: *DNA damage in human bronchial epithelial cells following treatment with a complex mixture compared to the negative control (Comet test)*

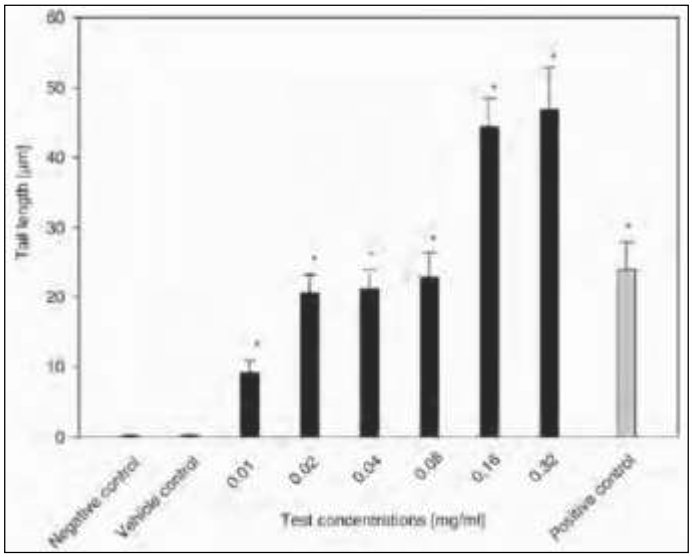
For Figs 22 and 23. The results are expressed using the "tail moment" which corresponds to the product of the length of the comet tail by the percentage of DNA in the tail; * P> 0.001. Two experiments were performed for each test



* P> 0.05. Source: Müller et al. 2002.

Figure 24: *DNA damage in human lymphocytes after treatment with a complex mixture compared to the negative control (micronucleus in vitro): micronucleated content per 1000 binucleated cells*

The presence of an external metabolism system (S9 mixture of rat hepatocytes) in the human cell culture system did not cause any change in the observed effects, when compared to experiments in the absence of the S9 mixture. Therefore, the author and his team concluded that the mixture acts as a direct genotoxic and that there is no detoxification by the external enzyme system.



* P> 0.001. Source: Müller et al. 2002.

Figure 25: *DNA damage in rat hepatocytes after treatment with a complex mixture compared to the negative control (comet test)*

Additionally, in vitro comet and micronucleus analysis on primary human cell cultures indicated that these assays can also be used for genotoxicological analyzes of complex mixtures.

A number of environmental chemicals have actions that mimic or change the normal hormones of the sex differentiation system. The fetus is particularly vulnerable (period when organs are developing). If the normal balance between estrogen and androgen is disturbed, it can lead to feminization of males, or masculinization of females. These sudden effects during the fetal development period are of particular importance because they are often irreversible.

Concise guidance is provided on absorption, distribution (including placental transfer) and excretion in humans and laboratory animals. Mention is made of kinetic factors which may influence the dose-response relationship, such as saturation of absorption mechanisms, protein binding, metabolic activation, detoxification and DNA repair processes.

Studies providing information on the metabolic fate of the agent in humans and in laboratory animals are briefly discussed and comparisons between human and animal data are made whenever possible.

Comparative information on the relationship between exposure and the dose reaching the target can be particularly valuable for extrapolation from one species to another. Data regarding acute and chronic toxic effects (other than cancer), such as organ toxicity, increased cell proliferation, immunotoxicity and endocrine effects are made. Effects on reproductive

function, teratogenicity, foetotoxicity and embryotoxicity are also briefly reported.

Tests for genotoxic effects are described because of the importance of genetic mutations and chromosomal damage in carcinogenesis [Vainio et al., 1992; Mc Gregor et al., 1999]. The value of the information provided on the characterization of the samples is reviewed and, where appropriate, commented on; for complex mixtures these comments are similar to those for animal carcinogenicity tests. The available data are subject to a critical interpretation by phylogenetic group depending on the results observed: DNA alterations, gene mutations, sister chromatid exchange, formation of micronuclei, chromosomal aberrations, aneuploidy and cell transformation in particular. The concentrations used are indicated,

This data is provided in the form of lists of test systems, results and benchmarks.

The tests for genotoxic and related effects presented in the monographs are also available as graphic activity profiles (GAP) prepared in collaboration with the Environmental Protection Agency (EPA) of the United States of America (see also Waters et al., 1987), using software[9] for microcomputer compatible with Microsoft WindowsR.

