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Report on the state of the art on integrated exposure and health assessment for exposure to carcinogens and neurodevelopmental disorders substances and recommendations for further development

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Executive Summary

The current paradigm for the assessment of the health risk of chemical substances focuses primarily on the effects of individual substances for determining the doses of toxicological concern in order to inform appropriately the regulatory process. Given the recently increased public awareness on the link between environmental condition and public health the policy-making and environmental management processes have an enhanced need for health and safety data. In Europe, this was signalled in the 6th Environmental Action Plan (EC, 2002), where for the first time the issue of environment and health was identified as a key determinant of sustainability. Since then, a number of legislative initiatives have been undertaken in the European Union with a scope to reducing the potential adverse effect of environmental pressure on public health as a key dimension for ensuring sustainability. Such regulatory processes include:

(a) Consumer Policy and REACH (the new chemical safety regulation in the European Union): need for data on chemical safety of consumer products (EC, 2007a-b))

(b) Environment & Health Action Plan: address mixture effects in various environmental matrices putting for the first time particular emphasis on indoor air (EC, 2004)

(c) Food safety: safety of chemicals in food contact materials/foodstuff (EC, 2004)

These policy instruments place varying requirements on health and safety data of chemicals in the environment. REACH focuses on safety of individual substances; yet all the other facets of public health policy that relate to chemical stressors put emphasis on the effects of combined exposure to mixtures of chemical and physical agents. This emphasis brings about methodological problems linked to the complexity of the respective exposure pathways; the beyond additive effect of mixtures (the so-called 'cocktail effect'); dose extrapolation, i.e. the extrapolation of the validity of dose-response data to dose ranges that extend beyond the levels used for the derivation of the original dose-response relationship; and the integrated use of toxicity data across species (including human clinical, epidemiological and biomonitoring data) (Sarigiannis et al, 2009).

In 2009 the European Environment Council recognized the major challenges posed to environmental health impact assessment by the need to develop scientific and regulatory approaches for the assessment of pollutants characterized by complex environmental pathways leading to multiple routes of human exposure and the assessment of the health impact associated to combined exposure to mixtures of chemical stressors via the environment. In response, the European Commission has produced a report (Kortenkamp et al, 2010) on the issue and it is currently examining the need for ad hoc regulatory actions to address the environment and health risk of chemical mixtures in the environment. In response to these policy and scientific needs, we view the assessment of 'complex' pollutants and mixtures thereof as a key dimension for integrated health impact assessment of environmental stressors. In particular, developing methodologies for quantifying the effects of exposures by multiple routes and to mixtures of substances is one of the main objectives of our current work on the estimation of health impact from exposure estimates. In this regard, our research aims at filling two important methodological gaps. Firstly, the quantification of effects across exposure routes, which has not been widely undertaken in previous EU studies, although methods have been tested in the US. Secondly, the fact that in previous studies, the effects of mixtures have been treated as being additive with no consideration of biological plausibility.

This report starts by giving an overview of the current state of science regarding the methodologies for quantifying (a) the health effects of human exposure to environmental chemicals by multiple routes and (b) the effects of chemical mixtures. This overview while capturing all current advances in these areas goes one step further: it evaluates the relevant work done in European projects as case studies and draws useful lessons towards the development of a comprehensive methodology. The report continues by providing a concise overview of the salient issues pertaining to biomarkers of exposure, effect, and susceptibility, encompassing recent advances using systems biology approaches and –omics technologies in order to demonstrate how information early biological events can be of practical use in risk assessment. Thus, a connectivity paradigm is forged, in order to provide a conceptual advancement in the method and practice of health risk and impact assessment by making integral use of biological and mechanistic information. Finally, in the way of example the report presents state-of-the-art methods for

health impact assessment of mixtures of specific chemical families such as pesticides and PCBs or of complex environmental mixtures combining multiple stressors such as second hand smoke.

Definitions, terms and abbreviations

Definitions for key events in the exposure to dose and health impact continuum are summarized below, compiled from four sources (Ott, 1990; 2005; US EPA, 1992a; Sexton et al., 1995a).

Active Substance: Substances or micro-organisms, including viruses, having general or specific action against harmful organisms or on plants, parts of plants or plant products (Directive 91/414/EEC).

Additivity: In the context of mixture toxicology additivity refers to a "non-interaction" situation where the toxicity of the mixture resembles the effects expected to occur when all mixture components act without diminishing or enhancing each other (DEPA 2009).

ADI: Acceptable Daily Intake (mg per kg body weight per day)

Adverse effect: A biological effect that causes change in morphology, physiology, growth, development or life span which results in impairment of functional capacity to compensate for additional stress or increase in susceptibility to the harmful effects of other environmental influences (IPCS, 1994).

Applied dose: Applied dose is the amount of the agent directly in contact with the body's absorption barriers, such as the skin, respiratory tract and gastrointestinal tract, and therefore available for absorption. Information is rarely available on applied dose, so it is calculated from potential dose based on factors such as bioavailability.

ARfD: Acute Reference Dose (mg per kg body weight per day)

Biocides: Active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means (Directive 98/8/EC). The directive lists 23 product types such as disinfectants and algaecides used for the treatment of swimming pool water, hospital waste, container disinfectants, drinking water disinfection, wood, leather and construction material preservatives, slimicides, rodenticides, avicides, molluscicides, piscicides, insecticides, repellents, antifouling products. Products covered by other directives are excluded.

Biological effect: A measurable response to dose in a molecule, cell or tissue is termed a biological effect. The significance of a biological effect, whether it is an indicator or a precursor for subsequent adverse health effects, may not be known.

Biologically effective (target) dose: The biologically effective dose is the portion of the delivered dose that reaches the site or sites of toxic action. The link, if any, between biologically effective (target) dose and subsequent disease or illness depends on the relationship between dose and response (e.g., shape of the dose-response curve), underlying pharmacodynamic mechanisms (e.g., compensation, damage, repair), and important susceptibility factors (e.g., health status, nutrition, stress, genetic predisposition).

Common Mechanism of Toxicity Group: Pesticides have a common mechanism of toxicity if they act the same way in the body, i.e. if the same toxic effect occurs in the same organ or tissue by essentially the same sequence of major biochemical events.

Delivered dose: The delivered dose is the portion of the internal (absorbed) dose that reaches a tissue of interest.

Dose: Once the agent enters the body by either intake or uptake, it is described as a dose. Several different types of dose are relevant to exposure estimation. All these different dose measures are approximations of the target or biological effective dose.

Exposure concentration: Exposure is the concentration of an agent in a carrier medium at the point of contact with the outer boundary of the human body. The concentration is the amount (mass) of a substance or contaminant that is present in a medium such as air, water, food or soil expressed per volume or mass. Assessments are often not at exposure or exposure concentration, since that information alone is not very useful unless it is converted to dose or risk. Assessments therefore usually estimate how much of an agent is expected to enter the body. This transfer of an environmental agent from the exterior to the interior of the body can occur by either or both of two basic processes: intake and uptake.

Exposure pathway: An exposure pathway is the physical course taken by an agent as it moves from a source to a point of contact with a person. The substance present in the media is quantified as its concentration.

Exposure route: Exposure route denotes the different ways the substance may enter the body. The route may be dermal, ingestion or inhalation.

Intake: Iintake is associated with ingestion and inhalation routes of exposure. The agent, which is likely to be part of a carrier medium (air, water, soil, dust, food), enters the body by bulk transport, usually through the nose or mouth. The amount of the agent that crosses the boundary per unit time can be referred to as the "intake rate", which is the product of the exposure concentration times the rate of either ingestion or inhalation. For inhalation, intake may be calculated for any time period. For ingestion, intake is usually expressed as the amount of food or water consumed times the pollutant concentration in that medium during a certain time period.

Internal (absorbed) dose: The amount of the agent absorbed, and therefore available to undergo metabolism, transport, storage or elimination, is referred to as the *internal* or ab*sorbed* dose. Bioavailability has been used to describe absorbed dose.

Mixture: A mixture is a combination of several chemicals with which organisms come into contact, either simulaneously or sequentially. According to (DEPA 2009) “binary mixtures” are combinations of two agents, “multi-component mixtures” are combinations of three or more chemicals whereas “complex mixtures” are mixtures of unknown composition, e.g. from environmental media. Feron et al. (2004) propose use the term “specified combinations” if all constituents of the mixture are known.

NOAEL: The No Observed Adverse Effect Level is the highest dose or concentration at which a certain adverse effect is not found in exposed test organisms where higher doses or concentrations resulted in the adverse effect

Pesticides residues: means residues, including active substances, metabolites and/or breakdown or reaction products of active substances currently or formerly used in plant protection products as defined in Directive 91/414/EEC, which are present in or on the products covered by Annex I to this regulation, including in particular those which may arise as a result of use in plant protection, in veterinary medicine and as a biocide (Regulation (EC) NO 396/2005).

Plant Protection Products: Active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to protect plants or plant products against harmful organisms or prevent the action of such organisms, in so far as such substances or preparations are not otherwise defined below, influence the life process of plants, other than as a nutrient (e.g. growth regulators), in so far as such substances or products are not subject to special Council of Commission provisions on preservatives, destroy undesired plants, or destroy parts of plants, check or prevent undesired growth of plants (Directive 91/414/EEC).

Potential (administered) dose: Potential or administered dose is the amount of the agent that is actually ingested, inhaled or applied to the skin. The concept of potential dose is straight forward for inhalation and ingestion, where it is analogous to the dose administered in a dose-response experiment. For the dermal route, however, it is important to keep in mind that potential (or administered) dose refers to the amount of the agent, whether in pure form or as part of a carrier medium, that is applied to the surface of the skin. In cases where the agent is in diluted form as part of a carrier medium, not all of the potential dose will actually be touching the skin.

Preparation: Mixtures or solutions composed of two or more substances of which at least one is an active substance, intended for use as plant protection product (Directive 91/414/EEC).

Sources: The point or area of origin for an environmental agent is known as a source. Agents are released into the environment from a wide variety of sources, which are often categorized as *primary sources* including point sources (e.g., incinerator) versus area sources (e.g., urban runoff), stationary sources (e.g., refinery) versus mobile sources (e.g., automobile) and anthropogenic sources (e.g., landfill) versus non-anthropogenic sources (e.g., natural vegetation) and *secondary sources* including condensation of vapors into particles and chemical reactions of precursors producing new pollutants.

Synergism: When an observed combination effect is larger than expected according to an additivity assumption there is synergism.

Uptake: Uptake is associated with the dermal route of exposure, as well as with ingestion and inhalation after intake has occurred. The agent, as with intake, is likely to be part of a carrier medium (e.g., water, soil, consumer product), but enters the body by crossing an absorption barrier, such as the skin, respiratory tract or gastrointestinal tract. The rates of bulk transport across the absorption barriers are generally not the same for the agent and the carrier medium. The amount of the agent that crosses the barrier per unit time can be referred to as the *uptake rate.* This rate is a function of the exposure concentration, as well as of the permeability and surface area of the exposed barrier. The uptake rate is also called a *flux.*

Methodologies for quantifying health effects of combined exposure to multiple stressors

Complexity of exposure assessment due to pathways and routes of exposure

Exposure is "an event that occurs when there is contact at a boundary between a human and the environment with a contaminant of specific concentration for an interval of time" (National Academy of Sciences, 1991). This contact may exist through inhalation, ingestion or penetration of the skin surface. According to the definition, exposure is related directly to the pollutant of interest, to the individual, and to the time and duration of exposure (Lioy, 1990). Determining the risk posed by environmental pollution to public health requires a knowledge of five fundamental components: the sources of pollutants, the transport of pollutants from sources to humans, the exposures of humans to pollutants, the doses received by those who are exposed, and the adverse health effects resulting from the doses (Ott, 1985). As the output of each model's component serves as input to the next, the absence of valid information on any component can impair the ability to make accurate assessments of public health impacts from environmental pollution.

Exposure assessment is defined as "determination of the emissions, pathways and transformations of pollutants in order to estimate the concentrations/doses to which humans are or may be exposed" according to the Commission of the European Communities (CEC) Directive 93/67/EEC (CEC, 1993). Moreover, exposure assessment can be used to determine the distributions of exposures within the population and the contribution of various microenvironments and pathways to the integrated exposure as well as in identifying subgroups with relatively high exposures (CEC, 1991). This can be a complex endeavor requiring analysis of many different aspects of the contact between people and hazardous substances (Table 1).

Table 1. Different aspects of the contact between people and pollution that are potentially important in exposure analysis (Sexton et al., 1995b).

Agent(s)	biological, chemical, physical, single agent, multiple agents, mixtures
Source(s)	anthropogenic/non-anthropogenic, area/point, stationary/mobile, indoor/outdoor
Transport/carrier medium	anthropogenic/non-anthropogenic, area/point, stationary/mobile, indoor/outdoor
Exposure pathways(s)	eating contaminated food, breathing contaminated air, touching residential surface
Exposure concentration	mg/kg (food), mg/litre (water), μg/m^3 (air), μg/cm^2 (contaminated surface), % by weight,
Exposure route(s)	inhalation, dermal contact, ingestion, multiple routes
Exposure duration	seconds, minutes, hours, days, weeks, months, years, lifetime
Exposure frequency	continuous, intermittent, cyclic, random, rare
Exposure setting(s)	occupational/non-occupational, residential/non-residential, indoors/outdoors

Exposed population	general population, population subgroups, individuals
Geographic scope	site/source specific, local, regional, national, international, global
Time frame	past, present, future, trends

Exposure assessments contribute to a number of health-related assessments, including risk assessment, status and trends analyses, and epidemiological studies. Based on the current consensus of the scientific community (U.S NRC, 1991a, 1991b; U.S. EPA, 1992a), exposure is defined in terms of contact with the visible exterior of the person (skin and openings into the body, such as mouth and nostrils). Exposure assessments often rely implicitly on the assumption that exposure can be linked by simple parameters to ambient concentrations in air, water, and soil. However, total exposure assessments that include time and activity patterns and microenvironmental data reveal that an exposure assessment is most valuable when it provides a comprehensive view of exposure pathways and identifies major sources of uncertainty.

The release of an agent into the environment, its ensuing transport, transformation and fate in various environmental media, and its ultimate contact with people are critical for understanding how and why exposures occur.

In the most general sense, exposure assessment involves the quantification of a link among a source of contamination, transport, and trans-formation among a set of environmental media, and human contact with an exposure medium (U.S. EPA, 1992b; McKone & Daniels, 1991). Environmental media include outdoor air, indoor air, ground-surface soil, root-zone soil, plants, groundwater, and surface water in a contaminated landscape, as well as carpets, furniture, and walls in the residential environment. Exposure media include substances with which we have direct contact, such as outdoor air, indoor air, food, household dust, home-grown foods, animal food products, and tap water.

Although exposure assessments are done for a variety of reasons, the quantitative exposure estimate can be approached in three different ways.

1. The exposure can be measured at the point of contact (the outer boundary of the body) while it is taking place, measuring both exposure concentration and time of contact and integrating them (point-of-contact measurement).

2. The exposure can be estimated by separately evaluating the exposure concentration and the time of contact, then combining this information (scenario evaluation).

3. The exposure can be estimated from dose, which in turn can be reconstructed through exposure has taken place (reconstruction).

These three approaches to quantification of exposure (or dose) are independent, as each is based on different data. The independence of the three methods is a useful concept in verifying or validating results. Each of the three has strengths and weaknesses; using them in combination can considerably strengthen the credibility of an exposure or risk assessment.

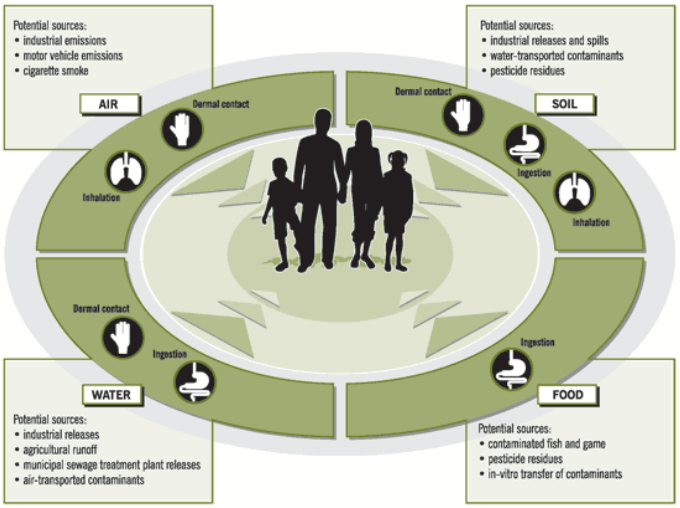

Figure 1. Multiple sources and exposure pathways (Sexton et al., 1995b)

Exposure pathways define a link between an environmental medium and an exposure medium. An exposure pathway is the course a chemical, biological, or physical agent takes from a known source to an (often unknown) exposed individual. An exposure pathway describes a unique mechanism by which an individual or population is exposed to chemical, biological, or physical agents at or originating from a source. Each exposure pathway includes a source or release from a source, an exposure point, and an exposure route. Exposure routes are inhalation, ingestion, and dermal uptake (Figure 1). Because exposure is defined in terms of contact with the visible exterior of the organism (skin, mouth and nostrils), we can view a human, an animal, or a plant as having a hypothetical outer boundary separating internal living tissues from the outside surfaces. Thus, exposure is the condition of a chemical contacting the outer boundary of an organism, and exposure over a period of time can be represented by a time-dependent profile of the exposure-medium concentration. Intake is the process by which a chemical is physically moved through an opening in the outer boundary of an exposed individual. Potential dose is the amount of chemical in the air inhaled, in water or food or ingested, or in a material applied to the skin surface. Absorbed dose is the amount of contaminant penetrating the exchange boundaries of an organism after contact. Absorbed dose is calculated from the intake and absorption efficiency. For human populations, it is typically expressed as the mass of contaminant absorbed into the body per unit mass per unit time, such as mg/kg-day.

In the exposure assessment framework for toxic agents, the link among a source, environmental media, and exposure media; the time history of concentration of the agent in an exposure medium; the route of contact (inhalation, ingestion, or dermal contact); and the frequency and duration of contact are all important components of the quantification of human exposure. For agents with large time variations of exposure concentration, the duration and frequency of contact become very important in defining cumulative contact and potential dose. The duration and frequency of contact depend on activity patterns. An activity pattern is simply a time budget of an individual's activities over some period of time. Activities can be described in terms of an activity type (e.g., recreational, personal care, etc.), temporal variation, and location. Data on activity patterns can be derived from video recordings, from diaries that participants in time–activity surveys complete, or from telephone surveys that request respondents to recall time–activity behaviors.

For many agents that are ubiquitous in several environmental media, total exposure may reflect concurrent contacts with multiple media instead of continuous or multiple contacts with a single medium. Multi-media pollutants give rise to the need to address many types of "multiples" in the quantification of exposure and dose, such as multiple media (air, water, soil); multiple exposure pathways (or scenarios); multiple routes (inhalation, ingestion, dermal); and multiple target tissues for dose and effect.

Thus, aggregate exposure is defined as the quantitative exposure assessment to a single agent from all potential exposure pathways. In contrast, cumulative exposure is defined as the total doses of multiple substances received from multiple sources. In the case that the multiple substances interact at the level of metabolism, then we need to deal with mixtures effects, a topic which is described in a following chapter of this report.

The overall objective of aggregation of exposure is the estimation of the biologically effective dose. The biologically effective dose is the portion of the delivered dose that reaches the site or sites of toxic action. It should be noted that for many xenobiotics the attributable health outcomes are primarily related to the parent compound; in this case, metabolic processes are usually detoxification processes, reducing the overall biologically effective dose of the toxicant. On the contrary, in some xenobiotics like benzene, intermediate metabolites are responsible for the observed health outcomes, such as benzene-related leukemia. In this case, metabolism is a toxicity potentiation process, that relative rate of which may affect the overall toxicity body burden.

A close view to Figure 2 can help us to better understand the complexity of aggregate exposure and the methodology that is needed for a comprehensive risk assessment. The graph presents the average daily profile of benzene in a typical South-European urban setting outdoors, as much as indoors, taking into account the average time-activity patterns of individuals in Europe. Outdoor concentration through the day varies due to the daily variation of traffic volume and the meteorological conditions (wind speed and direction, mixing boundary height etc.); indoor concentrations are mainly affected by the existence of strong benzene sources, including smoking being the dominant among them. Environmental media concentrations can easily be tracked over time, either by monitoring devices, or by air quality modeling. Personal exposure may be tracked either by personal samplers, or by summing up the integrals of time spent in each microenvironment times the air concentration in the respective microenvironment. However, as mentioned above, benzene carcinogenicity is not due to the parent compound, but rather to the intermediate metabolites. In this case, although information on the media and exposure concentration is necessary for implementing the full risk assessment process; however, this information does not suffice for correct risk assessment, since the time course profile of benzene metabolite is completely different than the respective ambient and indoor air concentrations (as shown in the plot). Moreover, based on the magnitude of exposure/pattern and the metabolic/elimination rate, possible bio-accumulation should not be excluded over time, further complicating the risk assessment process. Obviously, when additional exposure sources either from the same (e.g smoking) or (e.g. ingestion through food and water, skin contact with disinfectant products) different routes of exposure need to be aggregated (e.g. ingestion through food and water, skin contact with disinfectant products), use of a toxicokinetic model becomes even more necessary.

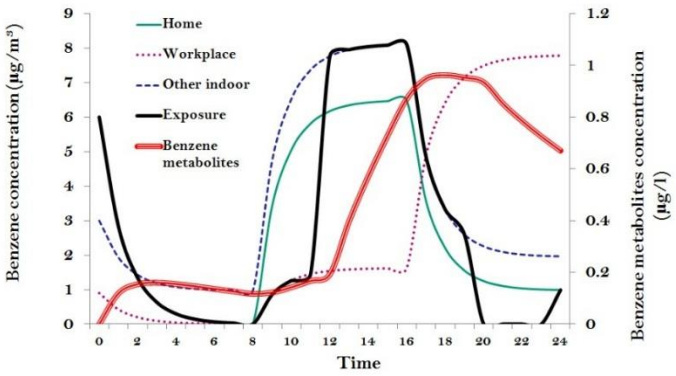

Figure 2. Daily variation of benzene environmental concentrations, personal exposure and metabolites internal dose.

Regardless of their effect or origin, the behavior of xenobiotics in the body can be described by general terms and models reflecting the mechanisms by which exposure occurs and the body handles the

chemical (Figure 3). These toxicokinetic or Physiology Based PharmacoKinetic (PBPK) models are very important in chemical risk assessment because they allow estimates to be made of how much of a chemical will be retained in the body, at what level, and for how long under various assumptions of absorption.

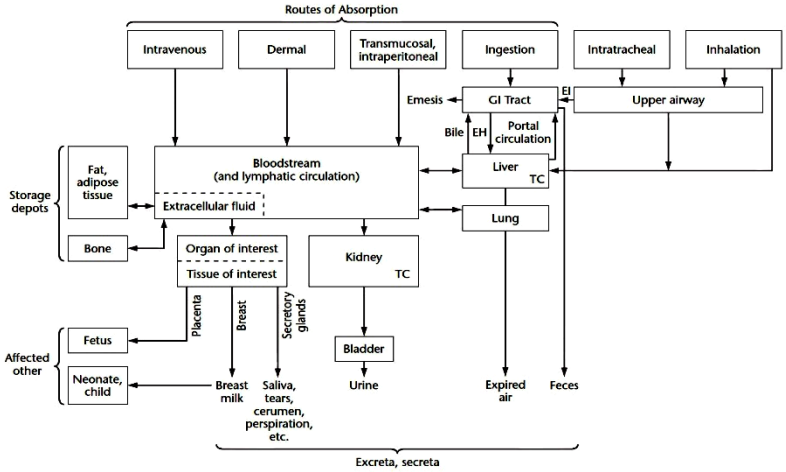

Figure 3. Conceptual representation of a toxicokinetic model (Robson and Toscano 2007)

The importance of PBPK modeling in exposure assessment due to different multimedia pathways and routes of exposure arises from two main reasons:

- Biologicaly effective dose to one chemical may have a completely different time profile depending on the route of administration, strongly depended on the metabolic pathway. In this case, the derived biologically effective dose is completely different to the estimation derived by just summing the overall intake by all routes of exposure.
- PBPK models offer a clear advantage when biomarkers (which are physiological, cellular, or molecular indicators used to evaluate xenobiotic exposures and potential effects in a population) are used to identify the exposure burden of aggregation. Considering that the observed biomarker is usually a urine metabolite and the urine sample is taken after the exposure event, the only way to link this to the overall exposure is reverse modeling through a toxicokinetic model (Georgropoulos et al, 2009). Considering the differences in the bioavailability through the exposure routes as mentioned above, the assessment of the overall exposure burden or the contribution of a specific unknown source among others with known contribution is feasible only by using a PBPK model.