Positive results obtained from tests on prokaryotes, lower eukaryotes, insects and mammalian cell cultures suggest that genetic and related effects may exist in mammals. The results of these tests may also provide information on the types of genetic effects produced and the role played by metabolic activation. Some effects observed are clearly genetic (such as gene mutations and chromosomal aberrations), while others are more or less closely associated with genetic phenomena (unscheduled DNA synthesis, for example). The tests carried out in vitro to demonstrate tumor promoting activity and cell transformations may reveal modifications which do not necessarily result from genetic alterations, but which may be directly linked to the process of carcinogenesis. A critical study of these tests has been published [Montesano et al., 1986].

A genetic or other activity observed in laboratory mammals and in humans is considered to be of greater relevance than the same activity observed in other organisms, if it is established that an agent or mixture induces gene and chromosomal mutations in the whole mammal. This indicates that it may have carcinogenic activity, although this is not necessarily detectably expressed in one or all of the species studied.

The relative activity observed in tests for mutagenicity and related effects is not a reliable indicator of carcinogenic activity. Negative results obtained by mutagenicity tests in specific tissues from animals treated in vivo are less conclusive, in particular because they do not exclude the possibility of an effect in tissues other than those examined.

Furthermore, negative results obtained by means of short-term genotoxicity tests cannot be considered as decisively excluding the carcinogenicity of agents or mixtures which would act by other mechanisms, such as, for example, the effects appearing through receptors, cellular toxicity with regenerative proliferation and proliferation of peroxisomes [Vainio et al., 1992]. Factors which can skew results in short-term tests have been examined extensively [Montesano et al., 1986].

When available, data on mechanisms of carcinogenesis that do not cause structural alterations at the gene level are also provided.

The quality of epidemiological studies, concerning effects on reproduction and genetic and related effects in humans, is evaluated according to criteria identical to those used for epidemiological studies relating to cancer.

Structure-activity relationships are also described, where they may be useful in the evaluation of carcinogenesis of a given agent.

As for biological agents (viruses, bacteria, parasites), and other relevant data for carcinogenicity, we use the descriptions of infectious pathology, molecular biology (integration and expression of viruses and any genetic alteration observed in human tumors.), as well as other observations, such as the cellular and tissue response to infection, the immune response and the presence of tumor markers.

General Conclusion

"What is the significance of an acceptable daily intake established by a single substance, when the toxic and carcinogenic effects of mixtures of two substances are no longer known, that we are all exposed to dozens of substances acting by multiple inputs, that many substances act at mono-molecular doses and that formidable synergies have been demonstrated, amplifying up to a thousand times the effects of substances that are not very active in isolation? "[Jean HUSS. pollution_sante_jean_hess.htm].

Many questions remain to be asked about the experimental approach, in particular in the field of complex mixtures.

So far, the number of mixtures in the environment to which a direct toxicity assessment has been carried out has been limited. Current methodologies for the assessment of risk to human health generally treat mixtures as single mixtures, deriving the combined toxicity of different components primarily from single-chemical studies.

The presence of mixtures of pollutants in the general environment and the work environment is an unresolved toxicology and environmental health problem, despite its urgency. Are the effects independent, additive, synergistic, or antagonistic?

The toxic effect will depend on the dose of the product entering the body, the route of entry, the cumulative nature of the doses and effects, the metabolic capacities (genetic heritage, age, sex, etc.), the state of personal health, current conditions (fatigue, stress, etc.). Although knowledge of the substances is still to be developed, there is even less data on mixtures (preparations) to assess the possible synergistic, antagonistic and additive effects when several substances are present simultaneously (case of pollutants environmental, cigarette smoke, etc.).

To strengthen the scientific basis of mixture toxicology, studies were performed to determine biological concepts and fundamental mathematical formulas for extrapolation of low doses.

Therefore, extrapolation of these doses should be considered as a key issue in the assessment of possible health risks from exposure to chemical mixtures, such as chemical mixtures in the atmosphere, disinfection by-products of drinking water, the combined intake of additives, etc.

Notable developments include the production of new programs applicable to the study of mixture (CombiTool, BioMol, modeling reaction network), to the functional application of genomics and proteomics to the studies of mixture, to the use of nanochemical probes for forming in vivo images of physiological processes within cells; and applying the optical probe for complex sample analysis.

Certainly, the intervention of biologists, biomathematicians and bioengineers in mixture toxicology is essential for the development of this science. Studies on complex mixtures make use of multidisciplinary knowledge.

The risk associated with complex mixtures remains a challenge. Before the results of the toxicity test can be used to adjust the risk assessment calculations, it is important to fully appreciate the chemical composition and understand the mechanism of the chemical interactions observed in animals chronically exposed to it. low doses of chemical mixtures.

[9]. EPA / IARC GAP software and databases can be accessed free of charge on the website www.epa.gov/gapdb.

The current development of exposure biomarkers allows the evaluation of the internal dose of exposure to toxic substances, integrating all the media and contact routes, thus allowing a precise assessment of the risk to human health.

These improvements in practices will have to evolve as research progresses in this field, by integrating new approaches. We can cite, by way of example, a more detailed knowledge of the exposome (all the exposures) but also of the mechanisms of toxicity making it possible to study or predict the effects of mixtures by experiments carried out on real mixtures and also thanks to the progress in molecular biology, epigenetics and high-throughput techniques (genomics, proteomics, transcriptomics, metabolomics).

For many chemicals, there is no relevant information on the mode of action. This is why it is difficult to predict the interactions of chemicals in mixtures, especially their long-term effects. Studies are needed to define criteria that could predict the potentiation or synergy of effects.

The problem is even more complex concerning ecotoxicology. It is difficult (if not impossible) to understand all possible modes of action in complex biological communities, and ecologically relevant parameters are generally vague and not as specific (e.g. toxicity to particular organs, etc.) than in human toxicology. In any case, it is necessary to improve knowledge and methods for assessing the risks associated with mixtures. Finally, the risk associated with complex mixtures remains a challenge. For the moment, the toxicity of mixed by-products is excluded (not accessible), we are only interested in the parent compounds.

Despite the difficulties encountered, the quantitative health risk assessment approach, through its ability to provide estimates in the field of low doses, including predictively, has emerged as a major tool for managing the quality of our physical environment.

As it relates to human health, it is essential that a public health professional be involved in this type of study. Interdisciplinarity is desirable because the scientific objects handled, very diverse, go far beyond the socio-health field. In addition, upstream collaboration would make it possible to plan the use in risk assessment of the results of studies and research from the development of their protocol.

Complementary to these reflections, the development and implementation of innovative socio-ecological intervention research must be supported by an evaluation process using methods from different but complementary disciplines (epidemiology, social sciences, political sciences, economics, etc.). According to the conclusions of Plano (2010), this diversity of quantitative, qualitative, critical, mixed approaches allows a more complete understanding of the problem studied, the validation / invalidation of all or part of the results, the illustration of the context, examining the processes and / or experiences within the intervention.

This plural methodological stance must be integrated from the training process in public health research.