The following step after estimating the biologicaly effective dose to link this to the possible health outcomes. The link, between biologically effective (target) dose and subsequent disease or illness depends on the relationship between dose and response (e.g., shape of the dose-response curve), underlying pharmacodynamic mechanisms (e.g., compensation, damage, repair), and important susceptibility factors (e.g., health status, nutrition, stress, genetic predisposition).

The three distinct varieties of the exposure-response relationship (Robson and Toscano 2007) need to be distinguished conceptually. These are the toxicological dose-response relationship, the clinical dose or exposure response relationship, and the epidemiological exposure-response relationship.

The most fundamental building block of toxicology is the dose-response relationship demonstrable in the laboratory, often called the *toxicological* dose—or exposure-response relationship. The fundamental principle is that the physiological response depends on the amount of the agent in the blood and presented to the tissue. As exposure increases at the tissue level, the response (for example, smooth muscle contraction, inflammation, cell injury or other outcomes) increases up to the maximum that the tissue can sustain.

The toxicological exposure-response relationship is usually studied in the laboratory in isolation, to characterize each important effect. However, in the intact animal or person, several toxicological exposure-response relationships develop at the same time. Some result in visible toxicity earlier than others. In a given individual, exposure to an increasing amount of a toxic substance leads to the progressive appearance of new and usually more severe health problems that finally lead to death—a sort of stepladder to lethality.

This gives rise to another type of dose- or exposure-response relationship, which might be termed the *clinical* exposure-response relationship. At a given level of exposure, often referred to clinically (if colloquially) as a *threshold,* one can usually expect a given constellation of symptoms and signs. This clinical exposure-response relationship depends importantly on susceptibility. In a given exposure situation, one person because of personal susceptibility may show one symptom and another, a different symptom. At relatively low levels of lead toxicity, some patients show elevated uric acid levels because of reduced renal clearance; however, many do not. The detection of the expected clinical response depends on the sensitivity of clinical examination and laboratory tests. Clinical tests are often inadequate for early detection of equivocal cases because they are designed to make specific diagnoses in people known to be sick in a way that strongly suggests a particular type of disease.

Biomonitoring

Estimating a population's potential risk from chemical exposures is based on a function that takes into account the chemical's toxicity, the opportunity for exposure, exposure assessment, and the population likely to be exposed. Exposure assessment is usually the factor of greatest uncertainty, given the paucity of exposure data and incomplete knowledge of exposure mechanisms (especially in the event of mixed exposures) of most chemicals. A number of approaches have been developed to reduce this uncertainty. One approach, often called *molecular epidemiology,* identifies and assesses the relationship between biological markers and health outcomes. Biological markers, or biomarkers, are physiological, cellular, or molecular indicators used to evaluate xenobiotic exposures and potential effects in a population.

Biomarker data can stand alone or augment more traditional risk assessment approaches such as questionnaires. Before conducting an epidemiological study using biomarkers, we need to understand the relationship between the specific biomarker and the toxicokinetics of a specific chemical. This process is defined as *biomarker validation* and includes both laboratory and population components.

Biomarkers are categorized by whether they measure exposure, effect, or susceptibility. In the case of biomarkers of exposure, these biomarkers are better at integrating individual differences than more traditional exposure measures. Biomarkers of exposure identify and measure chemical residues in tissue or body fluids, metabolites of the xenobiotic or physiological outcomes that are effects of exposure, often unrelated to the toxic effect of concern in humans. For example, a biomarker might be the concentration of a chemical in blood, the excretion of a metabolite of the chemical in urine over twenty-four hours, or the degree of inhibition of an enzyme known to be affected by the chemical. These data provide information on an individual's total exposure from all sources, preceding the time of the analysis. Biomarkers cannot distinguish between the contribution of various absorption pathways to the internal dose that is reflected in the biomarker level. Samples over time are used to identify population trends. Biomarker data can be used to compare exposures in different subpopulations, such as children, adolescents, or the elderly, or residents of different geographical areas. Ultimately, better information about a population's exposure results in better decisions to protect public health and assist in the prioritization of research and intervention programs.

Biomarkers of effect characterize the impact of exposure to chemicals or contaminants on a targeted system such as the blood. As a result, molecular, cellular, or even systemic effects can be observed before clinical symptoms occur. For example, recovery of DNA adducts from blood or urine may reflect the risk of genotoxicity. Not all individuals with a given biomarker of effect will develop the disease, and this distinction is important to communicate to potentially affected groups. Biomarkers of effect can indicate preclinical effects observed between exposure and disease and ultimately serve as surrogates for disease for a population.

Biomarkers of susceptibility can potentially characterize how populations respond to exposures. In addition, biomarkers of susceptibility can identify potentially sensitive population subgroups. For example, studies of genetic polymorphism can identify persons with enzyme types more likely to be affected by a chemical. Susceptibility biomarkers can be used to identify population subgroups potentially at greater risk from a given exposure so that protective measures can be taken. They may also be important in assessing the mechanism of toxicity.

The limitations of using biomarkers in the risk assessment process include characterizing the specificity and sensitivity of the biomarker for the xenobiotic, understanding the metabolism of the xenobiotic, and accounting for individual differences within a population. The primary objective of molecular epidemiology is to identify associations between biomarkers and potential risk. As more biomarkers are developed and data collected, these associations increasingly will be able to provide risk assessors with a better understanding of risk.

Literature review of chemical mixtures toxicology studies

Kortenkamp et al (2009) performed an extended literature review on the toxicological studies regarding the combined effects of chemicals relevant to human and mammalian toxicology. Emphasis was placed on experimental studies that strive to understand mixture effects in terms of the toxicity of its individual components. Their conclusion was that, well-designed mixture experiments assess observed effects against an explicitly stated additivity assumption, derived from the concepts of dose addition or independent action. These additivity expectations should be calculated on the basis of dose-response data of the individual chemicals in the mixture. The mixture ratio employed in the combination experiment should ensure that all (or most) of the components contribute significantly to an overall mixture effect, if at all present.

Carcinogenicity

In their 1989 report on toxicants in drinking water, the US National Academy of Sciences (NRC 1989) recommended the use of independent action for the estimation of risks from mixtures of carcinogens, with the implication that carcinogenesis is a stochastic process, fulfilling the basic assumptions behind independent action. However, it is complicated to assess whether there is empirical support for this idea in the scientific literature describing the joint effects of several carcinogens, mutagens and other genotoxic chemicals. To a large degree, these difficulties can be traced to the various definitions, concepts and terms that have evolved in this field for the purpose of assessing combination effects of carcinogens. Not all of these approaches are compatible with the concepts and terms in other areas of mixture toxicology. The way in which the problem has been framed in the carcinogens literature has had an impact on the experimental design of many key studies, particularly with respect to expected combination effects in the case of additivity. In many cases, assessments of the type of combination effect in terms of dose additivity or additivity according to independent action are not possible, because of a lack of data about dose-response relationships for individual carcinogens.

As an overall conclusion regarding the long-term animal studies, the empirical findings support the idea that syncarcinogenesis (including additive effects in the sense of "working together", as well as "augmentational" effects) is likely to occur with combinations of substances that target the same organs or tissues. This applies to the skin (Nakahara and Fukuoka 1960, Schmidt et al. 1976, Schmähl et al. 1977, Cavalieri et al. 1983), the urinary bladder (Tsuda et al. 1977), the airways and the lung (Kimizuka et al 1987, 1993, Nesnow et al. 1998) and the liver (Berger et al. 1987, Elashoff et al. 1987, Fears et al. 1988). Syncarcinogenesis does not require similarity in chemical structures, nor does it matter whether administration is sequential or simultaneous. However, syncarcinogenesis is not observed when carcinogenic substances are combined that target different organs.

Hasegawa et al. (1991) used a short-term initiation-promotion model with glutathione-S-transferase-positive hepatic foci to investigate the effects of a five-component mixture of different heterocyclic aromatic amines, after initiation with diethylnitrosamine (DEN). At the highest tested doses, all chemicals individually produced foci. When combined at 1/5 of these doses, but not at 1/25, combination effects in excess of the arithmetic sum of effects of the single chemicals were observed, which the authors interpreted as synergisms. This study indicates that the heterocyclic aromatic amines can produce hepatic foci when combined at doses where each single agent is without statistically significant effect, in line with the idea of syncarcinogenesis as "augmentational" effects.

Beside the "augmentational effects", Ruediger (2006) listed various mechanisms that might lead to attenuations of carcinogenic effects of chemicals. Mechanisms for which there is good experimental evidence include: inhibitions of metabolic activations of procarcinogens, induction of metabolic inactivation, slow-down of cell cycle progression, and induction of apoptosis. He pointed out that all these proposed mechanisms of cancer suppressing effects of a chemical are not linked to the carcinogenicity of a substance. Rather, an inhibitory effect appears to be possible only if the suppressing component has a weaker carcinogenic potency than the other chemical in the combination.

Conclusively, there is overwhelming evidence that carcinogens work together to exert tumorigenic responses after sequential or simultaneous exposure. Joint carcinogenic action occurs when carcinogens are combined at doses that individually are without observable effects. It is to be expected with combinations of carcinogens that target the same organ or tissue. Suppressions of carcinogenic effects have also been described, and such effects are highly likely when the inhibitory agent is either not carcinogenic to the tissue in question, or has a much lower potency than the second carcinogen. Due to the specific ways in which the question of "syncarcinogenesis" has been framed, there are very few studies that allow assessments of combination effects in terms of additivity expectations derived from DA or IA. The few examples that permit such evaluations indicate agreement with DA or IA. This suggests that many "syncarcinogenic" effects do not represent true synergisms in terms of responses greater than expected according to DA or IA, but rather are consistent with those additivity concepts.

Oncogene mutation in pancreatic and colon cancers in in Mediterranean cohorts

4,4'-DDT, 4,4'-DDE and some PCBs are carcinogenic to animals and possibly to humans. Thus, studies on the mechanisms involved in cancer development under the influence of these compounds are important. Evidence on the possible role of these compounds in the mutations of K-ras gene has been obtained from Mediterranean cohorts. One case selected for study has been pancreatic cancer due to the high frequency of K-ras mutations in the development of this illness (Porta et al., 1999). The relation between serum concentrations of selected organochlorine compounds and mutations in codon 12 of the K-ras gene in patients with exocrine pancreatic cancer was investigated (Porta et al., 1999). Cases were prospectively identified in five hospitals from Barcelona. Mutations in K-ras were analyzed by polymerase chain reaction and artificial restriction fragment length polymorphism. Cases of pancreatic cancer with wild-type K-ras (n = 17) were frequency matched for age and sex to cases of pancreatic cancer with a K-ras mutation (n = 34, case-case study). These 51 cases were further compared with 26 hospital controls (case-control comparison). Serum concentrations of 4,4'-DDT were significantly higher in pancreatic cancer cases with a K-ras mutation than in cases without a mutation (odds ratio for upper tertile 8.7 (95% CI: 1.6–48.5), p = 0.005). For 4,4'-DDE the corresponding figures were 5.3 (1.1–25.2, p = 0.031). These estimates held after adjusting for total lipids, other covariates, and total PCBs. A specific association was observed between a glycine to valine substitution at codon 12 and both 4,4'-DDT and 4,4'-DDE concentrations (odds ratio 15.9, p = 0.044 and odds ratio 24.1, p = 0.028, respectively). A similar pattern was shown for the major di-ortho-chlorinated PCBs (PCB138, PCB153, and PCB180), even after adjustment for 4,4'-DDE, but without a specific association with spectrum. Concentrations of 4,4'-DDT and 4,4'-DDE were similar among wild-type cases and controls, but significantly higher for K-ras mutated cases than for controls ($p<0.01$). The results of this study support that organochlorine compounds such as 4,4'-DDT, 4,4'-DDE and some PCBs could play a role in the pathogenesis of exocrine pancreatic cancer through modulation of K-ras activation.

Further insight into these mechanisms was obtained from a case–control study designed to assess the risk of colorectal cancer with exposure to these chemicals, and their potential interactions with genetic alterations in the tumors (Howsam et al., 2004). A total of 132 cases and 76 hospital controls were selected from a larger case–control study in Barcelona (Howsam et al., 2004). Point mutations in K-ras and p53 genes in tissue samples were assessed by polymerase chain reaction/single-strand conformation polymorphism by immunohistochemical methods. An elevated risk of colorectal cancer was observed at higher concentrations of mono-ortho PCB congeners PCB28 and PCB118; odds ratios for middle and higher tertiles were 1.82 (95% CI: 0.90–3.70) and 2.94 (95% CI: 1.39–6.20), respectively. HCB, αHCH, and 4,4'-DDE showed non-significant risk increases. Risk associated with mono-ortho PCBs was slightly higher for tumors with mutations in the p53 gene but was not modified by mutations in K-ras. Mono-ortho PCBs were further associated with transversion-type mutations in both genes. The trend and magnitude of the observed association and the identification of a molecular fingerprint in tumors led to a hypothesis that exposure to mono-ortho PCBs contributes to human colorectal cancer development.

Mutagenicity and genotoxicity

Mutagenicity in the narrow sense of the word can be defined as the induction of heritable changes in the DNA sequence of the affected organism, whereas genotoxicity is often used in an overlapping, but wider sense, including chromosome mutations, chromosomal aberrations and sister chromatid exchanges. The

induction of micronuclei is also judged to be a genotoxic effect. There is a fair amount of data available on the combined effects of mixtures of chemicals that induce mutagenic and genotoxic effects, but again only a limited number of these studies are informative with respect to the type of combination effect (CA or IA). As with the carcinogenicity studies discussed earlier, there is a dearth of mixture experiments with mutagencity and genotoxicity as the endpoints for evaluation that allow clear assessments of the usefulness of CA or IA as prediction concepts. Some publications however show that genotoxic and mutagenic agents, combined in sufficient numbers, can work together at very low concentrations to produce mixture effects (Kortenkamp et al., 2009).

Developmental neurotoxicity

There are approximately 200 chemicals which have been found to be neurotoxic in humans [Landrigan and Grandjean, Lancet, 2008], and for many more there is at least some evidence of neurotoxicity deriving from animal studies. Of these, several are developmental neurotoxicants. However, of over 80,000 chemicals on the market, only a handful (about 200) have undergone developmental neurotoxicity testing according the established guidelines [Makris et al., EHP, 2009]

Several lines of evidence suggest that the developing brain is much more vulnerable to toxicants than the adult one, as neurotoxicity is observed at much lower exposure levels. This is the case, for example, of metals as lead or methylmercury. In some cases, developmental exposure to neurotoxicants results in morphological alteration of the CNS, with accompanying changes in functions [Costa et al. 2004]. However, in several instances, functional changes may be the result of more subtle biochemical/molecular alterations without major structural abnormalities. Thus, similarly to cancer, neurobehavioral and neurodegenerative symptoms may appear several years later the contaminant's exposure, in the absence of pathological signs. Exposure to developmental neurotoxicants has been suggested to be associated with a number of developmental disabilities (learning disabilities, attention-deficit hyperactivity disorder, dyslexia, sensory deficits, mental retardation, and autism spectrum disorders) which are diagnosed in children at an alarming increasing rate worldwide [Landrigan Curr Op Pediatrics 2010, Miodovnik]. In addition exposure to neurotoxicants at low doses in critical fetal or developmental stages might increase vulnerability to later neurotoxic insults: such "double-hit" hypothesis has been proposed to explain the possible etiological role of some pesticides in Parkinson disease.

Most toxicological studies focus on a single agent and either do not measure or do not adjust for potential confounding or modifying effects of other chemicals. Although such an approach has identified toxicities associated with various chemicals, it does not reflect real-world scenarios in which humans are exposed to multiple chemicals (Cory-Slechta 2005). Human exposure to chemical mixtures is particularly widespread in socioeconomically disadvantaged populations (Naess et al. 2007) and among populations living near hazardous waste sites (Hu et al. 2007). The customary approach of examining chemicals in isolation may constrain our ability to understand neurologic and behavioural sequelae (Bellinger 2008a; Cory-Slechta et al. 2008). Despite the importance of examining joint exposures to toxicants, few epidemiological studies have done so up to now.

The extensive use of pesticides leads to exposure risk of the general population. Several studies have shown the presence of different classes of pesticides and their residues in the environment, in food and human tissues worldwide, with children showing comparable high exposure (cit). As these chemicals may have similar or different mechanisms of action it is extremely difficult to prove in human populations the adverse health effect of one single agent. Unfortunately there are a limited number of animal studies on mixtures of pesticides: interestingly, even compounds sharing the same mechanisms of action (i.e organophosphates and carbamates) or compounds belonging to the same class of pesticides (i.e. chlorpyrifos and diazinon, two organophosphates) seem to target different neural pathways at low doses. In vivo, in vitro and PBK models should reproduce exposure to real world mixtures where the potential interactive effects of chemicals on biological functions may be assessed. This require the combination of well characterized toxicological and phenotypic endpoints with the novel toxicogenomic approach to identify responses to chemicals individually and in simple combinations.to evidence differences or similarity in the toxicity mechanisms and in the health outcome of the different compounds.

Table 2. Different aspects of the contact between people and pollution that are potentially important in exposure analysis (Sexton et al., 1995b).

Category	Chemical	Evidence of developmental neurotoxicity	
		Animals	Humans
Metals	Methylmercury	***	***
	Lead	***	***
	Manganese	**	**
	Arsenic	**	**
Solvents	Ethanol	***	***
	Toluene	***	***
Pesticides	Organophosphates (various)	***	**
	Organochlorines (dieldrin)	*	–?–
	Herbicides (paraquat)	*	–?–
	Fungicides (maneb)	*	–?–
Other contaminants	PCBs	***	**
	PBDEs	***	*
	Phtalates	**	**
	Bisphenol A	**	–?–
	PFOS/PFOA	*	–?–

Methylmercury (MeHg)

Methylmercury is probably one of the most studied developmental neurotoxicant, because of several episodes of human poisoning over the years, and to the still present low-level exposure to this organometal through the consumption of contaminated fish. The first evidence of the deleterious effects of MeHg exposure on brain development emerged from Japan in the mid 1950s, where children born from mothers living around Minamata Bay which consumed MeHg-contaminated fish, presented severe neurological deficits, while their mothers appeared unaffected or suffered only mild symptoms (Harada M 1995). About a decade later, another extensive episode of poisoning occurred in Iraq, due to consumption of MeHg-contaminated grain [Bakir et al. Science 1973]. In both cases, while damage in adults was restricted to the cerebellum and the visual cortex, diffuse damage was reported in the developing brain [Burbacher et al 1990 Neurotox Teratol]. It was estimated that the nervous system during early development in utero has a five-fold greater vulnerability to MeHg. Signs and symptoms in MeHg-poisoned children included spastic paresis, mental retardation, movement disorders, seizures, primitive reflexes, and speech difficulty [Castoldi et al 2008]. The mechanisms of MeHg developmental neurotoxicity have been studied extensively, and appear to involve different pathways and key cellular processes [Clarkson and Magos, 2006].

As low-level contamination of fish is persistent and may be responsible for developmental neurotoxic effects, particularly in populations with high seafood consumption, three major longitudinal studies have examined the potential effects of low-level MeHg exposure in New Zealand, the Seychelles, and the Faroe Islands [35–37]. Two of these studies (in New Zealand and the Faroe Islands) reported a correlation

between maternal levels of MeHg and subtle neurobehavioral deficits in the offspring. In particular, a small decrease in IQ points and deficits in memory attention and visuospatial perception were noted in both studies

In the Seychelles study, such relation between MeHg exposure and neurodevelopmental effects was not found [38]. Concomitant exposure to polychlorinated biphenyls (PCBs) in the Faroe Islands population, because of consumption of whale meat and whale bubbler, may be an important confounder, as both may have independent neurological effects in this population .. Exposure limits for MeHg have been set, with provisional tolerable weekly intake values ranging from 0.7 to 1.6 ug/kg, depending on the regulatory agency [FAO 2011].

Lead (Pb)

Pb is a neurotoxic metal both in adults and in children. In adults, the main effects of Pb poisoning are peripheral neuropathies, and at higher concentrations (100 ug/dL in blood) encephalopathy. In contrast, the developing CNS is uniquely sensitive to the effects of Pb, even at much lower blood levels. In the 1970s, the blood Pb action level in children was 60 ug/mL, the level associated with clinical signs of toxicity in adults [Miodovnik 2011]. In those years, epidemiological studies clearly showed an association between body burden of Pb in children and adverse neurobehavioral outcomes, namely, lower academic performance and shortened attention span [Needleman et al., Nwe Engl J Med, 1979]. The phasing out of leaded gasoline and the limitation on smokestack Pb emissions caused Pb blood level in children in the USA to decrease by 80% in the period 1978–1991. Over the years, the level of concern for blood Pb have decreased to 25 ug/mL, then in 1991 to 10 ug/mL, where it stands today. However, blood Pb levels as low as 2 ug/mL have been associated with declines in IQ and various adverse behavioral effects [Lanphear et al, 2005], and there is widespread belief that there is no proven safe lower limit for Pb exposure [Grandjean, The Lancet, 2010].

Animal studies in multiple species have confirmed that developmental Pb exposure causes similar cognitive dysfunctions, learning impairment, and distractibility [Bull et al, Neurotoxicology 1983]. In vivo and in vitro studies have shown that Pb may disrupt the blood-brain barrier by injuring astrocytes, with a secondary damage to the endothelial microvasculature [52]. Developmental Pb exposure has been shown to target the hippocampus, cerebral cortex, and cerebellum. At the molecular level, Pb is known to interfere with the regulatory action of calcium in cell functions. [Simons, Neurotoxicology 1993]. One important enzyme shown to be activated by low concentrations of Pb is protein kinase C (the classical isoforms), with ensuing perturbations of cellular homeostatic mechanisms including cell proliferation [].

Arsenic: an emerging neurotoxicants?

Arsenic is a naturally occurring essential trace element found in soil deposits and water. Both natural and human sources contribute to the ubiquitous presence of arsenic in the environment; as such, several millions of people are exposed to this toxic metalloid in varying concentrations depending on location. Consistent exposure to arsenic results in different health problems including renal, cardiovascular, reproductive, hepatic, and neurological disturbances (*Brinkel et al. 2009)*. It's well established that arsenic is a toxin in high doses and a co-carcinogen in moderate doses, but the last decade of research has shown arsenic also to be a neurotoxin in low doses. Developmental and continuous arsenic exposure induces significant deficits in long-term memory in children, as measured by the Wechsler Intelligence Scale for Children (Calderon J et al., Env Res 2001). Other research has revealed considerable deficits in learning and memory after arsenic exposure in both rodent models and in humans (Rodriguez et al. 2002; O'Bryant et al. 2011. Additionally, developmental arsenic exposure alters cerebellar morphology in the brain and disrupts cell cycle dynamics of neuroepithelial cells in vitro (Sidhu et al. 2006).

Study of the effects of mixture of metals

Metals are very common neurotoxicants occurring within the environments of children. Concomitant exposure to several metals may have more severe (i.e., synergistic) effects on neurodevelopment and cognition than expected based on effects of exposure to each metal alone (Wright et al. 2006). The problem of metal mixtures and their potential additive or synergic effects on brain and behaviour has been poorly investigated so far in rodent models. A recent prospective study carried out on a cohort of children of Mexico City found evidence of increased lead toxicity among young children with high manga-

nese coexposure (Henn, BC et al. EHP, Jan 2012). These findings highlight the importance of understanding health effects of mixed exposures to metals, particularly during potentially sensitive life stages of development such as early childhood.

Polychlorinated biphenyls (PCB)

Evidence for PCB-induced neurotoxicity includes studies of exposed human population worldwide (Jacobson and Jacobson 1997, 2003; Grandjean et al 2001). These studies consistently show learning, memory and behavioural deficits that extend into school age, but also increased risk of neurodegenerative diseases (Petersen et al., 2008, Neurotoxicology). The greater risk is associated to in utero and lactational exposure when the pregnant/lactating woman is consuming PCB-contaminated food. A number of experimental studies in non human primates and laboratory rodents have confirmed the specific susceptibility of the developing brain to PCB. There have been many attempts to identify the individual congeners responsible for the neurotoxic effects of PCBs but the controversy remains unsettled. Some evidences seem to suggest that only non-coplanar PCBs have neurotoxic effects while others report neurotoxic effects after exposure to coplanar PCBs, dioxins and AHR ligands in general (Seegal et al. 2005). In laboratory rodents the effects of mixtures seem more significant than the effects of single congeners, and gene vulnerability might in turn play an additive role (Curran et al, EHP, 2011).

Pesticides: the case of Organophosphorus (OP) Insecticide

OPs make up approximately 50% of all insecticides used in the world (Colborn, 2006); http://epp.eurostat.ec.europa.eu), and are the subject of intensive investigation for their suspected developmental neurotoxicity. Starting from 2004, several epidemiological studies involving agricultural and urban communities have indicated that developmental OP exposure may affect children's neuropsychological maturation (Rosas and Eskenazi, 2008).

These compounds, largely used in agriculture, as well as in the home and garden for pest control, induce their acute neurotoxic effects through AChE inhibition and consequent cholinergic hyper stimulation. However, increasing evidences indicate that, similarly to other environmental chemicals, OPs exert developmental toxicity at low doses with mechanisms different from those observed at higher doses.

An increasing body of literature suggests that developmental exposure to OPs (though most work has been carried out with a single compound, chlorpyrifos), at dose levels causing little inhibition of AChE, results in biochemical and behavioural abnormalities. Experimental studies in rodents indicate that pre- or postnatal exposure to chlorpyrifos affects various cellular processes (e.g., DNA replication, neuronal survival, glial cell proliferation), noncholinergic biochemical pathways (e.g., serotoninergic synaptic functions, the adenylate cyclase system), and causes various behavioural abnormalities (e.g., locomotor skills, cognitive performance and social-emotional resonses). [Dam, Seidler and Slotkin, 1998; Roy et al., 1998; Crumpton et al., 2000; Slotkin and Seidler, 2007; Ricceri et al 2006; Tait et al. 2009; Venerosi et al. 2012 Neurotoxicology).

Experimental data clearly indicate that the acute toxicity of OPs is influenced by age, with young animals being more sensitive to the effects of exposure. Depending on the OP, low detoxication by cytochromes P450, by paraoxonase-1, or by carboxylesterase may account for the differential age-related acute toxicity (Mortensen et al. 1996). As these enzymatic systems are believed to show a developmental curve also in humans, young children would be expected to be more sensitive than adults to acute OP toxicity.

These findings, together with results of biomonitoring studies that indicate exposure of children, particularly in inner cities and in farming communities, to OPs (Lu et al. 2004), have led to regulatory restrictions on the residential use of certain OPs (e.g., diazinon, chlorpyrifos), and to heightened concern for their potential neurotoxic effects in children. For example, a series of studies in different cohorts in New York City and California have reported associations between developmental exposure to chlorpyrifos and other OPs and neurodevelopmental abnormalities in the domains of reflexes and cognitive performance, as well as in morphology of specific brain areas as assessed by NMRI (Rauh et al. 2012).

Exposure to Ops is widespread in Europe and Mediterranean countries, often in combination with other pesticide classes, such as pyrethroids and carbamates. Given the neurotoicty of these compounds and the recent evidences tht they might also have endocrine disrupting activity, by interfering with brain and

behavioural sexual dimorphism as well as with Thyroid Hormones, appropriate models to understand the mechanisms of combined exposure and health outcomes are strongly needed.

Studies of neurotoxicity in Mediterranean cohorts.

Poor social behavior and Attention-Deficit Hyperactivity Disorder

Poor social behavior and attention-deficit hyperactivity disorder (ADHD) have been examined in infants as early indicators of developmental neurotoxicity. Positive associations of these disturbances in pre-schoolers of 4 years and prenatal exposure to HCB were identified in two birth cohorts in Ribera d'Ebre and Menorca (Spain; 1997–1999; n = 475; Ribas-Fitó et al., 2007b). The California Preschool Social Competence Scale and the ADHD were scored by each 4-year-old-child's teacher. Children's diet and parental socio-demographic information was obtained through a questionnaire. All prenatal HCB exposure categories were associated with an increase in the risk of having a poorer Social Competence and ADHD, but only those children with HCB concentrations above 1.5 ng/ml at birth had a statistically significant increased risk of having a poor Social Competence = 4.04 (1.76–9.58) and ADHD = 2.71 (1.05–6.96) (relative risk (standard error)). No association was found between prenatal HCB and the cognitive and psychomotor performance of these children. No association was found for HCB concentrations of these infants at 4 years and the test scores.

Early evidence of deleterious effects of PBDEs and neurodevelopment has also been compiled from a study of pre- and postnatal PBDE concentrations and neurodevelopment in children at age 4 years from Menorca (Gascon et al., 2011). The effects of PBDEs were isolated from those of PCBs, DDT, DDE and HCB. At 4 years, children were assessed for motor and cognitive function (McCarthy Scales of Children's Abilities; MCSA), attention-deficit, hyperactivity and impulsivity (ADHD-DSM-IV) and social competence (California Preschool Social Competence Scale). PBDE concentrations were measured in cord blood (n = 88) and in serum of 4 years olds (n = 244). Among all congeners analyzed only BDE47 was quantified in a reasonable number of samples (limit of quantification, LOQ = 0.002 ng/ml). Exposure to BDE47 was analyzed as a dichotomous variable: concentrations above the LOQ (exposed) and concentrations below (referents). Motor and cognitive function scores were all lower in children classified as 'exposed' compared to those classified as 'referents'. Postnatal exposure to BDE47 generally showed stronger effects on cognitive functions than prenatal exposure, although none were statistically significant (β coefficient (95% CI) of the total score = −2.7 (−7.0, 1.6) and −1.4 (−9.2, 6.5), respectively). Postnatal exposure to BDE47 was significantly related to an increased risk of symptoms on the attention deficit subscale of ADHD (RR (95% CI) = 1.8 (1.0, 3.2)) but not to hyperactivity symptoms. A higher significant risk of poor social competence symptoms was observed as a consequence of postnatal BDE47 exposure (RR (95% CI) = 2.6 (1.2, 5.9)). Adjustment for other organochlorine compounds did not influence the results.

Cognitive skills

Early life exposure to organochlorine compounds is suspected to have deleterious effects on neuropsychological development which may involve decreases in cognitive or psychomotor skills. In the Mediterranean cohorts, several studies give ground to this hypothesis and evidence that most of the neural damage occurs as a consequence of prenatal exposure.

Studies on 4-year-old children from the cohort of Menorca in which organochlorine compounds were measured at delivery and at 4 years were used for further assessment of the critical exposure age of infant development. Examination of the neuropsychological development using the MCSA at 4 years of age showed that 4,4'-DDT cord serum concentration at birth was inversely associated with verbal, memory, quantitative, and perceptual-performance skills (Ribas-Fitó et al., 2006). Children whose 4,4'-DDT concentrations in cord serum were >0.20 ng/ml had mean decreases of 7.86 (standard error, 3.21) points in the verbal scale and 10.86 (standard error, 4.33) points in the memory scale when compared with children whose concentrations were <0.05 ng/ml. These associations were stronger among girls. Prenatal exposure to higher concentrations of 4,4'-DDT was associated with a decrease in preschoolers' cognitive skills. No association was found for 4,4'-DDT concentrations of these infants at 4 years and the test scores.

A study involving 14-month-old children from two Mediterranean areas (Sabadell and Valencia) and one Atlantic area (Gipuzkoa) (n = 1391; Forns et al., 2012a) showed an impairment of psychomotor development (coefficient = −1.24, 95% confidence interval = −2.41, −0.07) at higher exposures to PCB congeners PCB138 and PCB153 (determined in cord blood serum), but found no evidence for effects on cognitive development at higher prenatal concentrations of these compounds. Child neuropsychological assessment was grounded on administration of the test of Bayley Scales of Infant Development. A specific study on the lactation exposure of these individuals using breast milk concentration data and a physiologically based pharmacokinetic model confirmed that the observed psychomotor scores were related to prenatal exposure and not to breast feeding (Gascon et al., 2013). These results are consistent with a previous study also based on Mediterranean populations in which breast feeding was observed to have a protective function in the development of cognitive skills (Ribas-Fito et al., 2007a).

Examination of the possible influence of genetic variability in the 4-year-old infants from this cohort showed a significant relation with GST genes (GSTP1, GSTM1, and GSTT1) (Morales et al., 2008). Genotyping for this study was conducted for the coding variant Ile105Val from GSTP1 and for null alleles from GSTM1 and GSTT1. Linear regression models were used to measure the association between organochlorine compounds and neurodevelopmental scores by GST polymorphisms. DDT cord serum concentration was observed to be inversely associated with general cognitive, memory, quantitative, and verbal skills, as well as executive function and working memory, in children who had any GSTP1 Val-105 allele. GSTP1 polymorphisms and prenatal DDT exposure showed a statistically significant interaction for general cognitive ($p = 0.051$) and quantitative ($p = 0.018$) skills, executive function ($p = 0.009$) and working memory ($p = 0.017$). There were no significant associations between DDT and cognitive functioning at the age of 4 years according to GSTM1 and GSTT1 polymorphisms. The results indicated that children with GSTP1 Val-105 allele were at a higher risk of the adverse cognitive functioning effects of prenatal 4,4'-DDT exposure.

Further examination of prenatal exposure to organochlorine compounds and impaired neuropsychological development at 4 years of age showed no statistically significant effects of the sum of prenatal PCBs on MCSA scores. Nevertheless, individual congener analyses yielded significant detrimental effects of prenatal PCB153 on the majority of MCSA scores, while no effects were observed for other PCB congeners. The levels of PCBs at 4 years of age were not associated with neuropsychological development. Thus, prenatal exposure to low-level concentrations of PCBs, particularly PCB153, were observed to be associated with an overall deleterious effect on neuropsychological development at 4 years of age, including negative effects on executive function, verbal functions and visuo-spatial abilities, but not on motor development (Forns et al., 2012b).

These studies have been extended to 11 years by examination of the neuropsychological development using the continuous performance test-II (CPT-II). This test was administered on 393 11-year-old children from the Menorca birth cohort. The results showed that a number of socio-environmental factors during prenatal life and early childhood, such as socio-demographic characteristics, breast feeding, maternal nutritional supplementation with folic acid and vitamins, and exposure to some organochlorine compounds, for example, 4,4'-DDE and PCB levels at 4 years, may influence inattentive and hyperactive/impulsive symptomatology during pre-adolescence (Forns et al., 2012c). As far as is known, this is the first study reporting some relationship between low neuropsychological development, in this case at 11 years, and exposure to organochlorine compounds at preschooler ages instead of in utero. Confirmation from other independent studies is needed.

Evidence from exposure to PBDEs and impaired neuropsychological development has also been obtained from the Mediterranean cohorts. So far, only a few small studies have evaluated such effects. PBDE concentrations in colostrum and infant neuropsychological development at 12 to 18 months of age were examined in 290 women recruited from Sabadell (Gascon et al., 2012). Mental and psychomotor development in the children was assessed with the test of Bayley Scales of Infant Development. The sum of the seven most common PBDE congeners (BDEs 47, 99, 100, 153, 154, 183, 209) and each congener separately were determined. Increasing Σ7PBDEs concentrations showed an association of borderline statistical significance with decreasing mental development scores (β per log ng/g lipid = −2.25; 95% CI: −4.75, 0.26). BDE209, the congener present in the highest concentrations, appeared to be the main congener responsible for this association (β = −2.40, 95% CI: −4.79, −0.01). There was little

evidence for an association with psychomotor development. After adjustment for other persistent organic pollutants, the BDE209 association with the mental development score became slightly weaker (β = −2.10, 95% CI: −4.66, 0.46). The findings suggest an association between increasing PBDE concentrations in colostrum and a poorer infant mental development, particularly for BDE209, but require confirmation in larger studies. The association, if causal, may be due to unmeasured BDE209 metabolites, including hydroxylated PBDEs, which are more toxic, more stable, and more likely to cross the placenta and to easily reach the brain than BDE209.

Reproductive and developmental toxicity, teratogenicity

A series of papers on the effects of combinations of anti-androgens on male offspring exposed during development *in utero* has been published. In all these studies (Narotsky et al., 1995; Lee et al., 2006; Hass et al., 2007; Metzdorff et al., 2007; Howdeshell et al., 2007; Rider et al., 2008; Christiansen et al., 2009), explicit additivity expectations formed the basis for mixture effect assessments.

Respiratory toxicity

Comparatively few studies have been conducted with mixtures of toxicants affecting the respiratory system. Noteworthy are the results of model experiments published by Cassee and colleagues (Cassee et al. 1996; Cassee, Groten, &Feron 1996) on the effects of combinations of formaldehyde, acetaldehyde and acrolein on the respiratory system of the rat. While the results indicated some effects of additivity in high doses, no clear conclusion was derived for lower exposure doses.

Although it is quite well established that ultrafine particulate matter can exacerbate the respiratory toxicity of corrosive gases, experimental studies that recapitulated such combined effects in animals could not be located.

Endocrine disruption

Mixtures of estrogenic chemicals

In studying endocrine disrupter mixtures, many researchers have followed what has been called a "whole mixture approach" where a combination of many chemicals is investigated as if it were a single agent, without assessing the individual effects of all the components. This type of experiment is useful for studying complex mixtures, or on a case-by-case basis, but leads to difficulties in extrapolating from one mixture to the other because small variations in composition may lead to significant changes in its toxic effects. But whole mixture approaches do not answer whether chemicals act in an additive, antagonistic or synergistic fashion. However, one of the major difficulties in assessing endocrine disrupters is uncertainty about their potential to act together in an additive or synergistic manner.

The available evidence shows clearly that dose (concentration) addition proved to be a valid tool for the prediction and assessment of combination effects of estrogen mixtures, while independent action led to underestimations of the observed effects.

Mixtures of androgen receptor antagonists and other anti-androgens

In general, mixtures of anti-androgens followed dose addition (Wolf et al., 2004; Jarfelt et al., 2005; Hass et al., 2007; Howdeshell et al., 2007; 2008; Rider et al., 2008; Christiansen et al. 2009) for a variety of endpoints typical of disruption of androgen action. This held true even for mixtures composed of anti-androgens that display a variety of mechanisms of action. No example could be identified, where independent action provided a mixture effect prediction that was more conservative than dose addition, and at the same time proved to be in good agreement with experimental data.

Combination effects between different classes of endocrine disrupters

Comparatively little work has been carried out with mixtures of different classes of endocrine disrupters, such as estrogenic agents combined with anti-estrogenic chemicals, or endocrine disrupters combined with other toxicants. In terms of design and data assessment, these studies differ from those discussed so far, because not all components present in the mixture may induce the effect chosen for analysis. In these cases, a "modulatory" influence of toxicants on the effects of other chemicals was studied. It is important to realise that the magnitude of such effect modulations cannot be predicted by adopting additivity concepts such as concentration addition or independent action (Kortenkamp et al., 2009).

Deviations from expected additivity suggestive of synergisms or antagonisms

Deviations from expected additivity were observed quite rarely (Kortenkamp et al., 2009). Notable are the observations of Nesnow et al. (1998) who analysed mixture effects of five poly-cyclic aromatic hydrocarbons on lung tumours in A/J mice, with mixture ratios representative of ambient air levels of these carcinogens. At low doses, greater than additive effects were seen, at high doses the observed responses fell short of additivity expectations which were derived from independent action in an effect surface analysis. However, the observed deviations were rather small.

Another example is the study by Walker et al (2005) who employed a two year rodent cancer bioassays with female Harlan Sprague-Dawley rats given 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3,3',4,4',5-pentachlorobiphenyl (PCB-126), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or a mixture of the three compounds. The three chemicals, both singly and in combination induced hepatic, lung, and oral mucosal neoplasms. A re-analysis of the data, without utilizing the WHO TEF values, but by employing the concept of dose addition directly showed that the experimentally observed tumour incidences fell short of those anticipated by dose addition ([Kortenkamp et al., 2009).

There are a few examples from the area of endocrine disruption that indicated antagonisms in the joint effects of estrogenic agents (Rajapakse et al. 2004, Charles et al. 2007), but these deviations were rather small. Similarly, the study by Hass et al. (2007) on the feminizing effects of androgen receptor antagonists on male offspring of dams dosed during gestation indicated a weak synergism with respect to induction of nipple retention. Similar deviations from additivity were not observed with other endpoints of evaluation that were used in the same study.

PBPK in mixture toxicology

General

Human exposure to chemicals is rarely, if ever, confined to a single compound. Therefore, the study of chemical mixture toxicology has gained a great deal of momentum in the last two decades. Studying chemical mixtures is an extremely complex task because of the astronomical number of possible combinations. Such numbers certainly preclude any systematic experimental assessment of toxicology of all potentially troublesome chemical mixtures. In the past 15 years or so, physicologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling has been applied to the toxicological interactions of chemical mixtures, and a comprehensive review on these studies is conducted by Reddy et al (2005). As is generally the case in the evolution of a new area, the progress in the application of PBPK modeling to chemical mixtures has followed different phases from simple binary pharmacokinetic and pharmacodynamic interactions to more and more complex mixtures. First, PBPK modeling of binary chemical mixtures became necessary because of pharmacological or toxicological interactions. Second, as investigators became interested in mechanisms of toxicological interactions, the advances of physiologically based pharmacodynamic (PBPD) modeling formed a natural course of development of this area. Third, when more and more sophistication was incorporated into PBPK modeling, it is inevitable that PBPK modeling of complex chemical mixtures were attempted and novel approaches developed. Since 1996 when the Food Quality and Protection Act was enacted, the United States Environmental Protection

Agency (US EPA), under the Congressional mandate, began active consideration of cumulative risk assessment. Out of necessity, toxicological inter-actions must be taken into consideration. Thus, the application of PBPK modeling in cumulative risk assessment has become an active area of research endeavor (US EPA 2003).

Earlier Days: PBPK Modeling of Binary Mixtures

Although numerous drug interaction studies have been reported in the scientific literature in past decades (Mozayani and Raymon 2004), the earliest applications of PBPK modeling to chemical mixtures did not occur until the mid-1980s. Because of the necessity for extrapolation from animal experimentation to humans for many toxicants, the advances of PBPK modeling in the area of toxicology has far outpaced its application in pharmacology. An earlier review of PBPK modeling of chemical mixtures (Mumtaz et al. 1993) indicated that the "first example" of PBPK modeling of a "chemical mixture" actually involved one chemical, n-hexane, and its metabolites, methyl n-butyl ketone (MnBK) and 2,5-hexanedione (2,5-HD); thus, it is a kind of "one-chemical mixture." This particular PBPK model for n-hexane and its metabolites incorporated three inhibitory interactions: (1) hexane and MnBK are competitive substrates for w-1 oxidation; (2) MnBK and 2,5-HD are competitive substrates for a oxidation; and (3) 2,5-HD acts as a product feedback inhibitor (Andersen and Clewell 1984). The findings of this modeling study were intriguing and they explained some of the most interesting and complex toxicological and pharmacokinetic behaviors of n-hexane in animals (Mumtaz et al. 1993). However, this PBPK modeling work was never published in a peer-reviewed journal; the principal reason was that the investigators involved were never happy enough with the PBPK modeling results. Interestingly, after about 20 years, the n-hexane PBPK modeling work was revisited (Dennison 2004). Logically, some of the earliest investigations on PBPK modeling were on binary mixtures.

Example 1: Dibromomethane and Isoflurane

One of the reasons for the delay in the completion of n-hexane work was the difficulties encountered during the experimental and PBPK modeling processes. In those earlier days, as an alternative to mimic the interaction between n-hexane and its metabolite, MnBK, Clewell and Andersen (1985) studied, using PBPK modeling, a binary mixture of dibromomethane (DBM) and isoflurane. The rationale, as given later in a review (Mumtaz et al. 1993) by Clewell, was that (1) DBM was selected as a "surrogate" of MnBK because of its high tissue solubility and the ease of monitoring its metabolism, and (2) isoflurane was selected as a "surrogate" of n-hexane because of its poor tissue solubility and its rapid clearance by exhalation. The Clewell and Andersen (1985) publication was, in general, a review article, although some original experimental and modeling data were apparently included. Many studies described therein utilized the same basic PBPK model of styrene, shown in Figure 4, as a template. A plot of PBPK model simulation versus experimental data showed similar kinetics for the formation of carboxyhemoglobin (HbCO), resulting from the metabolism of DBM to CO, following DBM exposure alone, or in combination with isoflurane in rats. Not much specifics on experimentation and modeling (i.e., animals, exposure regimen, interaction model structure, etc.) were given in this particular publication. Somewhat more details were available in the 1993 review (Mumtaz et al. 1993).

Example 2: 1,1-Dichloroethylene and Trichloroethylene

The interactive PBPK model of 1,1-dichloroethylene (DCE) and trichloroethylene (TCE), the related discussions on different types of enzyme inhibitions, and the incorporation of competitive inhibition into the liver compartment represent truly the first comprehensive publication (Andersen et al. 1987) in the peer-reviewed journal on PBPK modeling of a chemical mixture. Using liver injury [aspartate transaminase (AST)] from DCE as an index, the pharmacokinetics of DCE alone in Fischer 344 rats as well as that in the presence of TCE were compared between PBPK model simulation and experimental results obtained from gas uptake pharmacokinetic studies. For PBPK modeling, Andersen et al. (1987) constructed a PBPK model for each chemical (i.e., DCE or TCE) individually and then linked the two models via the mass balance equation for the liver through enzyme inhibition (see next section for details). The kinetics of each chemical was described by a set of five mass balance differential equations for tissue compartments (fat,

muscle/skin, viscera, and liver) and the chamber atmosphere. Thus, the basic template for each PBPK model was again based on that of the styrene model shown in Figure 4 (Ramsey and Andersen 1984).

Physiological constants and partition coefficients were either available in the literature (Gargas et al. 1986) or, in the case of partition coefficients for TCE, experimentally determined using vial equilibration methods (Sato and Nakajima 1979). One of the most distinguished features of a PBPK model describing chemical interactions in chemical mixtures in the Andersen et al. (1987) article on DCE and TCE, as well as in a large number of subsequent papers on chemical mixtures, is the incorporation of enzyme inhibition as mechanistic basis for interactions. Thus, in many ways, the Andersen et al. (1987) article and its related descriptions of different types of enzyme inhibition is the pioneering effort both experimentally and conceptually in the area of PBPK modeling of chemical mixtures. The section below will provide the detailed description of the fundamentals on the mechanistic basis of enzyme inhibition.

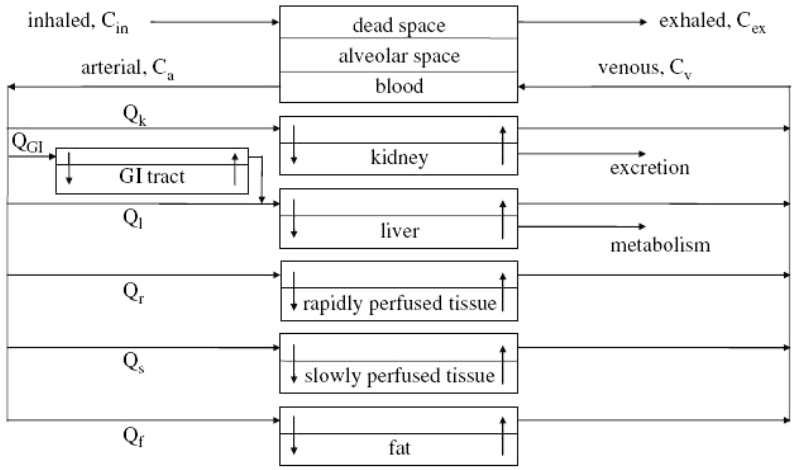

Figure 4. A graphical representation of a PBPK model for volatile organic chemicals such as styrene (Reddy et al., 2005). The variables C_{in} and C_{ex} are the concentrations of chemical in inhaled and exhaled air, respectively, C_a and C_v are the concentrations of chemical in the arterial and venous blood, respectively, and Q_k, Q_{GI}, Q_l, Q_r, Q_s, and Q_f are the flow rate of blood through the kidneys, GI tract, liver, rapidly perfused tissue, slowly perfused tissue, and fat, respectively.

Figure 5 is a general schematic for multiple mechanisms of enzyme inhibition during coexposure of two substrates (Andersen et al. 1987). E is the enzyme in free form; in the case of DCE and TCE, it is cytochrome P450 2E1 (CYP2E1). S1 and S2 are two substrates; in Example 2 above, they are DCE and TCE. The products formed from S1 and S2, respectively, are P1 and P2. All constants are dissociation equilibrium constants. The enzyme binding constants for substrates 1 and 2, KM1 and $_{KM2,}$ are also the inhibitory binding constants when one substrate serves to inhibit the metabolism of a second substrate. In that sense, accordingly, KM1 $^{equals}{}_{KMI1}$ and KM2 equals KMI2.

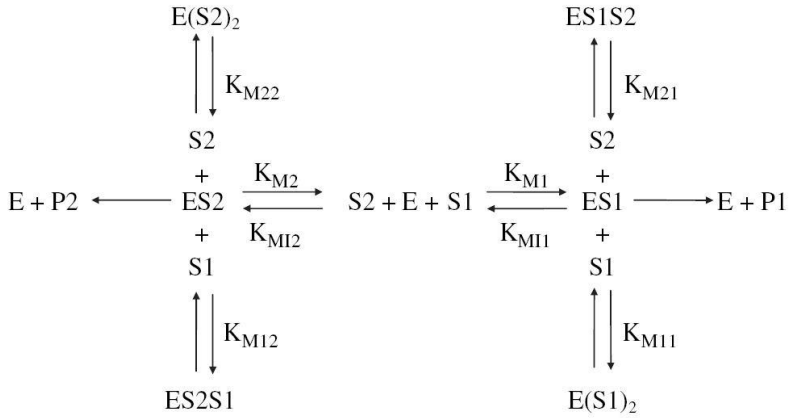

Figure 5. A general schematic for multiple mechanisms of enzyme inhibition during exposure to two substrates. E is the free enzyme; S1 and S2 are competing substrates for products P1 and P2; *K*M1 and *K*M2 are the substrate binding constants (they are the same as the inhibitory binding constants $_{KM1}$ and $_{KMI2}$). All constants are dissociation equilibrium constants. (Reddy et al., 2005).