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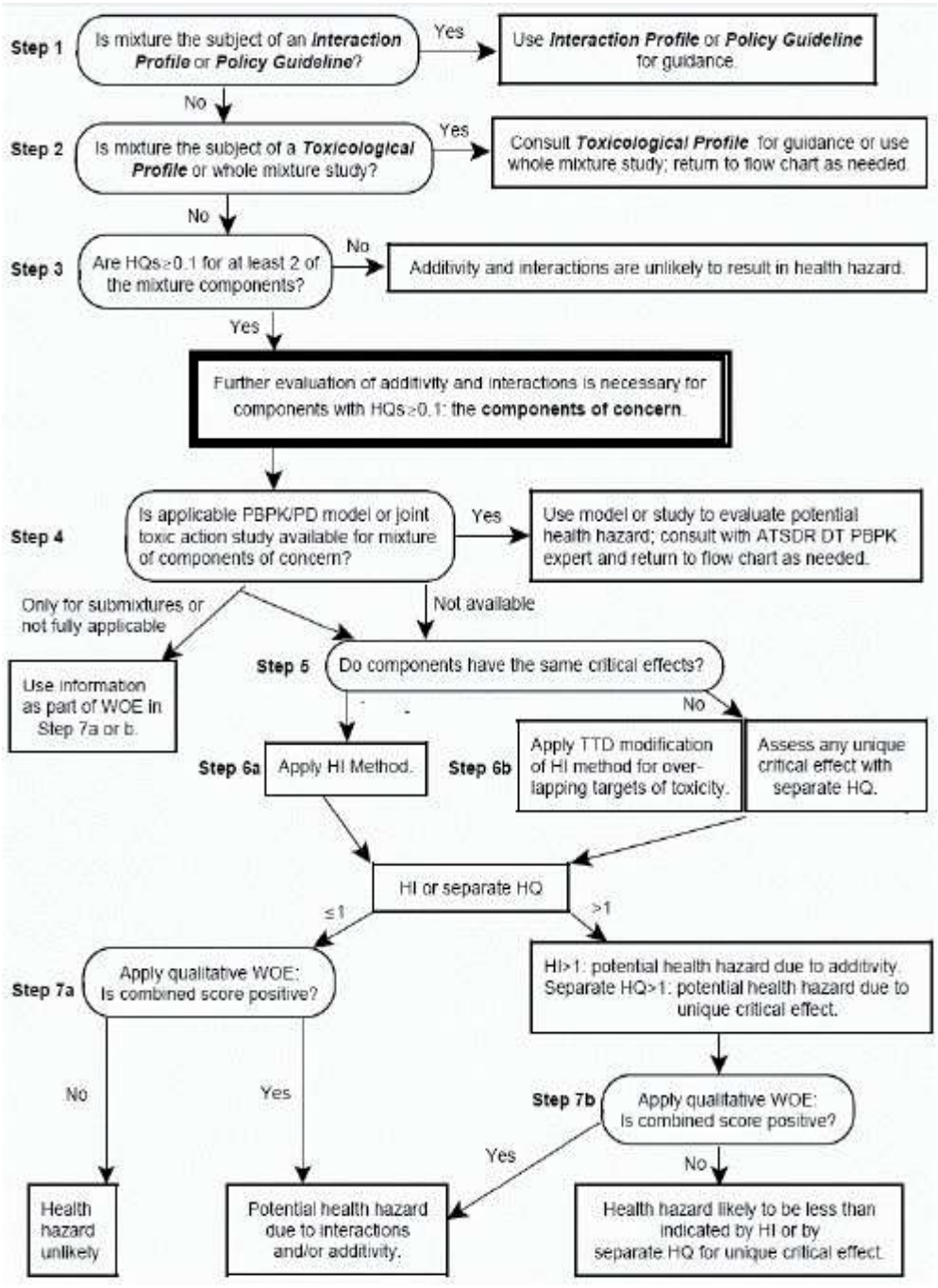
APPENDIX 1: Synoptics of the ATSDR

Strategy for Exposure-Based Assessment of Joint Toxic Action of Chemical Mixtures:

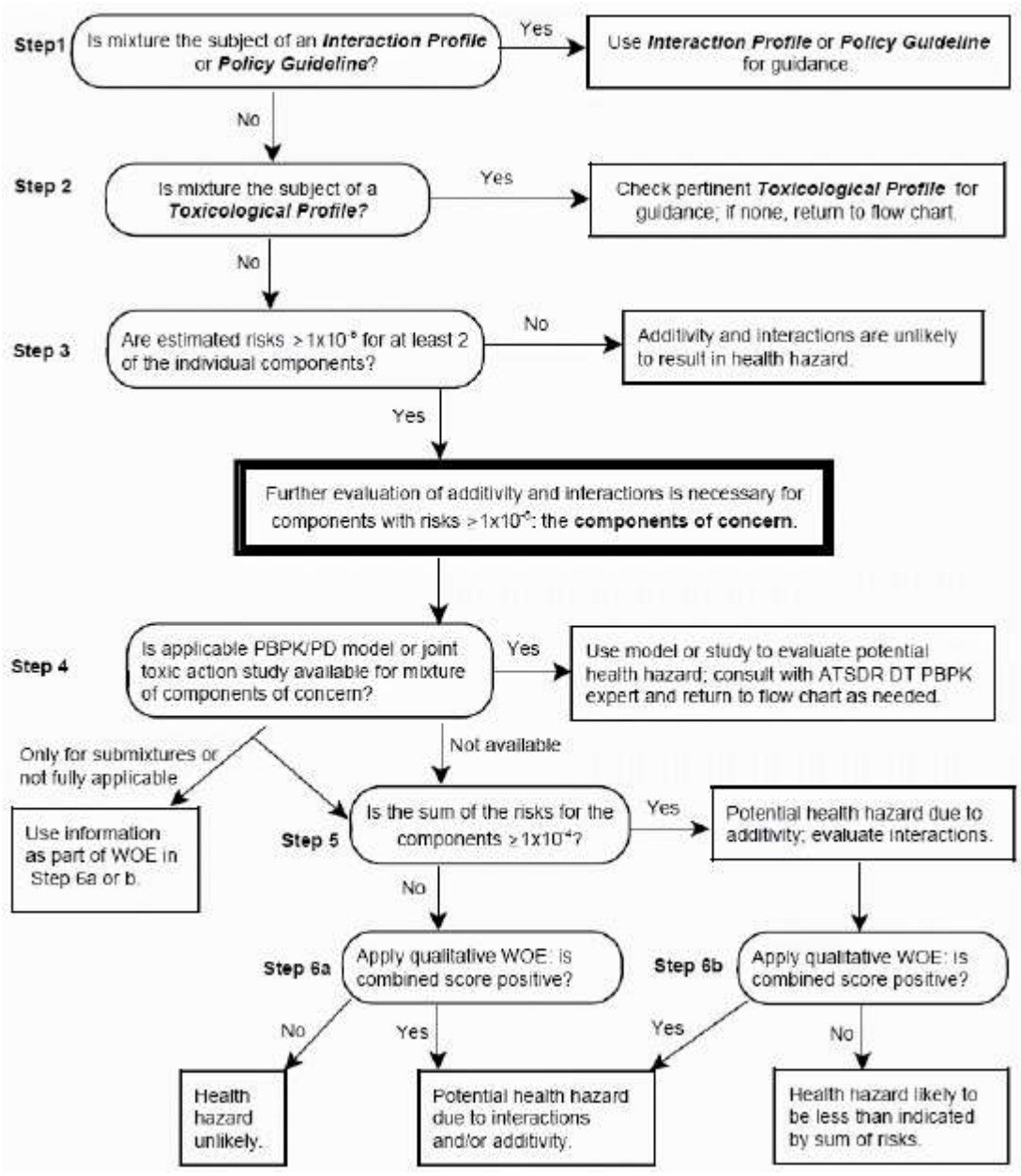
Noncarcinogenic Effects



Source: ATSDR, 2004.

Strategy for Exposure-Based Assessment of Joint Toxic Action of Chemical Mixtures:

Carcinogenic Effects



Source: ATSDR, 2004.

APPENDIX 2:

BINWOE score calculation factors describing the nature and intensity of interactions between two substances according to the WOE method

Factor f	Classification criteria	Sign or value
f1	**Nature of the interaction**	**Direction**
	Additive	0
	Potentiation	+
	Antagonism	-
	Unknown	0
f2	**Understanding of the mechanisms**	**Weighting**
	Mechanism known and well characterized	1
	Mechanism that can be determined from knowledge of the mechanisms of action of similar substances	0.71
	Inadequate or ambiguous data on the mechanism of action	0.32
f3	**Significance of the effects**	**Weighting**
	Demonstrated	1
	Deduced from that of other similar substances	0.71
	Not very obvious	0.32
Other parameters		**Weighting**
f4	Same times and duration of exposure	1.0
	Different time and duration of exposure	0.79
f5	In vivo data	1.0
	In vitro data	0.79
f6	Same route of exposure	1.0
	Different exposure routes	0.79

Source: INERIS, 2006.

APPENDIX 3:

Pairs of chemicals for which a toxic interaction has been identified

Substance 1	Substance 2	Final decision
Acetylsalicylic	Ethyl alcohol	Supra-additivity
Ethyl alcohol	Methyl alcohol	infra-additivity
Ethyl alcohol	Aluminum, Soluble salts	Supra-additivity
Ethyl alcohol	Carbon disulphide	Supra-additivity
Ethyl alcohol	Copper dust and mist	Supra-additivity
Ethyl alcohol	N, N-Dimethylformamide	Supra-additivity
Ethyl alcohol	Xylenes (o, m, p)	Supra-additivity
Elemental arsenic and inorganic compounds (except arsine)	Elemental cadmium and compounds	Supra-additivity