Central to the successful operation of the PBPK model for pharmacokinetic interactions in the binary chemical mixture of DCE and TCE is the incorporation of the set of equations related to different types (competitive, noncompetitive, and uncompetitive) of enzyme inhibition into the liver compartment mass balance differential equation as shown below:

$$V_L \frac{dC_{L1}}{dt} = \frac{dAMT_{L1}}{dt} = (Q_L C_{a1}) - (Q_L C_{vL1}) - \frac{V_{\max 1} \cdot C_{vL1}}{K_m(T_1) + C_{vL1}(T_2)}$$ equation I

$$T_1 = 1 + \frac{C_{vL2}}{K_{M12}} + \frac{(C_{vL2})^2}{K_{M12} \cdot K_{M22}} + \frac{(C_{vL1})(C_{vL2})}{K_{M12} \cdot K_{M12}}$$ equation II

$$T_1 = 1 + \frac{C_{vL2}}{K_{M21}} + \frac{(C_{vL1})}{K_{M11}}$$ equation III

Since this set of equations is very important for the understanding of a large portion of publications involving PBPK modeling of chemical mixtures, it is essential to fully appreciate how these equations came about and what do they mean. Therefore, we will go through the conceptual and algebraic derivation of the above equations. Based on the general schematic for multiple enzyme inhibition during co-exposure to two substrates in Figure 5, we first write the rate equation for the product of interest (in this case, we concentrate on P1 and S1):

$$v = k_1[ES1]$$ equation V

Next, we write the conservation equation for enzyme,

$$E_{total} = E_{free} + ES1 + ES2 + ES1S2 + ES1S1 + ES2S1 + ES2S2$$ equation V

Then we solve the conservation equation above in terms of ES1, the factor of interest from the rate equation IV, according to the following steps:
We write all the equations for the equilibrium dissociation constants (Figure 5)

$$K_{M1} = \frac{(E_{free})(S1)}{(ES1)}$$ equation VI

$$K_{M21} = \frac{(ES1)(S2)}{(ES1S2)}$$ equation VII

$$K_{M11} = \frac{(ES1)(S1)}{(ES1S1)}$$ equation VIII

$$K_{M2} = \frac{(E_{free})(S2)}{(ES2)}$$ equation IX

$$K_{M12} = \frac{(ES2)(S1)}{(ES2S1)}$$ equation X

$$K_{M22} = \frac{(ES2)(S2)}{(ES2S2)}$$ equation XI

Rewriting Eqs. VI to VIII in terms of concentrations,

$$(E_{free}) = \frac{(ES1)K_{M1}}{(S1)}$$ equation XII

$$(ES1S2) = \frac{(ES1)(S2)}{(K_{M21})}$$ equation XIII

$$(ES1S1) = \frac{(ES1)(S1)}{(K_{M11})}$$ equation XIV

Recasting Eqs. IX to XI is a bit more involved, we start out with rearranging Eq. IX and substitute Eq.XII into this one:

$$(ES2) = \frac{(ES1)(K_{M1})(S2)}{(S1)(K_{M2})}$$ equation XV

Similarly, Eqs. X and XI become

$$(ES2S1) = \frac{(ES2)(S1)}{(K_{M12})}$$ equation XVI

$$(ES2S2) = \frac{(ES2)(S2)}{(K_{M22})}$$ equation XVII

Sustituting Eq. XV into Eqs. XVI and XVII completes the process of transforming all forms of enzyme and enzyme/substrate complexes in terms of ES1, we obtain:

$$(ES2S1) = \frac{(S1)(ES1)(K_{M1})(S2)}{(K_{M12})(S1)(K_{M2})}$$ equation IIXX

$$(ES2S2) = \frac{(S2)(ES1)(K_{M1})(S2)}{(K_{M22})(S1)(K_{M2})}$$ equation XX

Substituting all these various forms of enzyme and enzyme substrate complexes, we get:

$$E_{total} = E_{free} + ES1 + ES2 + ES1S2 + ES1S1 + ES2S1 + ES2S2$$
$$= \frac{(ES1)K_{M1}}{(S1)} + (ES1) + \frac{(ES1)(K_{M1})(S2)}{(S1)(K_{M2})} + \frac{(ES1)(S2)}{(K_{M21})} + \frac{(ES1)(S1)}{(K_{M11})}$$
$$+ \frac{(S1)(ES1)(K_{M1})(S2)}{(K_{M12})(S1)(K_{M2})} + \frac{(S2)(ES1)(K_{M1})(S2)}{(K_{M22})(S1)(K_{M2})}$$

Factor out (ES1)/S1):

$$E_{total} = \frac{(ES1)}{(S1)}\left[\begin{array}{l}(K_{M1}) + (S1) + \frac{(K_{M1})(S2)}{(K_{M2})} + \frac{(S1)(S2)}{(K_{M21})} + \frac{(S1)^2}{(K_{M11})} \\ + \frac{(S1)(K_{M1})(S2)}{(K_{M12})(K_{M2})} + \frac{(S2)^2(K_{M1})}{(K_{M22})(K_{M2})}\end{array}\right]$$

Grouping all the terms in the brackets in terms of S1 and K_{M1}:

$$E_{total} = \frac{(ES1)}{(S1)}\left[\begin{array}{l}(S1) + \frac{(S1)(S2)}{(K_{M21})} + \frac{(S1)^2}{(K_{M11})} + (K_{M1}) \\ + \frac{(K_{M1})(S2)}{(K_{M2})} + \frac{(K_{M1})(S2)^2}{(K_{M22})(K_{M2})} + \frac{(S1)(K_{M1})(S2)}{(K_{M12})(K_{M2})}\end{array}\right]$$

Now rewright blocking for S1 and K_{M1}:

$$E_{total} = \frac{(ES1)}{(S1)}\left[\begin{array}{l}(S1)\left(1 + \frac{(S2)}{(K_{M21})} + \frac{(S1)}{(K_{M11})}\right) \\ +(K_{M1})\left(1 + \frac{(S2)}{(K_{M2})} + \frac{(S2)^2}{(K_{M22})(K_{M2})} + \frac{(S1)(S2)}{(K_{M12})(K_{M2})}\right)\end{array}\right]$$

Let T_1 and T_2 be the terms, respectively, in the big parenthesis as shown below

$$T_1 = \left(1 + \frac{(S2)}{(K_{M2})} + \frac{(S2)^2}{(K_{M22})(K_{M2})} + \frac{(S1)(S2)}{(K_{M12})(K_{M2})}\right)$$
$$T_2 = \left(1 + \frac{(S2)}{(K_{M21})} + \frac{(S1)}{(K_{M11})}\right)$$

Then,

$$E_{total} = \frac{(ES1)}{(S1)}\left[S1(T_2) + K_{M1}(T2)\right]$$

Rearrange to solve for (ES1):

$$ES1 = \frac{E_{total}(S1)}{\left[S1(T_2) + K_{M1}(T2)\right]}$$

Sustituting into the original rate equation equation A.5.4-IV, we obtain:

$$v = k_1[ES1] = k1\frac{E_{total}(S1)}{\left[S1(T_2) + K_{M1}(T2)\right]}$$

Since $k1E_{total}$ is V_{max}, highest possible ratio, so

$$v = k1\frac{V_{\max} \times (S1)}{\left[S1(T_2) + K_{M1}(T2)\right]}$$ equation XX

Please note that we have successfully derived the last term in Eq. I and T_1 and T_2 come from the equations published in the Andersen et al. (1987) article. Note also that K_{M2} here equals to K_{M12} in the Andersen et al. (1987) paper.
The short statement below is the most important point to extract for the reader—the role of process—in establishing the sets of equations for this or any other interaction model based on enzyme inhibition.
It should be noted that it always follows this process: (1) Write the rate equation; (2) write the conservation equation; (3) derive dissociation constants; (4) solve conservation equation for enzyme species represented in the rate equation.

Even though there are many different mechanisms for enzyme inhibition and an excellent reference (Segal, 1975) is available, the final equation above carries, simplistically, the following three types of enzyme inhibition:
1. *Competitive Inhibition*. In Eq. XX when *T2*=1 and the inhibitor (in our case, the second substrate) is only affecting K_{M1}, competitive inhibition results. This type of inhibition includes the following scenarios as described in Segal (1975):
Substrate (S) and inhibitor (I, or a second substrate) compete for the same binding site.
S and I are mutually exclusive because of steric hinderance.
S and I share a common binding group on the enzyme.
The binding sites for S and I, though distinct, are overlapping.
The binding of I to a distinct inhibitor site causes a conformational change in the enzyme that distorts or masks the S binding site, or vice versa.
Noncompetitive Inhibition. In Eq. XX, when the inhibitor is affecting both K_{M1} and S1, noncompetitive inhibition results. In this type of inhibition, S and I (or a second substrate) are not mutually exclusive but ESI (in our case ES1S2) is catalytically inactive. In this case, S and I don't interfere with each other's binding, but the conformational change of the enzyme affect catalytic center (Segal, 1975). There are other variations of the above scenario.
Uncompetitive Inhibition. In Eq. XX, when the inhibitor is affecting only S1 (i.e., *T1*=1), uncompetitive inhibition results. In this type of inhibition, I (or a second substrate) only binds to the ES (in our case ES1) complex. When S binds, a conformational change of the enzyme occurs to unmask the I binding site. The resulting ESI (in our case ES 1S2) is catalytically inactive.
Other examples involving PBPK modeling of binary chemical mixtures included benzene and toluene (Purcell et al. 1990), mirex/phenobarbital/chlordecone and bromotrichloromethane (Thakore et al. 1991), ethanol and trichloroethylene (Sato et al. 1991), and toluene and m-xylene (Tardiff et al. 1993). These mixtures and their respective PBPK modeling have been reviewed previously by Krishnan et al. (1994a,b).
With the exception of two binary mixtures involving two drug pairs, 5-fluorouracil/sorivudine and triazolam/erythromycin (Ito et al. 1998; Kanamitsu et al. 2000), all of the other studies were on volatile organic solvents (VOCs). Since these VOC studies are quite similar to the ones already described above, we chose not to discuss further the individual publications.

Interaction Mechanisms: Enzyme Inactivation or "Mechanism-Based Inhibition"

We will specifically discuss the Ito et al. (1998) and Kanamitsu et al. (2000) articles on two cases of drug–drug interactions involving 5-fluorouracil/sorivudine and triazolam/erythromycin, because: (1) These studies are two of the relatively few PBPK modeling studies on drugs, and it is specifically on drug–drug interactions; (2) the interactions involved a unique mechanism which was implicated in at least 15 human fatalities in Japan; and (3) the PBPK modeling approach in these articles is somewhat limited involving mainly the liver with two related compartments: portal vein and systemic blood.

In 1993 in Japan, 15 patients with cancer and herpes zoster were treated with 5-fluorouracil (5-FU), an anticancer agent, and sorivudine, an antiviral drug, and died from 5-FU toxicity due to a drug–drug interaction. This drug interaction involved a key enzyme, dihydropyrimidine dehydrogenase (DPD), which is a rate-limiting enzyme in the metabolism of 5-FU. It turned out that sorivudine was converted by gut flora to 5-bromovinyluracil, which is then metabolically activated by DPD. The reactive species binds to DPD and renders the enzyme inactive irreversibly. This type of inactivation of the enzyme, unlike competitive or noncompetitive inhibition, is unique mechanistically. Several terms have been used to describe this unique mechanism: "mechanism-based inhibition," "mechanism-based inactivation," "enzyme-activated irreversible inhibition," "suicide inactivation," and "k_{cat} inhibition" has the following characteristics:
Preincubation time-dependent inhibition of the enzyme (time-dependence); No inhibition if cofactors necessary for producing the activated inhibitor are not present in the preincubation medium; Potentiation of the inhibition depending on the inhibitor concentration (saturation kinetics); Slower inactivation rate of the enzyme in the presence of substrate compared with its absence (substrate protection); Enzyme activity not recovered following gel filtration or dialysis (irreversibility); 1: 1 Stoichiometry of the inhibitor and the active site of the enzyme (stoichiometry of inactivation).

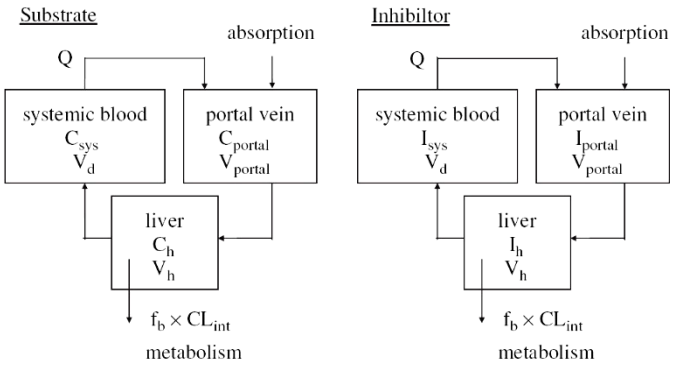

Figure 6. A conceptual physiological model for the time profiles of substrate and inhibitor concentrations in the plasma and liver. *Q* is the blood flow rate; $_{Csys}$ and $_{Isys}$ are substrate and inhibitor concentrations in systemic blood; *Vd* is the volume of distribution; *C*portal and $_{Cportal}$ are substrate and inhibitor concentrations in portal vein; $_{Vportal}$ is the volume of portal vein; *Ch* and *Ih* are substrate and inhibitor concentrations in the liver; *Vh* is the volume of the liver; *fb* is the unbound fraction in blood; $_{CLint}$ is the intrinsic metabolic clearance (Reddy et al., 2005).

Ito et al. (1998) also constructed a physiological model for the general "mechanism based inhibition" of a number of CYP isozymes. This model, shown in Figure 6 consists of three compartments: The liver, systemic blood and portal vein. The substrate (S) and inhibitor (I) share the same model structure. The mass balance equations are as follows:

For the substrate:

$$V_h \times \left(\frac{dC_h}{dt} \right) = Q \times C_{portal} - Q \times \frac{C_h}{K_p} - f_b \times CL_{\text{int}} \times \frac{C_h}{K_p}$$

$$CL_{\text{int}} = \frac{V_{\max}}{K_m + f_b \times \frac{C_h}{K_p}}$$

$$V_{\max} = V_{\max}(0) \times \frac{E_{act}(t)}{E_0}$$

$$V_{portal} \times \left(\frac{dC_{portal}}{dt} \right) = Q \times C_{sys} + V_{abs} - Q \times C_{portal}$$

$$V_{abs} = k_0 \times D \times F \times e^{-k_a \times t}$$

$$V_d \times \left(\frac{dC_{sys}}{dt} \right) = Q \times \frac{C_h}{K_p} - Q \times C_{sys}$$

For inhibitor:

$$V_h \times \left(\frac{dI_h}{dt} \right) = Q \times I_{portal} - Q \times \frac{I_h}{K_p} - f_b \times CL_{\text{int}} \times \frac{I_h}{K_p}$$

$$CL_{\text{int}} = \frac{V_{\max}}{K_m + f_b \times \frac{I_h}{K_p}}$$

$$V_{portal} \times \left(\frac{dI_{portal}}{dt} \right) = Q \times I_{sys} + V_{abs} - Q \times I_{portal}$$

$$V_{abs} = k_a \times D \times F \times e^{-k_a \times t}$$

$$V_d \times \left(\frac{dI_{sys}}{dt} \right) = Q \times \frac{I_h}{K_p} - Q \times I_{sys}$$

where Q represents blood flow rate, C_{sys} and I_{sys} represent concentrations of substrate and inhibitors, respectively, in systemic blood, V_d represents the volume of distribution in the systemic blood compartment, C_{portal} and I_{portal} represent concentrations of substrate and inhibitor, respectively, in the portal vein, D represents dose, V_{portal} represents the volume of portal vein, C_h and I_h represent concentrations of substrate and inhibitor, respectively, in the liver, V_h represents the volume of the liver, f_b represents the unbound fraction in blood, CL_{int} represents the intrinsic metabolic clearance, F_a (although the original article did not clarify this, F in the above equations really should have been F_a) represents the fraction absorbed from the gastrointestinal tract, K_m represents the Michaelis constant for the metabolic elimination, V_{max} represents the maximum metabolic rate, and K_p represents the liver-to-blood concentration ratio.

For active and inactive enzymes in the liver, Ito et al. (1998) provided the following differential equations:

$$\left(\frac{dE_{act}}{dt} \right) = -\frac{k_{inact} \times E_{act} \times f_b \times \frac{I_h}{K_p}}{k_{i,app} + f_b \times \frac{I_h}{K_p}} + k_{\text{deg}} \times (E_0 - E_{act})$$

$$\left(\frac{dE_{inact}}{dt}\right) = -\frac{k_{inact} \times E_{act} \times f_b \times \frac{I_h}{K_p}}{k_{i,app} + f_b \times \frac{I_h}{K_p}} + k_{\deg} \times E_{inact}$$

Ito et al. (1998) further defined that k_{deg} is the degradation rate constant (i.e., turn-over rate constant) of the enzyme. The initial conditions at t=0 are $E_{act} = E_0$ and $E_{inact}=0$. In the absence of an inhibitor, the enzyme level in the liver is at a steady state and the degradation rate ($k_{deg}E_0$) is equal to the synthesis rate, which is assumed to be unaffected by an inhibitor.

Subsequently, the same research group reported a similar "mechanism-based inhibition" of CYP3A4 by macrolide antibiotics, erythromycin, in a drug–drug inter-action with triazolam (Kanamitsu et al. 2000). These investigators used the above three-compartment physiological model and obtained quantitative predictions of the erythromycin/triazolam interaction. The predicted increase in triazolam AUC following erythromycin pretreatment was 2.0-fold (from 61.0 to 119nM·hr) and 2.6-fold (from 61.0 to 156 nM·hr) from model simulation. These model predictions were very close to the actual observed value, in vivo in human, of 2.1-fold increase (from 58.6 to 121 nM·hr) reported in the literature.

It should be noted here that a similar "suicide inhibition" phenomenon (i.e., an enzyme biotransforms a substrate to a reactive species which, in turn, "kills" the enzyme - a "suicide" from the perspective of the enzyme) had also been observed in CYP2E1 catalyzed metabolism of some volatile oraganic solvents such as cis and trans DCE (Andersen et al. 1987; Lilly et al. 1998).

PBPD Modeling of Binary Mixtures

Since the emphasis is on PBPK modeling and PBPK modeling of chemical mixtures is still at its infancy, we choose to only briefly discuss PBPD modeling by concentrating on two published examples on binary mixtures. For more details on the basic principles of PBPD modeling and specific information on the two case studies discussed below, the readers are encouraged to consult, respectively, El-Masri et al. (1996a,c). One of our earlier examples was the PBPK/PD modeling of a toxicological interaction between Kepone (also known as chlordecone) and carbon tetrachloride (CCl4) based on mechanisms of interactive toxicity and the application of computer technology in acute toxicity studies. Briefly, CCl4 is a well-known hepatotoxin. Following free radical formation through the P450 enzyme system, the toxicity of CCl4 can be an accumulation of lipids (steatosis, fatty liver) and degenerative processes leading to cell death (necrosis). Kepone is found in the environment as a result of photolytic oxidation of mirex, a pesticide used for the control of fire ants, or as a pollutant from careless and irresponsible discharge. At relatively low levels (e.g., 10ppm in the diet), even repeated dosing of Kepone in the diet up to 15 days caused no apparent toxicity to the liver. The toxicological interaction between Kepone and CCl4 was elucidated to be the impairment, by Kepone, of the liver's regeneration process. These mechanistic studies were summarized in a number of publications (Mehendale 1984, 1991, 1994).

El-Masri et al. (1996a) constructed a PBPD model based on the mechanism of toxicological interaction between Kepone and CCl4. This PBPD model was verified by literature information, and it was capable of providing time-course computer simulations of mitotic, injured, and pyknotic (dead) cells after treatment with CCl4 alone or with Kepone pretreatment. This PBPD model was further linked with Monte Carlo simulation to predict the acute lethality of CCl4 alone and in combination with Kepone. The second case study involved PBPK/PD modeling of pharmacodynamic interactions between trichloroethylene (TCE) and 1,1-dichloroethylene (DCE) regarding their binding and depletion of hepatic glutathione (GSH) in relation to the intrinsic hepatic GSH systhesis (El-Masri et al. 1996c). A PBPK/PD model was used to identify critical time point at which hepatic GSH is at a minimum in response to both chemicals. PBPK models for interactions leading to depletion of hepatic glutathione had been developed by several investigators (D'Souza et al. 1988; Frederick et al. 1992). Model-directed gas uptake experiments with DCE revealed that DCE was the only chemical capable of significantly depleting hepatic GSH. TCE exposure higher than

100 ppm to the rats obstructed the ability of DCE to deplete hepatic GSH, indicating metabolic competitive inhibition of DCE biotransformation to reactive metabolites. TCE exposure lower than 100 ppm was ineffective in inhibiting DCE from significantly depleting hepatic GSH. El-Masri et al. (1996c) further applied these quantitative analyses in establishing an "interaction threshold" between TCE and DCE.

PBPK Modeling of Higher-Order Mixtures

In the above section, we provided a glimpse of the development of chemical mixture toxicology in the "early days." As the field of PBPK modeling grows in parallel with the science of toxicology, the natural progression proceeded in two directions. First, when the toxicology of chemical mixture moves from descriptive work to mechanistic-based research, PBPK modeling transforms into PBPD modeling (discussed above). Second, investigators, driven by innate curiosity and practical need, begin to explore PBPK modeling of more and more complex chemical mixtures. Thus, in the next few sections, we will provide a few examples that reflect the application of PBPK modeling to the toxicology of more complex chemical mixtures.

PBPK Modeling of Ternary and Four-Chemical Mixtures

Pioneering efforts in the PBPK modeling of more complex chemical mixtures were from a research group led by Krishnan and various colleagues; two comprehensive reviews of work up to 1994 are available (Krishnan et al. 1994a,b). Earlier work from this group concentrated on interactions and PBPK modeling between two chemicals (Tardif et al. 1993, 1995; Pelekis and Krishnan 1997). As progress was made, these investigators began to build up the mixtures and devoted their effort to PBPK modeling of more and more complex chemical mixtures (Tardif et al. 1997; Haddad et al. 1999a,b, 2000). PBPK modeling of a ternary mixture on toluene, m- xylene, and ethylbenzene was studied and reported by Tardif et al. (1997), and the mechanism involved was competitive inhibition. The details of the conceptual interactive PBPK model and the equations with incorporation of competitive inhibition are provided below under the section for five-chemical mixture. Subsequently, Haddad et al. (1999a) applied this interactive PBPK model to the calculation of biological hazard index (BHI). BHI, defined as the biological level tolerable for exposure to mixtures, is traditionally calculated in an analogous way as the hazard index under additivity assumption (Ogata et al. 1993; Hadded et al. 1999a). However, Haddad et al. incorporated toxicological interaction by using PBPK modeling to obtain "simulation concentration" (SC) and modified the BHI calculation according to the following equation:

$$BHI = \sum_{i=1}^{n} \frac{SC_i}{BEI_i}$$

Where BHI and SC were defined before; BEI refers to the concentration or excretion rate of a biomarker in a healthy worker exposed to TLV. In doing so, Haddad et al. (1999a) applied interactive PBPK modeling of a chemical mixture into the risk assessment process. Using the same principle and similar technique, researchers at Colorado State University studied PBPK modeling of two ternary mixtures [trichloroethylene (TCE), tetrachloroethylene (PERC), methyl chloroform (MC) and toluene, ethyl-benzene, and xylenes] to respectively enhance the concept of "interaction thresh-olds" and modify and improve the "Mixture Formula" risk assessment by using an interactive PBPK modeling approach (Dobrev et al. 2001, 2002; Dennison et al. 2005a).

Haddad et al. (1999b) also studied the PBPK modeling of a four-chemical mixture involving benzene, toluene, ethylbenzene, and m-xylene. In general, the incorporation of the interaction mechanism, at the level of the liver metabolic enzyme inhibition, is similar to those described above for binary and ternary mixtures, albeit a bit more complicated. We will demonstrate again the principle and techniques involved in a later section on a five-chemical mixture.

The Concept of the "Interaction Threshold"

In 1996, El-Masri et al. introduced the idea of "interaction thresholds" as the minimal level of change in tissue dosimetry of two or more chemicals associated with a significant health effect (El-Masri et al. 1996b). When two or more interactive chemicals are studied together, theoretically there could be infinite interaction thresholds. However, if we specify certain occupational or environmental exposure concentrations for all the other component chemicals in the mixture except one, we may obtain an interaction threshold for that set of exposure conditions. This definition is important because human risk from exposure to multiple chemicals may not always obey the rule of additivity. Dobrev et al. (2001) estimated the interaction thresholds of three common volatile organic solvents—TCE, PERC, and MC—under different dosing conditions. First, an interactive PBPK model was built where PERC and MC were competitive inhibitors for TCE, the compound most extensively metabolized among the three. The model was developed and validated by gas uptake pharmacokinetic studies in Fischer 344 rats at relatively high doses of single chemicals, binary mixtures, and the ternary mixture. Using computer simulation to extrapolate from high to low concentrations, Dobrev et al. (2001) investigated the toxicological interactions at occupational exposure levels, specifically at around threshold limit value/time-weighted average (TLV/TWA). Since long-term toxicity/carcinogenicity of these three solvents is clearly associated with their metabolism, and TCE is the most extensively metabolized among them, this study focused on changes in internal TCE dose measures related to the mixture co-exposure. Using a 10% elevation in parent compound blood level as a criterion for significant interaction, interaction thresholds were estimated with two of the three chemicals held at constant concentrations. Under the above exposure conditions (i.e., TCE and PERC at their TLVs but varying MC concentrations), the interaction threshold for the ternary mixture was 50, 130, and 25 ppm for TCE, MC, and PERC, respectively. This work was later extended, using computer simulation (i.e., in silico toxicology), to human exposure to this three-chemical mixture and the estimation of interaction thresholds for humans (Dobrev et al. 2002). Increases in the TCE blood levels led to higher availability of the parent compound for glutathione conjugation, a metabolic pathway associated with kidney toxicity/carcinogenicity. The simulated change in production rates of toxic conjugative metabolites exceeded 17% for a corresponding 10% increase in TCE blood concentration, indicating a nonlinear risk increase due to combined exposures to TCE. This study (Dobrev et al. 2002) and the related discussion above reveal that evaluation of metabolic interactions and their thresholds illustrates a unique application of PBPK modeling in risk assessment of occupational exposures to chemical mixtures. It further underscores the importance of incorporating PBPK modeling into the cumulative risk assessment process.

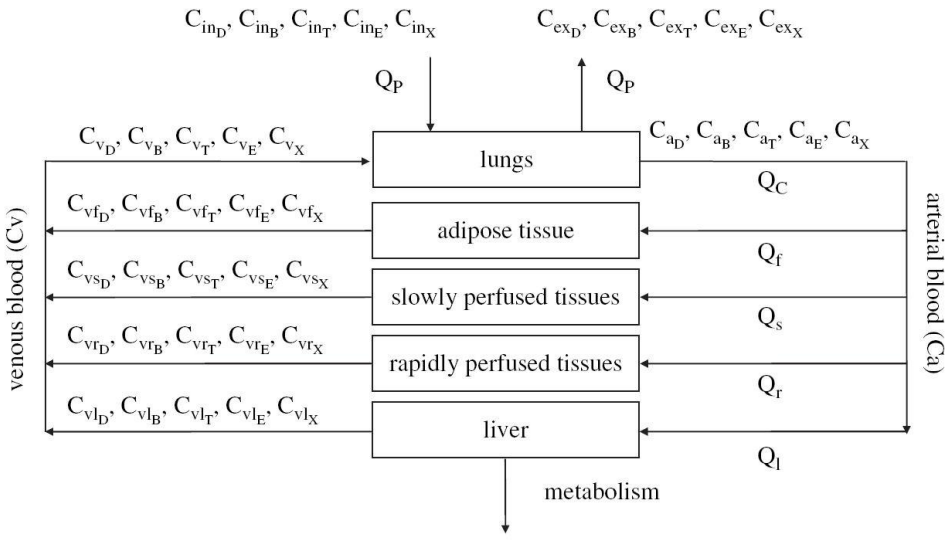

Figure 7. Conceptual interactive PBPK model for a five-component chemical mixture. D, dichloromethane; B, benzene; T, toluene; E, ethylbenzene; X, *m*-xylene. Pharmacokinetic interactions occur in the liver as competitive enzyme inhibition. *QP* is alveolar ventilation rate; *QC* is cardiac output; *Cv* and *Ca* are venous and arterial blood

concentrations of individual component chemicals; *Cvi* is venous blood concentration leaving tissue compartments; *Qi* is blood flow to tissues (f, adipose tissue; s, slowly perfused tissues; r, rapidly perfused tissues; l, liver); *C*in and *C*ex are inhaled or exhaled concentrations of individual component chemicals. (Reddy et al., 2005).

Development of a PBPK Model for Chemical Mixtures Involving Multiple Components

An interactive model for 5 chemicals (benzene, toluene, ethylbenzene, m-xylene, and dichloromethane) has also been completed (Haddad et al. 2000; Krishnan et al. 2002), leading to the idea that pharmacokinetic interactions of complex chemical mixtures, regardless of the number of components, may be predicted based on the PBPK modeling of binary mixtures of the component chemicals (Haddad et al. 2000; Krishnan et al. 2002). Since, to date, their studies represent the most complex inter-active PBPK model for a mixture with five reconstituted chemicals, we will provide more detailed discussion below. Also, their concept of predicting the kinetics of complex mixtures based on PBPK studies of binary mixture is interesting and revolutionary, and it warrants further discussion.

As shown in Figure 7, the conceptual PBPK model of the five-chemical mixture (Haddad et al. 2000) is similar to the styrene model in Figure 4; the only difference is the incorporation of the individual chemical concentrations in the arterial and venous blood as designated by subscripts D, B, T, E, X, representing the five chemicals. Because the mechanism of interaction was determined to be competitive enzyme inhibition of CYP2E1, the following Michaelis–Menten equations were incorporated into the mass balance differential equations in the liver compartment for the respective chemicals: benzene (B), toluene (T), ethylbenzene (E), m - xylene (X), and dichloromethane (D). RAM here represents rate of metabolism or velocity of the enzyme reaction.

$$RAM_B = \frac{V_{\max B} \times C_{vlB}}{K_{mB}\left(1 + \frac{C_{vlE}}{K_{iEB}} + \frac{C_{vlT}}{K_{iTB}} + \frac{C_{vlX}}{K_{iXB}} + \frac{C_{vlD}}{K_{iDB}}\right) + C_{vlB}}$$

$$RAM_T = \frac{V_{\max T} \times C_{vlT}}{K_{mT}\left(1 + \frac{C_{vlB}}{K_{iET}} + \frac{C_{vlE}}{K_{iET}} + \frac{C_{vlX}}{K_{iXT}} + \frac{C_{vlD}}{K_{iDT}}\right) + C_{vlT}}$$

$$RAM_E = \frac{V_{\max E} \times C_{vlE}}{K_{mE}\left(1 + \frac{C_{vlB}}{K_{iBE}} + \frac{C_{vlE}}{K_{iTE}} + \frac{C_{vlX}}{K_{iXE}} + \frac{C_{vlD}}{K_{iDE}}\right) + C_{vlE}}$$

$$RAM_X = \frac{V_{\max X} \times C_{vlX}}{K_{mX}\left(1 + \frac{C_{vlB}}{K_{iBX}} + \frac{C_{vlE}}{K_{iEX}} + \frac{C_{vlT}}{K_{iTX}} + \frac{C_{vlD}}{K_{iDX}}\right) + C_{vlX}}$$

$$RAM_D = \frac{V_{\max D} \times C_{vlD}}{K_{mD}\left(1 + \frac{C_{vlB}}{K_{iBD}} + \frac{C_{vlE}}{K_{iED}} + \frac{C_{vlT}}{K_{iTD}} + \frac{C_{vlX}}{K_{iXD}}\right) + C_{vlD}}$$

Experimentally, Haddad et al. (2000) did PBPK model simulations of the pharmacokinetics of components under two scenarios: (1) when one of the mixture components was substituted with another such as benzene in the BETX mixture was substituted with dichloromethane and (2) when another chemical was added to an existing four-chemical mixture model such as dichloromethane was added to the existing BTEX model. These investigators also did pharmacokinetic studies and specifically obtained blood kinetic data in rats on all the new binary mixtures with D added; thus, animal experimental data were generated on D-B, D-E, D-T, D-X binary mixtures. When competitive inhibition was incorporated into the interactive

PBPK model, the model simulation not only matched the newly obtained binary mixture kinetic data, but also matched earlier data for a variety more complex mixtures beyond binary combinations. Therefore, from their studies emerged the concept that predictability of pharmacokinetic and pharmacodynamic consequences of chemicals in more complex chemical mixtures is possible as long as there is the availability of quantitative data in the literature on binary chemical interactions (Haddad et al. 2000; Krishnan et al. 2002). So far their approach (Haddad et al. 2000; Krishnan et al. 2002) has worked for the volatile organic chemicals that they studied. Whether or not this concept has a broader application to mixed classes of chemicals in a mixture remained to be evaluated.

PBPK Modeling of Complex Chemical Mixtures.

As PBPK modeling of chemical mixtures progresses to involving more and more components, it is a natural course of development that investigators will attempt to tackle the real-world complex chemical mixtures. Verhaar et al. (1997) proposed the incorporation of lumping analyses (a chemical engineering technique used in petroleum engineering processes) and QSAR to PBPK modeling. The idea was that each of the three techniques would serve its unique function in the overall goal of predicting some aspects of the chemical mixtures of interest. Thus, QSAR analysis can be used to predict needed physicochemical and toxicological parameters for unknown compounds or for surrogate compounds (from lumping); lumping analysis can drastically reduce the complexity of the description of a mixture; and PBPK/PD modeling can be used to describe the pharmacokinetics, and possibly pharmacodynamics, of an ensemble of compounds or lumped pseudocompounds, including possible interaction effects. A detailed statistical/mathematical treatment on how to minimize errors in lumping was given in an appendix in this article. Verhaar et al. (1997) specifically suggested the application of these technologies (i.e., QSAR, lumping analysis, PBPK/PD modeling) to JP-5.

These ideas have now been applied with gasoline as the complex mixture (Dennison et al. 2003, 2004a,b, 2005b; Dennison 2004) developing both the PBPK modeling framework and lumping approach. Experimental work involved gas uptake pharmacokinetic studies in male Fischer 344 rats of single, multiple selected target components (benzene, toluene, ethylbenzene, o-xylene, and n-hexane), and two blends (summer and winter) of gasoline, as well as the volatile fractions of the gasoline. The target components were selected based on the prevalence and toxicological importance; the remainder of the hundreds of component chemicals were lumped into a pseudo chemical (Dennison et al. 2003; Dennison 2004). Technological development necessary for this effort included (1) modification of the gas uptake pharmacokinetic chamber system for more efficient incorporation of probes and (2) utilization of more efficient CO_2 absorbent. The PBPK model tracks selected target components and a lumped chemical group representing all nontarget components (Dennison et al. 2004a,b, 2005b; Dennison 2004). Competitive inhibition was the principal mechanism of pharmacokinetic interactions among these five selected target single chemicals and a pseudochemical from the lumped components. Computer simulation results from the six-chemical interaction model matched well with gas uptake pharmacokinetic experimental data from single chemicals, five-chemical mixture, and the two blends of gasoline (Dennison et al. 2003). Thus, for the first time, we have a PBPK model for a real-world complex chemical mixture.

Although the problems and tasks associated with finding a reasonable way to handle, and eventually predict, adverse health effects due to chemical mixtures are complex, human health problems are really the final manifestation of dynamic equilibria of multiple stressors of which chemical mixtures are but one element. Thus, the potential combinations of these multiple stressors may approach infinity. In order to circumvent the study of astronomically large number of combinations, the only logical way is to concentrate on the finite system—in this case, the human body. Dr. Craig Venter of the human genome fame stated: "...If we hope to understand biology, instead of looking at one little protein at a time, which is not how biology works, we will need to understand the integration of thousands of proteins in a dynamically changing environment. A computer will be the biologist's number one tool. . ." (Butler 1999). In line with this thinking, we believe that the only efficient way of studying chemical mixtures or multiple stressors is to understand our body in an integrated manner through biologically based computer simulation such as PBPK/PD modeling and very focused experimentation. In essence, this emphasis leads to a systems biology approach with tools from in silico toxicology.

In silico toxicology, by our defintion, means integrating computer modeling with focused, mechanistic, animal experimentation such that experiments which are impractical (e.g., too large, too expensive) or impossible (e.g., human experiments with carcinogens) to perform may be conducted by computer simulation. With this type of approach, once we have a "virtual human" in place, multiple stressors and their integrated adverse human health effects are nothing more than the perturbation of certain processes in the normal systems. In that sense, adverse health effects are therefore the manifestation of parameter changes in the computer simulation of the "virtual human." The classical compartmental pharmacokinetic models may be considered an embryonic form of "Virtual Human" (Yang et al. 2004b). Indeed, despite the crude nature of these models as compared with the human body, classical pharmacokinetics has contributed very significantly to the field of medicine. The advancement of PBPK modeling, as a result of "delumping" and the incorporation of physiology into the modeling process, results in a better "Virtual Human."

Utilization of computer modeling is essential in the studies of toxicology of chemicals, chemical mixtures, and multiple stressors. Biology will be well-served by the application of computer technology as an alternative research method to conserve resources and minimize the use of laboratory animals. In the past few years, tools such as "Reaction Network Modeling" and "Gene Network Modeling" have become available to support computer simulation at the molecular interaction level. For more information on the specifics of these newer modeling approaches in biomedical sciences, the readers are referred to a number of publications (Andersen et al. 2002; Klein et al. 2002; Liao et al. 2002; Reisfeld and Yang 2004; Liao 2004). Looking into the future, linkage of PBPK modeling with "Reaction Network Modeling" and/or "Gene Network Modeling" has the potential of providing a computer simulation platform for modeling complex biological systems from the whole organism down to the molecular interaction level.

Biomarkers integration over multiple routes of exposure to multiple stressors

The use of biomarkers in risk assessment

The aim of risk assessments is to provide society with estimates of the likelihood of illnesses and injury as a consequence of exposure to various hazards. Risk assessments are needed when social policy decisions are in dispute, when the health consequences of alternative policies in question are not subject to direct measurement (at least in a timely fashion), and when the scientific analysis of a hazard is not complete (Hattis and Silver, 1993). The assessment procedure involves the development of an exposure-response curve for the target species (e.g., humans), based on animal and human information, followed by the projection of the curves to estimate levels of exposure that may be considered safe (NRC, 1987). For risk assessments to be useful they should lead to projections that are close to the true risks. A strong scientific basis for conducting risk assessments is the best way to assure that projections are close to true risks or at least provide an honest depiction of the state of knowledge and the degree of certainty about risks (Bailar and Bailer, 1999). Risk assessment can be a qualitative or quantitative exercise at the individual or group (population) level. The term quantitative risk assessment (QRA) has been used to describe the response associated with a specific level of exposure (Bailer and Dankovic, 1997). The availability of adequate dose/concentration response data is a prerequisite to conducting a QRA.

A biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease (IPCS, 2001). IPCS (2001) has produced a very thorough overview of the use of biomarkers in risk assessment classifying them into markers of exposure, effect and susceptibility. If biomarkers are to contribute to environmental and occupational health risk assessments, they have to be relevant and valid. Relevance refers to the appropriateness of biomarkers to provide information on questions of interest and importance to public and environmental health authorities and other decision-makers. The use of relevant biomarkers allows decision-makers to answer important public health questions by being used in research or risk assessments in a way that contributes useful information that cannot be obtained better by other approaches, such as questionnaires, environmental measurements or record reviews. For example, chronic exposure to organochlorines is better indicated by serum organochlorine levels than by market-basket studies or industrial hygiene measurements, and early kidney damage may be better indicated by a battery of urinary biomarkers than by morbidity records. Relevance also pertains to whether the questions on which a biomarker can provide information are important questions; not merely ones that can be answered, but ones that should be answered (Muscat, 1996). Thus, the ability to measure a biomarker after exposure to a toxicant may not be as important a question as whether individuals with exposure to the toxicant are at increased risk of disease

The second characteristic of potentially useful biomarkers is validity. Validity of biomarkers has been widely discussed (Hemberg and Aitio, 1987; Schatzkin et al., 1990; Schulte and Perera, 1993; Boffetta, 1995; Bernard, 1995; Dor et al., 1999). It includes both laboratory and epidemiological aspects. Validity refers to a range of characteristics that is the best approximation of the truth or falsehood of a biomarker. It is a sense of degree rather than an all or-none state. The validity of a biomarker is a function of intrinsic qualities of the biomarker and characteristics of the analytic procedures (Dor et al., 1999) (see Tables 3 and 4 for an example of this distinction).

Additionally, three broad categories of validity can be distinguished: measurement validity, internal study validity and external validity (Schulte and Perera, 1993). Measurement validity (in terms of analytical chemistry, accuracy) is the degree to which a biomarker indicates what it purports to indicate. Internal study validity is the degree to which inferences drawn from a study actually pertain to study subjects and are true. External validity is the extent to which findings of a study can be generalized to apply to other populations. The use of invalid biomarkers can lead to invalid inferences and generalizations and ultimately to erroneous risk assessments.

Table 3. Factors affecting the validity and feasibility of biomarker studies: analytical procedures (Dor et al., 1999)

Sampling constraints (for example, timing requirements)
Number of samples necessary for an acceptable precision
Degree of invasiveness of the sampling procedure
Availability of storage methods after the sample is taken (to avoid the need for immediate analysis)
Controlling or reducing the contamination of the sample when it is taken and when it is manipulated in the laboratory
Simplicity, possibility of routine usage, and speed of the procedure
Trueness, precision and sensitivity
Specificity for the component to be detected: interference must be identified to avoid misinterpretation
Standardization of the procedure

Table 4. Factors affecting the validity of biomarkers: intrinsic characteristics of the biomarker (Dor et al., 1990)

Significance: exposure, effect, individual susceptibility
Specificity in relation to the pollutant or pollutant family
Sensitivity: capacity to distinguish populations with different exposure levels, susceptibilities or degrees of effect
Knowledge of its background in the general population
Existence of dose-response curves between exposure level and marker concentration
Estimation of the inter- and intra-individual variability
Knowledge of confounding factors that can affect marker

Although biomarkers have a long history in medicine and public health, the systematic development, validation and application of biomarkers is a relatively new field in environmental health (Shugart et al., 1992; Anderson et al., 1994), except for biological monitoring in occupational health (Hemberg and Aitio, 1987). When used in risk assessment, information from biological markers may replace default assumptions when specific information regarding exposure, absorption and toxicokinetics is unavailable or limited (Table 5) (Ponce et al., 1998). Although examples of how this biomarker information can be used are limited, a general framework can be adduced. Quantitative evaluations of the utility of biomarker information in risk assessment are rare (Bois et al., 1995; Ponce et al., 1998).

One compelling example of the use of susceptibility markers is the work of El-Masri et al. (1999). They investigated how changing glutathione-S-transferase theta (GSTTI) genotype frequencies would impact cancer risk estimates from dichloromethane by the application of Monte-Carlo simulation methods in combination with physiologically based pharmacokinetic (PBPK) models. They reported that average and median risk estimates were 23% to 30% higher when GSTTI polymorphism was not included in the models. This analysis was a major factor in the permissible exposure levels promulgated by the US Occupational Safety and Health Administration (OSHA, 1998). Goldstein (1996) has identified two important impediments to the development of biomarkers of value to risk assessment. The first is the over-reliance on mathematical models to the exclusion of monitoring data. This occurs because regulators have a need to make a decision and, for expedience, use models until better approaches are developed. However, once locked into a regulation, the existence of the model serves as a major inhibition to the development of more reliable methods of indicating exposure and effect, including biomarkers. The second is that ethical review boards may find it difficult to sanction research where participants are exposed in a scientific study to levels they are exposed to in the general environment or at work because the participation in the study is voluntary while the latter is generally involuntary.

The concepts and principles supporting the use of biomarkers in the assessment of human health risks from exposure to chemicals have been reviewed by the International Programme on Chemical Safety (IPCS, 1993). The IPCS has produced the concise guidelines for the monitoring of genotoxic effects of carcinogens in humans (Albertini et al., 2000). It has also issued monographs on the methodology for the assessment of human health risks in a wider context, which includes the use of biomarkers (IPCS, 1994, 1999).

Table 5. Use of biomarkers to refine risk assessment information (Ponce et al., 1998)

Variable	Use of biomarkers
Exposure	Establish exposure characteristics • Route of exposure • Peak of exposure • Rotal exposure
	Estimate cumulative exposure
Absorption	Establish absorption factors Inhalation Dermal exposure Ingestion
	Identify factors that influence absorption
	Identify interspecies differences
	Identify sensitive population characteristics
Toxicokinetics	Establish distribution kinetics
	Establish half-life in blood or body
	Identify interspecies differences
	Identify factors that influence distribution, metabolism or excretion
	Estimate cumulative exposure
	Estimate peak exposure variables • Time • Concentration
	Identify sensitive population characteristics
	Identify mechanism of toxicity at target organ
Toxicodynamics	Identify mechanism of toxicity at target organ
	Establish target organ potency
	Identify sensitive population characteristics
	Identify factors that influence target organ toxicity
	Identify interspecies differences

Hazard identification

Historically, hazard identification has been the driving force in risk assessment (McClellan, 1999). Various national and international organizations have recognized the role human biological markers of exposure and effect can play in the hazard identification step: both make use of such data in classifying carcinogens. Like other classic measures of exposure, there are limitations to the use of exposure biomarkers in epidemiological research for hazard identification (Schulte & Perera., 1993; Pearce et al., 1995). The major limitation is the general inability of biomarkers (with some exceptions) to indicate historic exposures. Additionally, their strength in integrating routes of exposure also may be a weakness by introducing confounding due to source, as full use of exposure biomarkers may also require understanding of those inherited and acquired factors that influence the level of exposure biomarkers (Vineis et al., 1990). In determining whether a xenobiotic is hazardous, biomarkers may yield a more accurate determination than approaches based on less sensitive measures of exposures (e.g., job titles, as exposure proxies). In situations where exposures occurred that were variable or intermittent and the effect of exposure is integrated, biomarkers that represent accumulation of exposure, such as hemoglobin adducts, might be useful (Perera, 1995). A biomarker approach may allow for clarifying exposure outcome relationships better than with classical methods, due to reduction in exposure measurement error.

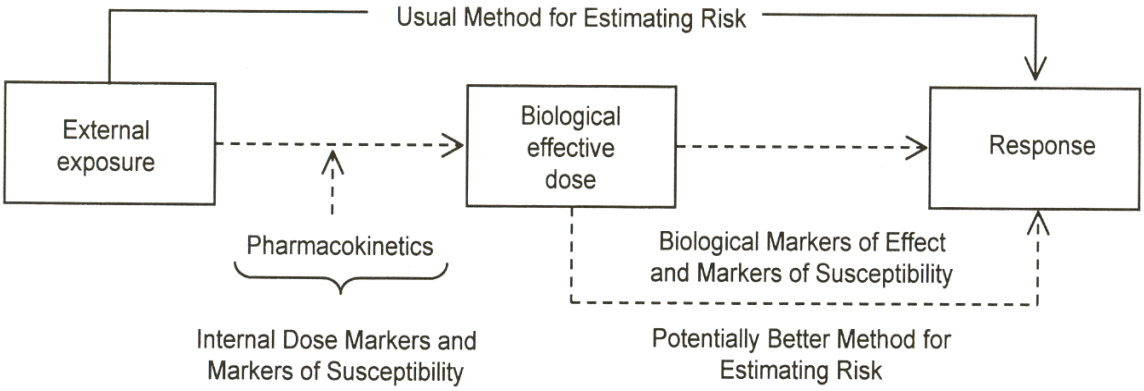

Figure 8. General approach for Risk estimation (IPCS, 2001)

The basic rationale for using exposure biomarkers is that they could provide, in some cases, a more accurate method for assessing exposure and, ultimately, risk (Figure 8) (Schulte & Waters, 1999). While use of biomarkers can reduce mis-classification, it is also possible that measurement error in the biomarker may contribute to bias in the measure of association (White, 1997; Saracci, 1997). Such error can be evaluated and its impact adjusted for, but, on balance, it is better to avoid or minimize it with good laboratory and epidemiological practices. The va1ue of biological markers in epidemiological studies that will be useful in the hazard identification step of risk assessment depends on the quality of the design and analysis of the studies.

Various reviews of biomonitoring and molecular epidemiological studies have been conducted and in some instances have found that a better design or analysis could have been applied (Bonassi et al., 1994; IARC, 1997). In 1994, Bonassi et al. reviewed three years of biomonitoring studies and found that only 5% of those studies adopted the best statistica1 techniques available. The major recommendations were to focus more on point estimates and confidence intervals instead of significant tests, utilize appropriate multivariate techniques and pay more attention to adjustment for confounding and evaluation of possible interaction between factors.