Elemental arsenic and inorganic compounds (except arsine)	Selenium and compounds	Supra-additivity
Nitrogen dioxide	Ozone	Supra-additivity
Elemental cadmium and compounds	Lead and its inorganic compounds, dust and fumes	Additivity
Elemental cadmium and compounds	Selenium and compounds	infra-additivity
Carbon dioxide	Carbon monoxide	Supra-additivity
Chromium III and compounds	Elemental cobalt and inorganic compounds	Supra-additivity
Chromium VI, some water-insoluble compounds	Ozone	Supra-additivity
Chromium VI, certain water-soluble compounds	Ozone	infra-additivity
Manganese, dust and compounds	Mercury, aryl compounds and inorganic compounds	Supra-additivity
Manganese, dust and compounds	Methyl isobutyl ketone	Supra-additivity
Manganese, dust and compounds	Lead and its inorganic compounds, dust and fumes	Supra-additivity
Mercury, aryl compounds and inorganic compounds	Selenium and compounds	Additivity
Mercury, all forms except alkyl compounds, mercury vapors	Selenium and compounds	Supra-additivity
Methyl ethyl ketone	Xylenes (o, m, p)	Supra-additivity
Nickel, soluble compounds	Vanadium pentoxide, fumes and respirable dust	Supra-additivity
Nickel, soluble compounds	Yttrium	Supra-additivity
Toluene	Xylenes (o, m, p)	Supra-additivity

Source: IRSST, 2005

APPENDIX 4 :

Toxicological profiles of mixtures proposed by ATSDR (2004)

- Arsenic, Cadmium, Chromium, Lead;
- Benzene, Toluene, Ethybenzene, Xylene;
- Lead, Manganese, Zinc, Copper;
- Persistent chemicals found in breast milk;
- Persistent chemicals found in fish;
- 1,1,1- Trichloroethane, 1,1- Dichlorethylene, Trichlorethylene, Perchlorethylene;
- Cesium, Cobalt, Polychlorinated biphenyls (PCB), Strontium, Trichlorethylene;
- Arsenic, Hydrazines, Jet Fuels, Strontium-90, Trichlorethylene;
- Cyanides, Fluorides, Nitrate, Uranium.

APPENDIX 5:

Interactions that could increase1 the overall toxicity of chemicals in a mixture and how they might be dealt with in a risk assessment

Interaction	Effect	How it could be taken into account
Chemical - Chemical		
Produce a new component	Additional toxicity may occur that would not be seen from the individual components.	Consider whether the conditions exist for the new component or complex to form and, if so, include this as an additional component in the risk assessment.
Produce a complex between components	This could affect the bioavailability of components. If the complex allowed a component to be transported to a target site that was not normally accessible, additional toxicity may occur.	
Toxicokinetic		
Absorption	Increased bioavailability leading to higher levels of the toxicant at the target site.	Ideally, sufficient data will be available to make a quantitative assessment of the impact of the interaction. If this is not possible, apply an additional assessment factor to take account of potential increases in the levels of a toxicant at its target site or to take account of prolonged exposure if clearance of the toxicant is delayed. The potential for toxicokinetic interactions to influence the relationship between external dose and the level of a toxicant at its target site should be taken into account before the potential for toxicodynamic interactions is considered.
Distribution	Competition for binding sites on plasma and intracellular proteins leading to higher levels of the toxicant at the target site. Owing to excess protein binding capacity in the body, this is most likely to occur at higher levels of exposure, except where high affinity transport mechanisms are involved.	
Metabolism	Saturation, induction or inhibition may produce greater levels of the toxicant or slower detoxification. Given the large capacity of the body to metabolize xenobiotic substances, these effects are most likely to occur at higher levels of exposure.	
Elimination	Slowed elimination could prolong the time the toxicant is available to act at its target site.	
Toxicodynamic		
	Toxicodynamic interactions are only likely to occur where components are at or above thresholds of effect but it will be difficult to predict the nature of any toxicodynamic interactions in the absence of information on mechanisms of toxicity.	Providing exposures to all components are below thresholds of effect (for groups of components that have similar effects, it may be necessary to derive thresholds on a group basis) there should be no toxicodynamic interactions. Where there are concerns for toxicodynamic interactions it may be necessary to adjust assessment factors for individual components to increase confidence that the amount of that component in a mixture is below its threshold of effect.

[1] Interactions that could decrease the overall toxicity of chemicals in a mixture have not been specifically considered in this table because they do not carry the same health concerns.

Source: IGHRC, 2009.