Exposure assessment

Exposure assessment has generally been considered the weak link in hazard evaluation and risk assessment (Dary et al., 1996). The exposure assessment component of risk assessment includes consideration of such issues as representativeness of exposure measurements for a population, differences in exposures within and between individuals, individual differences in uptake and biotransformation, identification of factors that control or modify exposures, exposure estimation methods applicable in the absence of direct measurements, and identification of the most relevant dose metric (the most re1evant measure of dose) for the agent under consideration (Schulte & Waters, 1999). The use of biomarkers in assessing exposure for risk assessment increasingly may include consideration of susceptibility factors in conjunction with exposure factors (gene-environment interaction), such as the presence of a specific genetic polymorphism for a metabolic enzyme (Bois et al., 1995; D'Errico et al., 1996). Such genetic differences may account for some interindividual variability in exposure markers.

Quite often epidemiological studies utilize exposure surrogates rather than direct measurement of exposures. For environmental studies, surrogates might include geographical location such as residence for a drinking-water or air pollution study, age of housing in studies of lead-based paint exposures, or proximity of residence to e1ectrical power 1ines. Occupationa1 studies use surrogates such as job title or job group, years worked at a plant, pounds of pesticide applied per week, and tasks performed when direct measurements are not available or are limited (Stewart et al., 1991; Go1dberg & Hemon, 1993). The use of quantified direct measurements of personal exposures can lower uncertainty in the risk

assessment process considerably compared to the use of such exposure surrogates (Schulte & Waters, 1999). Biomarkers may serve to evaluate the completeness of exposure assessment information by associating environmental or source information, exposure measurements, and epidemio1ogica1 and human activity data with internal dose (Dary et al., 1996). In some cases, biomarkers of exposure may be better than extemal measurements of exposure for situations where protective equipment has been used or when there is the possibility of dermal (or gastrointestinal) absorption.

Dose response assessment

One of the most controversial aspects of risk assessment is the extrapolation of higher-level exposure data to lower levels of exposure (Goldstein, 1996). In risk assessment the ascertainment of a dose-response relationship is crucial for ultimately determining the shape of the dose-response curve and for predicting a no-observed adverse-effect level (NOAEL). Exploring the lower end of the dose response curve through epidemiological studies is generally impractical, if not impossible, due to extraordinarily large sample sizes (Stayner, 1992). However, biomarkers can contribute to identifying a dose-response relationship at lower levels of exposure. The demand on environmental epidemiology to evaluate increasingly subtle health risks requires more accurate estimation of the quantity and timing of a toxicant reaching target tissue (Kriebel, 1994). Kriebel (1994) described a two-stage approach to derive estimates of dose from exposure data and then linked them to epidemiological models estimating disease risk. Such an approach incorporates physiological processes into epidemiological modeling and is possibly more valid than approaches with less detail. Biomarkers of exposure can be used as indicators of dose, which can then be assessed against classic measures of morbidity or mortality.
Often dose-response determinations are made by use of PBPK models. Examples of how biomarker data could be incorporated in PBPK models include: 1) calibration of the model (empirically determining the population values of kinetic parameters); 2) validation of the model (determining how well the model predicts data of another cohort); 3) prediction (applying the model to new cohorts, and predicting the internal doses associated with given exposure scenarios). These predicted measures of internal (biologically-effective) dose can then be used in dose-response modelling, in lieu of the external exposure measures, to predict disease risk. The internal dose measures may be better predictors of disease risk, especially when exposure-dose is nonlinear (e.g., due to capacity-limited metabolism). A PBPK model could also potentially allow for extrapolation from limited data, such as a short-term laboratory study to predict the biomarker concentrations that might be found in a population. For example, the model for carboxyhemoglobin formation (Andersen et al., 1991) from exposure to methylene chloride was calibrated against short-term human exposure but could be used to predict carboxyhemoglobin from long-term exposures.

Another use of biomarkers can be as outcome measures that correlate with exposure. Here exposure markers are not what is needed; rather, there is a need for effect markers. Effect markers are those that relate to or predict disease. A marker validated to predict disease can be used as a surrogate for disease. For example, specific types of chromosomal aberrations that appear to predict cancer risk on a group basis could be used as the outcome variables in a dose response analysis, as in the case of ionizing radiation exposure (Joksic & Spasojevic-Tisma, 1998).

Biomarker validity and validation

According to IPCS (2001), the ultimate driving force for whether biomarkers will contribute to environmental health efforts is the validity of the markers. Validity describes the extent to which a biomarker reflects a designated event in a biological system. Generally, these events are exposure, effects of exposure, disease and susceptibility. Only when validity at the laboratory and population level has been established is a biomarker ready for the full spectrum of environmental research and uses. As noted, most biomarkers have not had that level of validation. A broad effort is underway but the products of this activity are not available yet.

Validation of candidate biomarkers is an empirical process that can be approached by producing several different, but convergent lines of evidence. There is extensive literature on criteria for validating biological markers (e.g., WHO, 1975; Lucier and Thompson, 1987; Hemberg and Aitio, 1987; Schulte, 1989; Schatzkin et al., 1990; Margetts, 1991; Schulte and Mazzuckelli, 1991; Schulte & Perera, 1993; Schulte & Talaska, 1995; Boffetta, 1995; Ponce et al., 1998). In general, these criteria include understanding the natural history, biological and temporal relevance, pharmacokinetics, background variability, dose response and confounding factors (Schulte and Talaska, 1995). Biomarker validity is also dependent on reliability of the assay to measure the biomarkers. These criteria allow for the assessment of whether a biomarker represents an event that is in a continuum between exposure and resultant disease, and whether the biological specimen containing the biomarkers is appropriate and the marker reflects the time period of concern. Finally, by assessing confounding and effect modifying factors, it is possible to understand what other factors influence a biomarker or its assay.

The careful measurement of strong confounders and effect modifiers should be given as much attention as is given to measurement of the exposure and disease variables or biomarkers. Consideration should be given to mounting validation substudies to quantify measurement error in important covariates (Hatch and Thomas, 1993). Controlling measurement validity makes it possible partially to control study validity since measurement errors can produce biased estimates of regression coefficients used in statistical models of exposure and disease (Louis, 1988). Measures of association, such as the odds ratio, can be distorted, depending on the type of error and other characteristics, towards or away from the null hypotheses of no association between the biomarker and disease (or exposure).

The terminology to express measurement error traditionally used in biomarker measurements is different from that applied in analytical chemical laboratories. In epidemiological work involving biomarkers, the emphasis is on the biomarker level in the studied population rather than on an individual, and the measurement error includes the intra individual variation with time (White, 1997). According to White (1997) measurement errors for an individual can be defined as the difference between a person's measured biomarker (the biomarker "test") and the person's true biomarker. The true biomarker is the biomarker without laboratory or other sources of error and, if the measure can fluctuate over time, the true biomarker is an integration of its concentration over the time period of etiological interest.

Validity in the context of epidemiological research involving biomarkers can be defined as the relation of the biomarker test (the potentially mismeasured biomarker) to true biomarker in the population of interest. Parameters that describe the measurement error in the population are called measures of validity (White, 1997). Two indicators of measurement error are used to describe the validity of an observed measurement compared with the true measurement (Armstrong et al., 1994). The first is systematic error or bias that would occur on average for subjects measured. The second is subject error, which is additional error that varies from subject to subject. The subject error is also called imprecision or the measure of the variation of measurement error in the population. Precision can be assessed by a construct known as the validity coefficient. It ranges from 0 to 1 with the value one indicating that the observed measurement is a perfectly precise indicator of the true measurement (Armstrong et al, 1994). A validity study would be defined here as one in which a sample of individuals is measured twice: once using the biomarker test of interest and once using a perfect measure of the true biomarker (White, 1997). However, for most biomarkers such perfect measures of the true biomarker do not exist, and, in practice, validation of a method must rely on comparison to other (similarly non-validated) methods. Then the indicator of biomarker measurement error from the validity study can be applied to what is known about the association under study in the parent study to estimate the effects of biomarker error on the association of interest (White, 1997). While the impact of measurement error on exposure-disease associations has been studied extensively, the impact on estimates of interaction of two or more risk factors has been studied less thoroughly (Greenland, 1993). Assessment of interaction of multiple exposures, gene environment or gene-gene is an important issue in environmental epidemiology and all the more important with biomarkers depicting mechanistic events. There are numerous sources of measurement error in biomarkers; some of these are shown in Table 6.

Ultimately, validation requires the use of epidemiological study designs to assess at least one of three types of relationships: exposure-dose; biological effects-disease; and susceptibility influencing an exposure-disease relationship.

Table 6. Examples of sources of error in biomarker measurement in epidemiological studies (White 1997)

Errors in the laboratory method as a measure of the exposure of interest

- Method may not measure all sources of the biological true exposure of interest
- Method may measure other exposures that are not the true exposure of interest
- Methods may be influenced by subject characteristics (other than the true exposure) that the researcher cannot manipulate, e.g., by the disease under study or by other diseases

Errors or omissions in the protocol

- Failure to specify the protocol in sufficient detail regarding timing and method of specimen collection, specimen handling, storage and laboratory analytical procedures
- Failure to include standardization of the instrument periodically throughout the data collection

Errors due to variation in execution of the protocol

- Variations in method of specimen collection
- Variations in specimen handling or preparation
- Variations in length of specimen storage
- Variations in specimen analysis between batches (different batches of chemicals, different calibration of instrument)
- Variation in technique between laboratory technicians
- Random error within batch

In addition to measurement error, the uncertainty of the results is affected by biological variability within subjects, i.e., short-term variability (hour to hour, day to day) in biological characteristics due 10, for example, diurnal variation, time since last meal, posture (sitting vs lying down); medium term variability (month to month) due to, for example, seasonal changes in diet; and long-term change (year to year) due to, for example, purposeful dietary changes over time.

Studies that contribute to these types of validation and bridge the gap between laboratory experimentation and population-based epidemiology have been referred to as "transitional" studies (Hulka, 1991; Schulte et al., 1993; Rothman et al., 1995). They may be designed to evaluate exposures, health effects or susceptibility, and some may have the characteristics of pilot or developmental studies (Hulka & Margolin, 1992).
In this section the kinds of information and approaches to validate specific types of biomarkers are discussed. Characteristics of valid biomarkers are outlined in Table 7.

Table 7. Characteristics of valid biomarkers

Biomarker type	Characteristics of validity
Exposure	Consistently linked with exposure at relevant levels of exposure with confounding and background exposures assessed
Effect	Consistently linked with increased risk with confounding and effect modifying factors assessed
Susceptibility	Can distinguish subgroups at risk given specific exposure
Biomarkers of exposure may also be validated by establishing a constant link to an adverse health effect or to the concentration of the chemical in the target organ	

-Omics Markers in Mixture Toxicology

Introduction

Holistic approaches or 'omics' technologies are changing the way toxicology studies are performed and safety data are generated and interpreted. When speaking about 'Omics' technologies, we usually refer to genomics, proteomics, and metabonomics technologies. These new approaches provide the means to predict toxicity and to increase the understanding of the molecular events underlying a given toxicity. Moreover, toxicogenomics is helping scientists to integrate toxicology into the earlier discovery phases by including sensitive parameters that should help toxicologists recognize liabilities at lower doses (pharmacological rather than toxicological doses) or after short exposure times (acute rather than chronic exposures).

Several facets of public health policy that relate to environmental chemical stressors put emphasis on the effects of combined exposure to mixtures of chemical and physical agents. This emphasis brings about methodological problems linked to the complexity of the respective exposure pathways; the beyond additive effect of mixtures (the so-called 'cocktail effect'); dose extrapolation, i.e. the extrapolation of the validity of dose-response data to dose ranges that extend beyond the levels used for the derivation of the original dose-response relationship; and the integrated use of toxicity data across species (including human clinical, epidemiological and biomonitoring data).

The 'cocktail' effect becomes all the more complex due to the large number of possible combinations of chemicals and other (physical or biological) stressors possible in the environment. This has hampered the development of rigorous methodologies for tackling the issue of environmental mixture safety. Currently, our scientific understanding and science policy for environmental mixtures are based largely on extrapolating from and combining data in the observable range of single chemical toxicity to lower environmental concentrations and composition, i.e., using higher dose data to extrapolate and predict lower dose toxicity. More precise approaches to better characterize toxicity of mixtures are needed (Mason et al, 2007). A main obstacle to the development of effective mixture risk assessment methodologies is the possibly infinite ways of combining chemicals into actual environmental mixtures. It should be noted, however, that although in theory the number of combinations of chemicals or stressors is infinite, the number of biological processes is finite. Therefore, in considering an integrated approach for risk assessment, it makes more sense to work on the finite biological processes that may be affected by human exposure to these mixtures rather than the infinite combinations of chemicals and stressors (Liao et al, 2002).

Integrated health impact assessment of environmental stressor mixtures would need to follow a so-called "full chain" approach to take into account all relevant health stressors and their interaction. Application of the full chain approach entails considering all possible exposure pathways via the environment and lifestyle choices. It also encompasses considering the effects of co-exposure to relevant stressors and how risk modifying factors such as age, diet, gender and time window of exposure affects the final physiological response. It is clear that successful application of this approach poses demanding data requirements both in terms of environmental monitoring and in terms of biological and clinical data interpretation. What is most important is the need for comprehensive data interpretation of the molecular, biochemical and physiological processes that couple exposure to health outcome. This requires forging a new paradigm for interdisciplinary scientific work in the area of environment and health. We shall call this the connectivity paradigm for risk assessment, denoting an approach that builds on the exploration of the interconnections between the co-existence of multiple stressors and the different levels of biological organization that come to bear to produce the final adverse health effect (Workman et al, 2006). Connectivity marks a clear departure from the conventional paradigm, which seeks to shed light on the identification of singular cause-effect relationships between stressors and health outcomes. It entails creating a new way of combining health-relevant information coming from different disciplines, including but not limited to environmental science, epidemiology, toxicology, physiology, molecular biology, biochemistry, mathematics and computer science (Kitano, 2002). In this chapter the integration of these different information classes into a unique framework to better inform and support public health impact assessment of chemical mixtures in the environment is discussed.

The advent of new technologies reaping the fruit of advances in biological science and in the improved understanding of the mechanisms of response to toxicological insults at different biological levels provides novel possibilities to improve the current state of the art in health risk assessment. In particular, the combined use of -omics methodologies including genomics, proteomics and metabonomics helps to approach the source-to-outcome continuum more effectively. This continuum comprises source/stressor formation, assessment of environmental contamination, exposure profile identification and (external and internal) dose estimation, up to the induction of early biological events and finally, health effects/outcomes. The following table gives an overview of the biological parameters most frequently assessed by –omics technologies.

Table 8. Parameters most commonly assessed by omics technologies (Suter-Dick and Singer, 2008).

	Description	Biologic material	Analytical method	Parameter	Example	No. of analytes
Genomics/ Transcriptomics	Gene-expression analysis	Tissue, cell cultures	Microarray QPCR	mRNA (transcripts)	Induction of Proteins after cell damage	>10000
Proteomics	Protein-expression analysis	Tissue, cell culture, serum, urine		Protein products, including post-translational modifications	Increased P450 after Adduct formation	>1000
Metabonomics/ Metabolomics	Metabolic profiles of endogenous metabolites	Urine, serum	NMR, LC-MS GC-MS	Endogenous metabolites, small molecules	Adduct formation Reduction of intermediate products of the Krebs cycle due to mitochondrial damage	>500

Towards a connectivity paradigm

The translation of this integrated thinking into a new paradigm for modern toxicology is described in a pivotal report published by the US National Academy of Science (NAS, 2007) showing the way towards a systems toxicology approach for the 21st century. According to the report integrated assessment of the toxicity of chemicals includes several components as follows:

- chemical characterization
- toxicity testing,
- dose-response modeling and
- extrapolation modeling

Chemical characterization is meant to provide insights to key questions, including a compound's stability in the environment, the potential for human exposure, the likely routes of exposure, the potential for bioaccumulation, possible routes of metabolism, and the likely toxicity of the compound and possible metabolites based on chemical structure or physical or chemical characteristics.

Toxicity testing includes toxicity-pathway assays. The initial perturbations of cell signaling motifs, genetic circuits, and cellular-response networks are obligatory changes resulting from chemical exposure that might eventually result in disease. The consequences of a biologic perturbation depend on its magnitude, which is related to the dose, the timing and duration of the perturbation, and the susceptibility of the host. At low doses, many biologic systems may function normally within their homeostatic limits. At higher doses, clear biologic responses occur. They may be successfully handled by adaptation, although susceptible individuals may have an off-normal response. More intense or persistent perturbations may overwhelm the capacity of the system to adapt and lead to tissue injury and possible adverse health effects.

Dose-response models would be developed for environmental agents primarily on the basis of data from mechanistic, in vitro assays as described in the toxicity-testing component. The dose-response models would describe the relationship between concentration in the test medium and degree of in vitro response. In some risk contexts, a dose-response model based on in vitro results might provide adequate data to support a risk-management decision.

Extrapolation modeling estimates the environmental exposures or human intakes that would lead to human tissue concentrations similar to those associated with perturbations of toxicity pathways in vitro and would account for host susceptibility factors. Extrapolation modeling has three primary components. First, a toxicity-pathway model would provide a quantitative, mechanistic understanding of the dose-response relationship for the perturbations of the pathways by environmental agents. Physiologically based pharmacokinetic modeling would then be used to predict human exposures that lead to tissue concentrations that could be compared with the concentrations that caused perturbations in vitro. Third, human data would provide information on background chemical exposures and disease processes that would affect the same toxicity pathway and provide a basis for addressing host susceptibility quantitatively.

The following technologies are used to support the implementation of the connectivity approach to chemical risk assessment: genomics, proteomics, metabonomics and biology-based dose-response modeling. Genomics, or more appropriately, transcriptomics measures the modulation in the expression of the mRNA to determine perturbations at the molecular level due to xenobiotics. Differential gene expression means differentiated coding of proteins, which, in turn, leads to modifications of the protein equilibrium defined as the proteome. The analytical determination of the modulated protein profiles and the linkage with chemical exposure is called proteomics. Proteomics research uses advanced analytical techniques such as HPLC-MS (high performance liquid chromatography – mass spectrometry) and MS-MS or MS-TOF (mass spectrometry time of flight) systems to determine accurately the overall set of proteins comprising the proteome. The complex network of proteins, enzymes and other biological molecules determine human physiology and thus, the status of human health. Biological chemicals and xenobiotics alike get metabolized as a result of normal physiological processes; the products of the metabolic regulatory networks circulate in the body and are finally excreted in urine and feces. The whole set of metabolites in the human body can be analyzed using analytical techniques such as MS-TOF and NMR (nuclear magnetic resonance) following the metabonomics approach.

One of the most challenging aspects of implementing toxicogenomics (which encompass transcriptomics, proteomics and metabonomics) in risk assessment involves establishing the appropriate infrastructure to support and facilitate the management, integration, interpretation, and sharing of very large amounts of toxicogenomic data. An effective, flexible, and comprehensive knowledge base is required that is populated with phenotypically anchored toxicogenomic data, complemented with absorption-distribution-metabolism-excretion (ADME), histopathology, clinical chemistry, and toxicity data. The main paradigm for such database development currently used is the minimum information about a microarray experiment (MIAME) standard (Brazma et al., 2001). Although commercial databases are highly promoted, there is a lack of peer-reviewed publications critically assessing their utility, although over the last five years the latter have started to emerge (Fletcher et al., 2005; Ganter et al., 2005).

Independent verification and validation of toxicogenomics results will be needed to further demonstrate their utility and facilitate increased acceptance of -omics technologies in the scientific and regulatory communities. Another key step to wider acceptance of these scientific data is the ability to effectively

communicate and share laboratory data. Strategies to facilitate electronic data exchange between databases, such as microarray gene expression-markup language (MAGE-ML) (Spellman et al., 2002) and systems biology markup language (SBML) (Hucka et al., 2003), have now been developed and already facilitate effective electronic data exchange between compliant repositories. Ideally, these databases will provide access to the large, disparate, and robust toxicogenomic data sets required to develop the necessary computational algorithms and models needed to support quantitative risk assessment.

With few exceptions, most toxicogenomic studies to date provide a qualitative description of changes, with only anecdotal reporting regarding the implications to physiological outcomes, and limited contributions to elucidating mechanisms of toxicity (Cunningham and Lehman-McKeeman, 2005; Boverhof and Zacharewski, 2006). Reproducibility problems, quantification issues, and limited throughput compromise the utility of proteomics (Cox et al., 2005; Garbis et al., 2005) even though significant advances have been made over the last few years towards applying proteomics data for regulatory purposes by the US Food and Drug Administration (FDA). The lack of comprehensive peptide and metabolite reference databases also hinders the ability to elucidate mechanisms of toxicity associated with changes in protein and metabolite profiles (Cox et al., 2005; Kell, 2004).

The -omics have demonstrated their utility in classification and diagnostics (Pennie et al., 2004), but significant advances in deciphering mechanisms of toxicity and aiding in risk assessment have yet to materialise. The combination of the -omics techniques with biologically-derived models of cell function and physiological processes can significantly enhance our understanding of the biological mechanisms leading to onset or exacerbation of human pathologies linked to xenobiotic exposure, if appropriately anchored to phenotypic evidence. These results would also have to account for inter- and intra-individual variability of expression profiles, as well as being able to differentiate between adaptation and defence mechanisms and actual response that may lead to pathology. Computational toxicology methods and biological modelling help put together a systems approach for the derivation of dose-response functions and their effective application in risk assessment.

The combination of the –omics techniques with biologically-derived models of cell function and physiological processes can significantly enhance our understanding of the biological mechanisms leading to onset or exacerbation of human pathologies linked to xenobiotic exposure. Computational toxicology methods and biological modeling help put together a systems approach towards the derivation of dose-response functions and their effective application on risk assessment.

The above systems toxicology approach to mechanistically-based risk assessment of environmental chemical mixtures can be tackled with an integrated multi-layer computational methodology (Sarigiannis et al, 2009) that ideally comprises the following steps (Figure 9):

a) Characterization of exposure factors quantifying the parameters that affect human exposure to environmental chemicals, such as time-activity relationships, seasonal and climatic variation, consumer choice. These exposure factors can be used to derive aggregate and cumulative exposure models, leading in probabilistic exposure assessments.

b) Current toxicological state of the art combines estimations of biologically effective dose with early biological events to derive dose-effect models, which can be used in combination with the probabilistic exposure estimates to derive biomarkers of exposure and/or effect. Combined use of epidemiological, clinical and genetic analysis data may shed light on the effect risk modifying factors such as lifestyle choices and DNA polymorphisms. Observation of real clinical data and /or results of biomonitoring, if coupled with the exposure/effect biomarker discovery systems can produce biomarkers of individual susceptibility and thus, allow estimations of individual response to toxic insults. Toxicogenomics and in particular transcriptomics, is the catalyst for this kind of analytical and data interpretation process.

c) The integrated analysis of the biomarker data (including results on biomarkers of exposure, effects and individual susceptibility) results in the integrated assessment of risk factors. Using the information on risk factors with molecular dosimetry data (i.e. estimation of the actual internal and biologically effective dose of xenobiotic substance found in the target organ and, indeed, perturbing cellular response) population risk studies can be concluded, converting generic exposure profiles into

population risk metrics having taken into account inter-individual variability of response and exposure uncertainty.

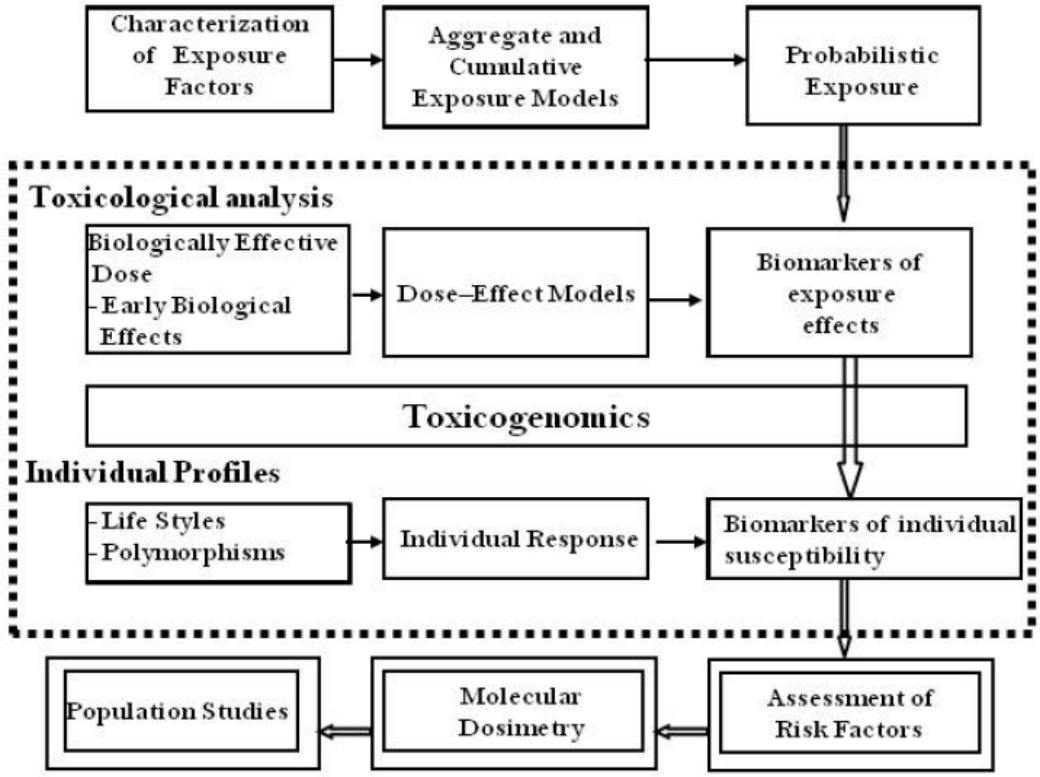

Figure 9. Multi-layer computational approach for integrated health impact assessment (Sarigiannis et al, 2009)

Technology Platforms

Suter-Dick and Singer (2008) give a very nice overview of the available technologies for implementing the –omics informed approach outlined above. In the following chapter, this overview is reported in order to provide a sound basis for understanding the necessary technological tools and the general principles they are based on.

Transcriptomics

Nucleic acids have the characteristic of having a four-nucleotide code with very well defined complementarity, which is used during the biological processes of replication and transcription. Microarray technologies have made use of this biochemical property and use highly specific sequences (generally called probes) fixed to a solid support for the quantification of the genes of interest (generally called targets). For all represented genes, this technology quantifies the amount of transcript present and is therefore called transcriptomics or genomics. These two terms are commonly used as synonyms.

Methods. For each target gene, one or several specific probes complementary to a stretch of its sequence are represented on the microarray. Most test systems (platforms) use 20- to 70-bases-long probes. The high spatial density of the microarrays allow tens of thousands of probes, covering virtually all known transcripts, to be represented in one single microarray and thus measured simultaneously. Hence, the whole transcriptome (transcriptome is the sum of all transcribed genes in a given tissue at a given time) for a given tissue can be assessed with one microarray experiment. For this, all transcripts (messenger RNA, mRNA) from a given tissue or cell culture are extracted, usually amplified, labeled, and

hybridized onto the microarray. Hybridized probes are measured (usually fluorimetrically) generating an output where the intensity of the signal detected for each probe is proportional to the amount of transcript (mRNA target) in the original sample. Some microarray platforms use two-color dyes for the simultaneous determination of the expression level in two samples, one being typically a baseline (control) and the other being the treated sample. In these cases, both the control and treated sample are hybridized together onto the same microarray exactly under the same hybridization conditions, thus minimizing the technical variability. However, the disadvantage of obtaining relative rather than absolute gene-expression values is that they always refer to the sample that was arbitrarily selected as baseline. In all cases, microarrays provide a (semi)-quantitative measurement of absolute or relative expression level for each gene represented on the microarray. Owing to the differences in the genetic code between the species and of the exact complementarity required between a probe on a microarray and the target in the tissue of interest, microarrays are highly species-specific. There are many commercial providers of microarrays including (but not limited to) Affymetrix, Applied Biosystems, Agilent, Illumina, and Codelink. Also, several laboratories in academia and industry use spotting devices to spot oligonucleotides and produce their own microarrays. In addition to the microarray platforms that rely on hybridization for the detection of mRNA-species, qRT-PCR (quantitative reverse transcriptase polymerase chain reaction) uses specific amplification of a transcript of interest as a means to determine the expression level of a gene. The amplified product is highly specific due to the use of specifically designed primers and can be quantified after each PCR-cycle by means of a fluorescent dye. Thus the reaction can be followed in real time (qPCR is often also called real-time PCR). In general, qPCR is considered to be more sensitive, specific, and accurate than most hybridization-based microarray platforms. Strictly speaking, qPCR is not an 'omic' application, since it can only be applied to a limited number of genes, rather than being a global, genome-wide approach. However, automation on 384-well plates and the use of commercially available microfluidics cards (e.g., from Applied Biosystems) allows us to measure up to 50 or 100 genes simultaneously using qPCR. Moreover, qPCR is currently the technology of choice for most scientists to corroborate results obtained using microarrays and plays therefore a major role in toxicogenomics.

Proteomics

Proteomics is the study of the proteome, defined as the sum of all proteins expressed in a given tissue at a given time. The proteome is more complex than the transcriptome, as it includes the study of post-translational modifications such as phosphorylation. Most proteomics platforms use the strategy of separation, quantification, and identification of the protein products to determine the proteome in a given sample. Proteins are more complex chemically than nucleic acids. First, it needs to be taken into consideration that there is no natural biochemical complementarity as with the genetic code, which precludes a simple hybridization process. Moreover, most proteins undergo extensive modifications after translation, such as cleavage of signalling peptides, phosphorylation/dephosphorylation, or glycosylation. In addition, treatment with xenobiotics can also lead to drug–protein covalent binding, a protein modification of relevance in toxicology. These post-translational modifications have a major impact on the function of the proteins. For example, the activity of many proteins is regulated through phosphorylation/dephosphorylation, which can cause dramatic biological changes, whereas the total protein content remains unchanged. In addition, proteins are present in serum and tissues with a very wide dynamic range: some proteins are highly expressed, while others are up to 10 orders of magnitude less abundant. For example, the serum concentration of albumin, the most abundant protein in serum, is about 10 billion times greater than that of interleukin-6. This complex situation puts the technologies around proteomics to a challenge, since such a dynamic range is extremely difficult to achieve.

Methods. Separation can be achieved by fractionation of cellular components (sub-cellular fractionation) and further by two-dimensional gel electrophoresis (2D-PAGE) or chromatographic methods. Generally, 2D-PAGE is considered the workhorse of proteomics, although it is very time-consuming and lacks sensitivity. Low-abundance proteins and highly hydrophobic proteins such as cytochromes P450 can usually not be assessed with this method. As a further development of 2D-PAGE, and similarly to the two-color dyes in microarrays, 2D-DIGE includes a fluorescence-labeling step of the proteins before the electrophoresis and the simultaneous separation (by 2D-PAGE) of two samples labeled each with a different color. The advantage of 2D-DIGE is that it allows relative quantification while controlling for technical variability, increasing thus the dynamic range and sensitivity of traditional 2D-PAGE.

Retentate chromatography–mass spectrometry (RC-MS), also known as SELDI (surface-enhanced laser desorption/ionization) is an alternative to 2D-PAGE-based methods. SELDI uses surfaces with different physicochemical properties to separate the proteins in a complex mixture. The adsorptive chromatographic support, placed on thin metal chips, acts as a bait to adsorb proteins in the sample. This is based on the fact that different chemical surfaces present affinities to groups of proteins with specific characteristics. A mass-spectrum profile is then created by desorbing the proteins with the laser from the MALDI (matrix-assisted laser desorption ionization) instrument. This technology is relatively easy to use, requires a small sample volume, and allows a higher throughput than 2D-PAGE. However, SELDI has several technical drawbacks and has been reported to suffer from large experimental variability. Identification of the proteins after 2D-PAGE is usually performed by digesting the protein spots of interest with trypsin and by determining the masses of the tryptic fragments with mass spectroscopic (MS) approaches. The mass spectrograms are like fingerprints that can be compared to public databases (DB) for identification purposes, thus this process is called peptide fingerprinting. After SELDI analysis, the identification of the identified possible 'markers' is rather difficult. Some researchers try to use this platform to identify 'fingerprints' without identifying the actual proteins, but this is not ideal. If identification is required, the peaks of interest must be isolated to allow subsequent peptide fingerprinting or tandem-MS to be performed.

A different approach to proteomics is the use of protein arrays. The most frequently used protein array is the antibody array. These arrays rely on the spotting of specific antibodies for the proteins of interest, and are conceptually similar to the DNA-microarrays. The antibodies fixed onto a solid support can capture through affinity binding the proteins present in the complex mixture hybridized onto the chip. The main limiting factor of antibody arrays is the need for specific, good-quality antibodies for a large number of proteins of interest.

Metabolomics/Metabonomics

Metabolomics studies the collection of all endogenous metabolites present in body fluid or tissue. While transcriptomic and proteomic analyses provide partial information regarding the biological processes occurring in a cell, metabolic profiling gives an overall picture of the cellular physiology. Moreover, changes in the metabolic profile reflect the reaction of the system to external stimuli, such as feeding or exposure to chemicals. These changes in the metabolic profile can be investigated in a cell, tissue, or organ. The metabolome is the final downstream product of the genome and is defined as the total quantitative collection of low-molecular-weight compounds (metabolites) present in a cell or organism that participate in metabolic reactions required for growth, maintenance, and normal function. Therefore, metabolomics refers to the study of the collection of all metabolites such as in a biological organism, which are the end products of the activity of expressed genes and proteins and their interactions. Some investigators define the metabonome as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification. Others consider the metabonome as the sum of the metabolomes of each individual cell, organ, and tissue of a complex organism. In any case, metabonomics measurements give an overall picture of the physiology of an individual and its reaction to a given treatment, which can be measured in body fluids such as serum and urine. However, there is still some disagreement over the exact difference between metabolomics and metabonomics and in practice both terms are often used interchangeably.

The applications of metabolomics include the characterization and prediction of drug toxicity diagnosis and monitoring of clinical disease, the evaluation of therapeutic intervention, and the understanding of the effects of genetic modifications. These are achieved by measuring the metabolite profiles, generally in biofluids (urine, serum, plasma), although metabolomics measurements can also be applied to tissues. The study of the metabolome faces similar challenges to the study of the proteome, since it cannot rely on simple chemical hybridization to separate, quantify, and identify metabolites of interest. Metabolites are even more chemically diverse than proteins and they are also present in a wide dynamic range of concentrations, with differences in concentration of up to nine orders of magnitude. It is a goal of researchers in this field to develop technologies that enable large-scale high-throughput screening, without losing sensitivity. However, there is currently no technology that fulfils all these requirements and

investigators are forced to evaluate the advantages and disadvantages of each platform for each experiment.

Methods. Metabolomics uses technologies such as H-NMR (proton nuclear magnetic resonance) or a chromatographic separation [liquid (LC) or gas chromatography (GC)] coupled to a mass spectroscopic (MS) determination. NMR spectroscopy is the only detection technique that does not rely on separation of the analytes and is not destructive, e.g. it allows the sample to be recovered for further analyses. It requires relatively little sample processing and the measurement times are short, allowing for relatively high throughput. All kinds of small molecules can be measured simultaneously in a complex mixture. However, it is relatively insensitive compared with mass spectrometry-based techniques and the processing of the NMR-spectra requires very specialized technical expertise and is relatively time-consuming. Additionally, the identification of metabolites of interest poses a challenge. The other technology commonly used for metabolic profiling is LC-MS or GC-MS. Complex samples are separated by liquid or gas chromatography and the masses of the components of each fraction are determined by MS. This method is more sensitive than NMR, but requires additional sample processing and the sample cannot be recovered for subsequent analyses.

Computational integration of environmental health data

In compliance to the methodologies described above, a computational platform which implements the essential mathematical frame for assessing health implications originated from environmental and consumer products contamination was developed. All methodological steps composing the platform are integrated in a single software tool using the acslXtreme dynamic simulation environment. The platform (a simplified conceptual visualization presented in Figure 10) is composed by 4 main components, named as:

- Emissions-Concentrations component. The emissions-concentration component is responsible for translating emissions from environmental sources (or migration from food contact materials) to media (ambient air, food) concentrations. Depending on the application and the purpose, the proper model is incorporated in the platform in each case (e.g. box model for indoor pollutants, migration model for food contact materials)
- Exposure component. The exposure compartment is responsible for composing the exposure pattern of the exposed individuals, relying to the information coming from the media compartments for given activity patterns (e.g. time spend in indoor locations, smoking activity, nutritional schedule). For inhaled xenobiotics, if over the given period of time, ***T***, the person passes through ***n*** locations, spending a fraction ***fn*** of the period ***T*** in location ***n*** where the concentration of the pollutant under consideration is ***Cn***, then the personal exposure for this period ***T***, represented by the concentration C_T, is given by (Hinwood et al. 2006) :

  $$C_T = \sum_n f_n \cdot C_n$$

  To evaluate the contribution of the sources to personal exposure, the amount of time a person spends in each location, along with the concentration of the compound in each location needs to be estimated a process usually performed by Time Activity Microenvironment Diaries (TAMD) kept by the exposed subjects (Jantunen et al. 1999). In the case of oral administration the estimation of chronic or single doses is easier, by multiplying the media concentration (food) with amount of food ingested, while for dermal exposure we take into consideration the concentration in the media, the conduct duration and the surface of contact.
- Internal dose – PBTK/PD (Physiology Based ToxicoKinetic/ToxicoDynamic) model component. This is the core of the whole platform. This compartment is responsible to describe the mechanisms of absorption, distribution, metabolism and elimination (ADME) of chemicals in the body resulting from acute and/or chronic exposure regimes, given by the exposure component. The PBTK model may implement all possible routes of exposure (inhalation, oral or dermal) for the xenobiotic or the xenobiotics mixture of interest. Thus, it is important to clarify that aggregation of exposure to a single contaminant from several exposure routes is not calculated by just summing the overall dose integrated in time by each route; aggregation of doses is calculated dynamically at the internal dose module of the computational platform, estimating the contribution of each route (incorporating the differences in uptake rate and the metabolic processes), reflecting in this way the differences in bioavailability and internal exposure dynamics relevant to the exposure route. Additionally, this component provides the bi-directional link of biological fluids (blood, urine) monitoring to exposure.
- Health impact component. In this component the internal dose is linked to possible health outcomes. To derive a quantitative health risk estimates we apply a multistage approach based on the decomposition of the dose-response relationship into different micro-relations each one describing a specific biologic process. Instead of trying to quantify the relation between dose and response probability directly, it is useful to decompose the causal relation between exposure history and health risk probability into biologically meaningful causal links called "micro-relations", to quantify these links, and then to estimate the full dose-response relation by composing its constituent micro-relations.

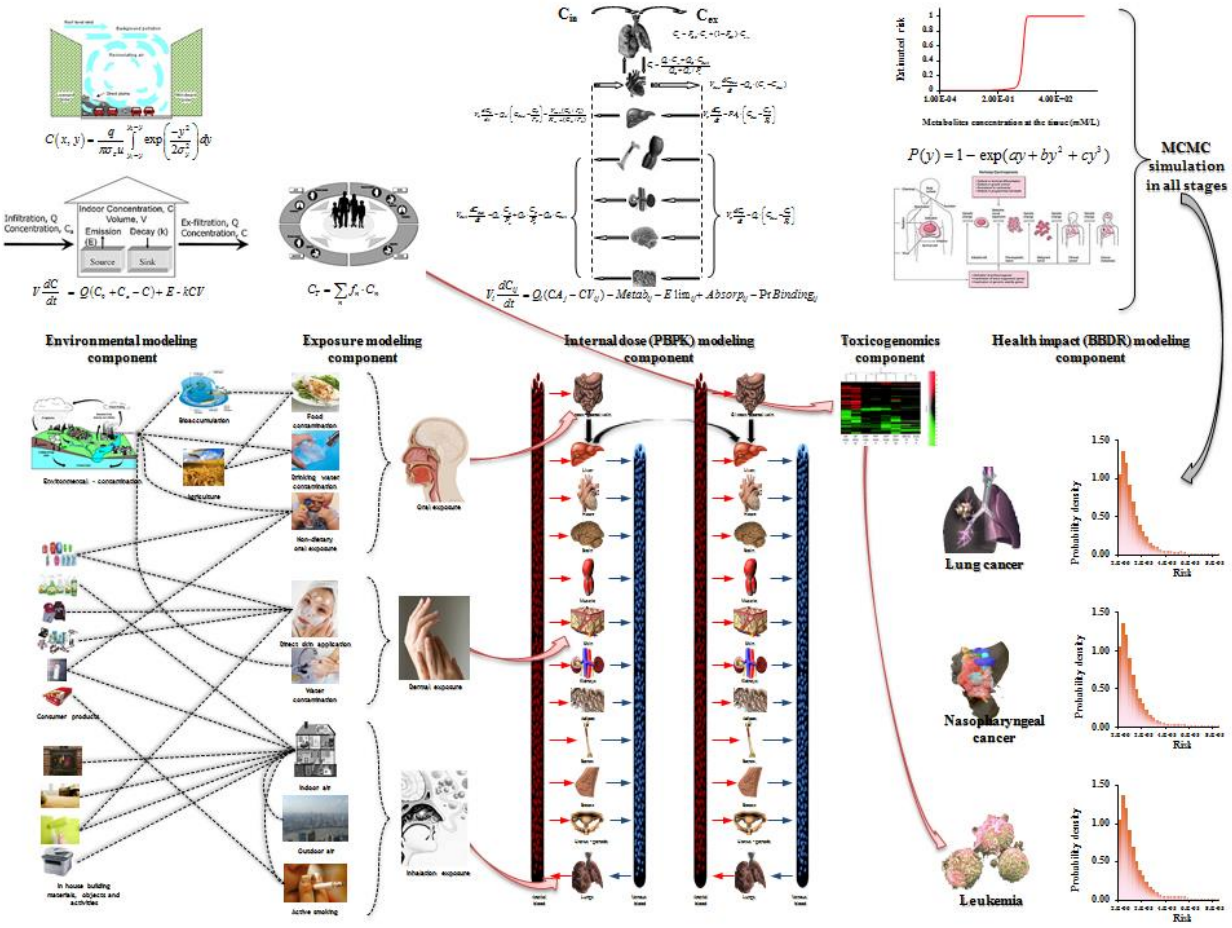

Figure 10. Conceptual representation of the computational platform

At this stage is important to clarify that the components are connected among them in a way that the output of the previous component is the input of the next one. In this way, with the integration through time, dynamic processes occurring either in environmental media, either in the human body are described in a continuous way. However, toxicogenomics data are a cross-cutting component, linked to anyone of the three latest components depending on the nature of the physical problem.

Besides the understanding of environmental-health continuum in a robust biological basis, additional advantages of such a mechanistic approach for assessing exposure are:

- The more detailed information about the several steps of the full chain approach are available, the more precise are the final estimations. On the contrary, on the non-mechanistic approaches, information related to a component of the full chain approach is usually a parameter that can hardly be incorporated in the calculations and is usually just another parameter that increases uncertainty.
- The effect of each component of the full chain approach represents a "real world" affecting parameter and due to its distinguished contribution to the final estimations, any policy related to that parameter can be separately quantified and consequently evaluated.
- The effect of policy measures related to contaminants with different sources or multiple pathways and routes of exposure can be precisely evaluated and cost-benefit analyses are feasible
- Synergistic effects of chemical mixtures can be incorporated

- The susceptibility of the population to the different environmental stressors is taken under consideration
- Uncertainty originated either from the heterogeneity of the data, either from real uncertainty parameters can be easily incorporated in the probability distributions
- Missing links from the full chain assessment may be calculated if the rest of the elements are known by reverse modeling algorithms.

Examples of combined exposure to multiple stressors

Environmental tobacco smoke

General concept

Environmental tobacco smoke is the most complex mixture of everyday exposure, including almost 4000 different contaminants, 60 of them classified as carcinogens. Under this frame, a mechanistic assessment was conducted including the carcinogenic potential of ETS based on some of the major Class I carcinogens, namely benzene, formaldehyde, NNK and arsenic. Benzene is known that induces leukemia, but the toxicity potential of benzene is affected by the co-exposure to toluene ethyl-benzenes and xylenes, which is the case on ETS. Formaldehyde causes nasopharyngeal cancer and although for the moment no further interaction with the other chemicals is considered, is strongly contributing to the cumulative cancer risk. NNK and arsenic, although they do not interact on the ADME level, they have a common health endpoint (lung cancer), being a typical example of "dose addition". However, in our methodology the addition of doses is not occurring by just summing external exposure doses, but is derived by the addition of the carcinogenic potential as derived by capturing internal exposure dynamics and the individual dose response functions. The pharmacokinetic behavior of these key carcinogens in second hand smoke and the respective dose-response functions are described here below.

Benzene model

PBPK model

The whole metabolic chain of benzene (Figure 11) was modeled starting from previously developed PBPK/PD models for benzene metabolism in mice (Manning et al. 2010) and its extrapolation to humans (Yokley et al. 2006). The model evaluates tissue levels of benzene, benzene oxide (BO), phenol (PH), and hydroquinone (HQ), as well as the total amounts of muconic acid (MA), phenylmercapturic acid (PMA), phenol conjugates, hydroquinone conjugates, and total catechol produced. For benzene oxide, phenol, and hydroquinone, the body is divided into five compartments: kidney; liver; fat; rapidly perfused tissues (RTP), and slowly perfused tissues (PPT). As for the benzene model the liver is subdivided into three compartments of equal volume according to the specific enzymatic distribution. The further metabolism of BO, PH and HQ is supposed to occur in the liver (main metabolism organ) and to a lesser extent in the kidney.

The PBPK/PD model results were validated through successive and complementary steps: first we validated the interaction mechanism among toluene, xylene and ethylbenzene; then the biokinetics of benzene and finally the metabolic chain of benzene. Each one of these validations was carried out by comparing the model results with independent experimental data reported in literature (Pekari et al. 1992; Tardif et al. 1997; Waidyanatha et al. 2004). Results show that the PBPK/PD model developed in the present study provides accurate estimations of the kinetics of the BTEX mixture in the human body. This also confirms that the mechanism of interaction based on the competitive inhibition between the four VOC's constituents the mixture describes the experimental data adequately. Some minor discrepancies from the empirical data are likely to be attributed to the use of reference physiological parameters in the absence of actual parameters and inherent variability in the experimental data.

The high potential toxicity of benzene metabolites associated to the leukaemia risk in humans, suggested taking into account more in detail the metabolic chain from benzene to its key metabolites through a more refined PBPK model for that chemical. The whole metabolic chain of benzene was modelled starting from previously developed PBPK models for benzene metabolism in mice and its extrapolation to humans. The model (Figure 12), evaluates tissue levels of benzene, benzene oxide (BO), phenol (PH), and hydroquinone (HQ), as well as the total amounts of muconic acid (MA), phenylmercapturic acid (PMA), phenol conjugates, hydroquinone conjugates, and total catechol produced. For benzene oxide, phenol, and

hydroquinone, the body is divided into five compartments: kidney; liver; fat; rapidly perfused tissues (RTP), and slowly perfused tissues (PPT).

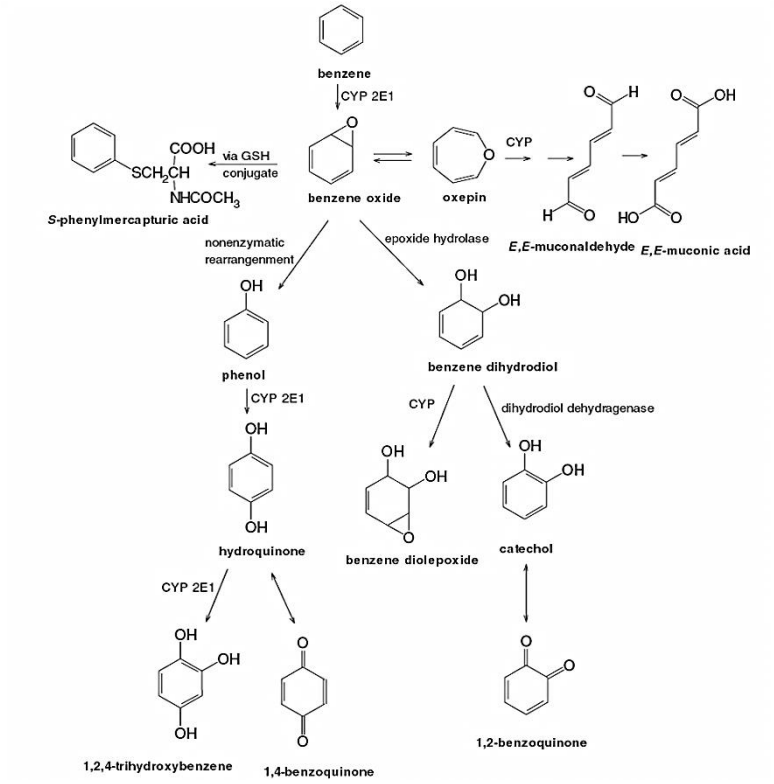

Figure 11. Metabolic chain of benzene to its primary metabolites

The model (Figure 12), evaluates tissue levels of benzene, benzene oxide (BO), phenol (PH), and hydroquinone (HQ), as well as the total amounts of muconic acid (MA), phenylmercapturic acid (PMA), phenol conjugates, hydroquinone conjugates, and total catechol produced. For benzene oxide, phenol, and hydroquinone, the body is divided into five compartments: kidney; liver; fat; rapidly perfused tissues (RTP), and slowly perfused tissues (PPT). As for the benzene model the liver is subdivided into three compartments of equal volume according to the specific enzymatic distribution. The further metabolism of BO, PH and HQ is supposed to occur in the liver (main metabolism organ) and to a lesser extent in the kidney. More in detail the followings metabolic transformations (mediated by CYP2E1) are supposed to occur in zone 3 of the liver as well as in the kidney:

benzene ➔ benzene oxide

phenol ➔ hydroquinone

phenol ➔ catechol

hydroquinone ➔ trihydroxy benzene

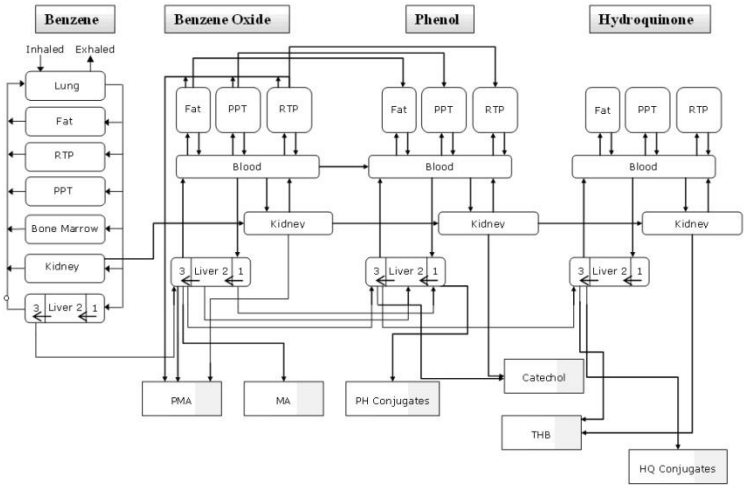

Figure 12. Conseptual representation of benzene PBPK model

The equations describing these metabolic reactions are given hereafter.

CYP2E1 activity in the liver (zone 3 of the liver):

$$RM_{BO,Liver3}^{BZ} = k_1 \frac{V_{2E1} C_{Liver3}^{BZ}}{D_L} C^{MP} \frac{T_L}{3}$$ *Benzene to Benzene Oxide*

$$RM_{HQ,Liver3}^{PH} = k_5 \frac{V_{2E1} C_{Liver3}^{PH}}{D_L} C^{MP} \frac{T_L}{3}$$ *Phenol to Hydroquinone*

$$RM_{CAT,Liver3}^{PH} = k_6 \frac{V_{2E1} C_{Liver3}^{PH}}{D_L} C^{MP} \frac{T_L}{3}$$ *Phenol to Catechol*

$$RM_{THB,Liver3}^{HQ} = k_7 \frac{V_{2E1} C_{Liver3}^{HQ}}{D_L} C^{MP} \frac{T_L}{3}$$ *Hydroquinone to Trihydroxy benzene*

where:

$$D_L = 1 + A^{BZ} C_{Liver3}^{BZ} + A^{PH} C_{Liver3}^{PH} + A^{HQ} C_{Liver3}^{HQ}$$

Since the kidney contains approximately 10% of the concentration of CYP2E1 found in the liver, it is assumed that relative to the metabolism in the liver, 10% of the metabolism mediated by CYP2E1 is in the kidney. The V2E1, that is the CYP2E1 specific activity as determined by the oxidation of p-nitrophenol to p-nitrocatechol, is scaled by 10% to give us the rate equations for the metabolisms using CYP2E1 in the kidney:

CYP2E1 activity in the kidney:

$$RM_{BO,Kidney}^{BZ} = k_1 \frac{V_{2E1} C_K^{BZ}}{10 D_K} C^{MP} T_K$$ *Benzene to Benzene Oxide*

$$RM^{PH}_{HQ,Kidney} = k_5 \frac{V_{2E1} C_K^{PH}}{10 D_K} C^{MP} T_K$$ *Phenol to Hydroquinone*

$$RM^{PH}_{CAT,Kidney} = k_6 \frac{V_{2E1} C_K^{PH}}{10 D_K} C^{MP} T_K$$ *Phenol to Catechol*

$$RM^{HQ}_{THB,Kidney} = k_7 \frac{V_{2E1} C_K^{HQ}}{D_K} C^{MP} T_K$$ *Hydroquinone to Trihydroxy benzene*

where:

$$D_K = 1 + A^{BZ} C^{BZ}_{Kidney} + A^{PH} C^{PH}_{Kidney} + A^{HQ} C^{HQ}_{Kidney}$$

and T_L and T_K are respectively the total mass of the liver and the total mass of the kidney. Assuming that tissue has the same density as water, we obtain:

$$T_j = V_j \times \frac{10^3 g}{1L}$$

where ***j*** could be the liver (L) or the kidney (K)

Since epoxide hydrolase, which mediates the metabolism of benzene oxide to muconic acid, is found in the centrilobular region (zone 3) of the liver, this metabolism is also supposed to occur in the zone 3 of the liver and it can be described by the first-order equation:

$$RM^{BO}_{MA,Liver3} = k_4 C^{BO}_{Liver3} \frac{V_L}{3}$$ *Benzene Oxide to Muconic Acid*

The metabolism of hydroquinone to its conjugates is assumed occurs in zone 3 since the glucuronidation capacity is greater in this region. It can be represented by the equation for glucuronidation from:

$$RM^{HQ}_{Conj,Liver3} = \frac{V_{HQ} C^{HQ}_{Liver3}}{K^{HQ}_m + C^{HQ}_{Liver3}} C^{MP} \frac{T_L}{3}$$ *Hydroquinone to its conjugates*

Since the sulfation takes place primarily in zone 1 of the liver, the conjugation of phenol was simulated occurring only in zone 1 and, according to, it is represented by the following equation:

$$RM^{PH}_{Conj,Liver1} = \left(\frac{V_{PH1} C^{PH}_{Liver1}}{K^{PH}_{m,1} + C^{PH}_{Liver1}} + \frac{V_{PH2} C^{PH}_{Liver1}}{K^{PH}_{m,2} + C^{PH}_{Liver1}} \right) C^{CP} \frac{T_L}{3}$$ *Phenol to its conjugates*

The metabolism of benzene oxide to phenol is nonenzymatic, so we assumed that this metabolism occurs in all compartments. This process is described by the first-order equation:

$$RM^{BO}_{PH,j} = k_2 C^{BO}_j V_j$$ *Benzene Oxide to Phenol*

where ***j*** is the compartment index.

Finally, glutathione S-transferase, which is required for the metabolism of benzene oxide to phenylmercapturic acid, is found in tissue such as the liver, kidney, muscle, and heart. Within the liver, glutathione S-transferase is found primarily in the plate limiting hepatocytes of the central vein. Thus, we consider first-order metabolism to occur in the slowly and rapidly perfused tissues, the fat, the kidney, the blood, and the third zone of the liver according to the following equation:

$$RM_{PMA,j}^{BO} = k_3 C_j^{BO} V_j$$

BBDR model

The final building block for addressing the source to health outcome continuum is the definition of mathematical models of human pathology. Pathology modeling has focused on a few ill-health conditions such as cancer and organ malfunction (e.g. heart disease) to date. This approach varies from a purely phenomenological one, including statistical descriptions of the link between toxic insult and health effect, to sets of differential equations describing mechanisms of action and partial differential equation models that take into account the presence of xenobiotics in specific parts of organs, up to more advanced mathematical techniques including cellular automata, neural networks and other artificial intelligence methods for quantifying the link.

According to Schollnberger et al (Schöllnberger et al. 2006), the earliest approaches to mathematically investigate cancer began in the early 1950s. Nordling (Nordling 1953) and Stocks (Stocks 1953) proposed that several successive mutations in a cell would be necessary to explain the fact that, for many carcinomas, the incidence rate varies as a power function of age. This has been quantitatively formulated by Armitage and Doll (Armitage and Doll 1954) in one of the best-known cancer models, a multi-stage model that accounts for the relationship between age and cancer incidence. The model reflects the number of stages needed for a normal cell to develop into a malignant cell. For the Armitage–Doll multi-stage model, no clonal growth was assumed. Because of discrepancies with the observed number of biological stages, Armitage and Doll further developed their model into one of two stages, with exponentially growing clones (Armitage and Doll 1957). The Armitage–Doll two-stage model has limitations in cancer risk assessment because it assumed deterministic cell growth. When the growth rate is small, it is more appropriate to use a stochastic model because the probability of extinction of clones, which is not considered in a deterministic model, cannot be neglected. This led to the development of stochastic cancer models (Knudson Jr 1971; Moolgavkar 1978; Moolgavkar and Venzon 1979; Moolgavkar and Knudson Jr 1981; Tan 1991). Stochastic cell growth of intermediate cells is assumed for the stochastic two-mutation model with clonal expansion. This two-step clonal expansion (TSCE) model is the best-known multi-step model and was developed by Moolgavkar, Venzon and Knudson (Moolgavkar 1978; Moolgavkar and Venzon 1979; Moolgavkar and Knudson Jr 1981), after whom it is known as the MVK model. In contrast with the Armitage–Doll model, there is a considerable amount of experimental data supporting the stochastic two-mutation model (Chen 1993).

Among the four VOCs considered in this work, benzene represents certainly the most potentially dangerous to human health. Chronic exposure to low levels of benzene may produce reversible decreases in blood cell numbers but, at higher levels, an irreversible bone marrow depression, with pancytopenia, may establish. This pathological condition is called aplastic anemia. Pancytopenia can occur also in the so-called myelodisplastic syndrome (MDS). Benzene MDS usually proceeds to leukemia, mostly acute myeloid leukemia (AML). The approaches taken to assess the carcinogenic risk from benzene exposure have been varied and have resulted in risk estimates that range considerably in magnitude. The U.S. EPA (USEPA 2000) used the Goodyear Pliofilm study (Rinsky et al. 1981; Rinsky et al. 1987) for their quantitative risk estimation. They estimated a range of $2.2 \cdot 10^{-6}$ to $7.8 \cdot 10^{-6}$ as the increase in the lifetime risk of an individual who is exposed for a lifetime to 1 ug/m^3 benzene in air. This is based on a linear model and extrapolates to air concentrations of 1.3 to 4.5 ug/m^3 for a risk level of 1 in 100,000. The approach used by Crump directly linked external exposure to cancer risk using the Area Under the Curve (AUC) as the dose metric. Finally, an empirical statistical D-R model based on Maximum Likelihood Estimation (MLE) is derived, based on experimental data about cancer incidence as function of the exposure. The dose-response model developed by Crump (Crump 1994) takes the following form:

$$P(x) = 1 - e^{\left[-\left(0.00145x + 0.00013x^2\right)\right]}$$

where $P(x)$ represents the cancer probability attributable to x mg/kg/day of administered benzene to male mice. This equation implies that, at very low administered doses, the risk varies linearly with dose. To extend it to humans the authors of this study assumed that the same administered quantities of benzene "produce equal cancer risk in humans and animals, independent of the route of exposure". A key weakness of this approach is that AUC might not be the best choice of dose metric for benzene since it does not distinguish between dose histories having different time evolution if they have the same integrated total dose. For these situations the risk estimate based on the AUC will produce the same risk, although many experiments have shown that different time patterns of benzene dose administration with the same AUC produce very different profiles of benzene metabolites (Crump and Allen 1984) and very different hematotoxic effects.

In this work we applied a method, originally developed by (Cox Jr 1996), based on the decomposition of the dose-response relationship into a set of causal micro-relations, each one describing a separate biologic process. Instead of evaluating the relationship between administered dose and cancer risk 'directly' through an empirical-statistical model, this relationship is thus decomposed into two different parts: the first one links the administered dose to the total amount of metabolites produced (internal dose), while the second connects the internal dose to the probability of cancer.

The first relation is provided by the results of the PBPK/PD model, which has already been validated against human biomonitoring data (Sarigiannis and Gotti 2008). The statistical relation between internal dose and cancer probability was calculated using a parameterized function from Crump and Allen (Crump and Allen 1984). In particular the administered dose was calculated assuming an average person of 70 kg (adult) who inhales 10 m^3 of air in 8 hours for an occupation period of 40 years over a life of 70 years.

The next step was to derive an empirical statistical relation linking the internal dose to cancer probability. This was found to be the following:

$$P(y) = 1 - e^{\left[-0.04296940\,y + 0.02633730\,y^2 - 0.00764081\,y^3\right]}$$

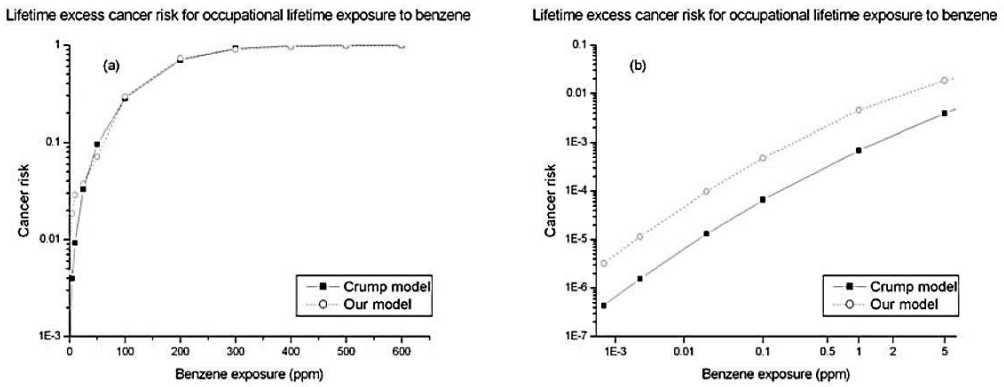

Figure 13. (a) lifetime excess cancer risk for 8hr/day lifetime inhalation exposure to benzene as predicted by Crump and Allen and our model. (b) the same at low doses

The standard error for the model parameters varied from 23% (for the linear term) to 9% (for the cubic term). This relationship incorporates the results of the PBPK/PD models that allow us to estimate the actual biologically effective dose (BED) of benzene metabolites in the bone marrow (the main target tissue for leukemia). BED of benzene metabolites is associated with the intake dose of both benzene and other VOCs present in the BTEX mixture. It has to be noted, however, that metabolic inhibition of benzene by co-exposure to toluene, ethylbenzene and xylenes is dose-dependent. This kind of biochemical interaction is usually low at very low levels of exposure. Thus, cancer probability, $P(y)$, can be linked mathematically to the average benzene exposure level, y, even though the carcinogenicity potency of benzene is attributed to its metabolites. In Figure 13 the estimated cancer risk as a function of the external benzene concentration as predicted by Crump and Allen model (solid line) and by our model (dashed line) is given.

Uncertainty and variability analysis

The methodology illustrated estimates the health risk associate to exposure scenarios of BTEX starting from the calculation of the internal dose of chemicals in the target tissues (through the application of a PBPK/PD model) and then applying a dose-response function to derive the associated health risk estimate. PBPK/PD models involve a large number of parameters each of which is subject to some degree of uncertainty and variability, which are translated into PBTK model outputs. Ignoring them in model parameters renders model results almost useless for risk assessment.

Variability typically refers to differences in the values of model parameters among individuals (inter-individual variability) or across time within a given individual (intra-individual variability). Uncertainty, on the other hand, essentially is a result of lack of knowledge and may have various sources. Toxicokinetic parameters are sometime known only with finite precision and the use of standard values, such as those in the International Commission on Radiological Protection report (ICPR 2002) are approximate values of the average for a human population.

To handle uncertainty and inter-individual variability we applied a Markov-Chain Monte Carlo approach on the most significant model parameters determined after a global sensitivity analysis on the whole model parameters dataset. This method is based on large repetition of model running using rather than fixed values of the model parameters a probability distribution for each of them and sampling randomly from its specific distribution. In this way a large number of different values for the model predictions are calculated representing a statistical distribution of the health risk for the population. To calculate the cancer risk derived from exposure to a mixture of BTEX, Monte Carlo simulations with 10000 iterations needs to be performed using distribution functions both for exposure values and for the most significant model parameters derived through global sensitivity analysis on the whole dataset of the model parameters. Global sensitivity analysis produced a list of the fifteen parameters/variables, which affected the most the final end-point (i.e. cancer risk).

The list is reported hereinafter and it includes physiological and biochemical parameters and exposure variables:

- Body weight
- Cardiac output
- Alveolar ventilation rate
- Fat content
- Maximum velocity of Michaelis-Menten metabolism for benzene in the liver and in the bone marrow
- Michaelis-Menten affinity constant for benzene in the liver and in bone marrow
- CYP2E1 specific activity
- Concentration of microsomal protein per gram of tissue in the liver
- Efficiencies of CYP2E1 for specific oxidation
- BTEX concentrations in the ambient air

The values of certain key model parameters such as the partition coefficients play a major role in determining the internal dose. As an example, the internal dose of benzene shows the lowest value compared to the others chemicals in venous blood and in lung but it shows definitely higher values than ethylbenzene in alveoli.

Table 9. Input parameter statistics for Markov-Chain Monte Carlo analysis

Parameter/Variable	PDF form	Min	Max	Average	Std. Dev.
	Lognormal	45.0031	128.5796	70.3144	11.8116

Alveolar ventilation rate (l/h/kg)	Normal	9.2363	26.6311	18.0351	2.5042
K_m:Michaelis-Mentent affinity constant for benzene in Liver (umol/liter)	Normal	0.0135	8.9530	4.4724	1.5553
V_{max}:Body surface-normalized maximal velocity of metabolism for benzene in Liver (umol/h/kg)	Normal	0.1854	30.2980	15.2787	5.1019
K_m:Michaelis-Mentent affinity constant for benzene in Bone Marrow (umol/liter)	Normal	0.0148	8.9297	4.4773	1.5756
V_{max}:Body surface-normalized maximal velocity of metabolism for benzene in Bone Marrow (umol/h/kg)	Normal	0.0300	29.6379	14.8325	4.8715
Fractional volume fat (%)	Normal	0.0732	0.2135	0.1432	0.0215
Cardiac output (liters/h/kg)	Normal	9.0768	26.5346	17.9597	2.4849
V2E1: CYP2E1 specific activity (umol/mg/hour)	Normal	2.00×10^{-6}	3.60×10^{-2}	1.81×10^{-2}	6.33×10^{-3}
CMP: Concentration of microsomal protein per gram of tissue in the liver (mg/g)	Normal	0.1717	115.8087	57.8942	19.0387
K_i: Efficiencies of CYP2E1 for specific oxidation (liters/umol)	Normal	0.0005	0.0826	0.0422	0.0119
Benzene (ug/m^3)	Lognormal	2.2652	26.3237	8.8267	4.8429
Toluene (ug/m^3)	Lognormal	9.3983	162.0743	43.3058	29.4848
Ethylbenzene (ug/m^3)	Lognormal	1.6986	44.6934	8.9174	7.7497
Xylene (ug/m^3)	Lognormal	6.7942	205.6319	38.3238	37.3683

For each of them a probability density function (PDF) was derived via a combination of literature data and prior distributions estimated from experimental measurements.

Formaldehyde model

PBPK model

All the inhaled amount of formaldehyde is absorbed by the upper respiratory system. Thus, internal dose modelling is meaningful only for the nasopharyngeal cavity, where is also located the target tissue. Regional flux of formaldehyde from inhaled air to the air-lining interface was estimated from steady-state CFD simulations of airflow and formaldehyde uptake that were conducted using three-dimensional, anatomically accurate reconstructions of the nasal passages of an adult male F344 rat and rhesus monkey. The locations of areas in which DPX were measured (Casanova et al. 1991; Casanova et al. 1994) were mapped into the rat and monkey CFD models, with the exception of the rat nasopharyngeal meatus, a portion of the dissected tissue that was included in the low-tumor region for DPX measurement, but was not included in the rat CFD model.

Steady-state airflow simulations were based on the results of previous studies (Kepler et al. 1998; Kimbell et al. 1997), conducted using FIDAP software. Simulations were carried out at 0.576 L/min in the rat and 4.8 L/min in the monkey, flow rates equivalent to twice the estimated minute volume in each species. Simulations of regional uptake by nasal walls were also conducted, and flux of formaldehyde to nasal walls was calculated using FIDAP software. Predicted flux in the rat low-tumor region was adjusted as follows to account for nasopharyngeal tissue missing from the CFD model that was collected from exposed rats as part of the low-tumor region in which DPX were measured. The weight of tissue in the low-tumor region of the CFD model was estimated by multiplying surface area and tissue thickness to estimate tissue volume and assuming that the density of the tissue is equal to that of water. The difference between the estimated weight and the weight of tissue collected (Casanova et al. 1994) was used to approximate the amount of missing surface area in the CFD model associated with the nasopharyngeal meatus. Formaldehyde concentration in air passing through this posterior portion of the rat nasal passages was expected to be low enough that flux in the nasophrayngeal meatus could be assumed to differ insignificantly from zero. Therefore, formaldehyde flux for a low-tumor region commensurate with the region dissected was approximated by the amount of formaldehyde predicted to be taken up by the low-tumor region as defined in the CFD model divided by the adjusted surface area.

The model used to describe the tissue disposition of formaldehyde and the formation of DPX was similar to that described by Hubal et al. (Cohen Hubal et al. 1997). Briefly, after entering the tissue, formaldehyde is cleared by a saturable pathway, a separate first-order pathway, or by pseudo first-order binding to DNA. The saturable pathway represents enzymatic metabolism of formaldehyde, which is primarily by formaldehyde dehydrogenase (Heck d'A et al. 1990), while the first-order pathway is assumed to represent the intrinsic reactivity of formaldehyde with tissue constituents. The pseudo first-order rate of binding of formaldehyde to DNA responsible for DPX formation was measured by Heck and Keller (Heck d'A and Keller 1988). No other routes of formaldehyde clearance are described in the model.

The model thus implicitly assumes that other potential routes of clearance, such as diffusion back across the tissue-air interface or into blood perfusing the respiratory epithelial tissue, are not quantitatively significant. These assumptions are based on the following considerations. a) Formaldehyde is highly soluble in water and reacts with tissue constituents. Quantitatively important desorption from tissue back into the air is thus judged to be unlikely. b) Heck et al. (Heck d'A et al. 1985) and Casanova et al. (Casanova et al. 1988) reported that inhalation of formaldehyde did not lead to measurable changes in the blood concentration of formaldehyde in F344 rats, rhesus monkeys, and humans. These findings do not preclude the possibility that some formaldehyde is cleared from the nasal mucosa by blood perfusion, but they do suggest that the amount cleared by perfusion, if any, is relatively small.

The rate of change of the concentration of formaldehyde in the mucosal tissue lining the nose of F344 rats or rhesus monkeys is defined by the formula:

$$\frac{dC_f}{dt} = \frac{Q \cdot E}{th} - C_f \left(\frac{V_{\max}}{K_m + C_f} + K_f + K_b \right)$$

where C_f is the concentration of formaldehyde in the tissue (pmol/mm^3), Q is the flux of formaldehyde from air into tissue (pmol/mm^2-min-ppm), th is the mucosal thickness of the tissue (mm), E is the inhaled concentration of formaldehyde, V_{max} is the maximum rate of formaldehyde clearance via the saturable pathway (pmol/mm^3-min), K_m is the concentration at which the enzyme is half-saturated (pmol/mm^3), K_f is the first-order clearance rate constant (min^{-1}), and Kb is the pseudo first-order rate constant for binding of formaldehyde to DNA to form DPX. The rate of change of the concentration of DPX is given by:

$$\frac{dC_{DPX}}{dt} = K_b \cdot C_f - K_{loss} \cdot C_{DPX}$$

where C_{DPX} is the concentration of DPX (pmol/mm^3), K_b is the pseudo first-order rate constant for binding of formaldehyde to DNA (min^{-1}), and K_{loss} is the first-order rate constant for the clearance of DPX (min^{-1}).

BBDR model

Exposure to formaldehyde is related to nasopharyngeal cancer. The initial step in this direction was the use of target tissue dosimetry data based on Formaldehyde-derived DNA protein cross-links (DPX) formation in the nasal respiratory epithelium, for the exposure ¬dose-response characterization step of the overall risk assessment process. Even without specific consideration of mechanisms of tissue response, this innovation markedly affected the predictions of low dose risk. The relationship between formaldehyde exposure concentration and DPX formation is highly nonlinear, with the rate of DPX formation increasing disproportionately as formaldehyde exposure levels increase. The non-linearity exists because some of the inhaled formaldehyde absorbed in the target region never exerts a toxic effect due to nonlinear clearance processes. Important parameter that may lead to nasopharyngeal cancer even in lower concentrations is the mucosal thickness (Conolly et al. 2000). The DPX data were used in place of exposure concentration as input to the LMS (Linear Multi-Stage) model and less risk was predicted for the lower exposure levels. The potential impact of target tissue DPX on formaldehyde risk assessment has also recently been considered by the U.S. EPA (USEPA 1991). Their proposed revision of the 1987 formaldehyde risk assessment (USEPA 1987) predicts a lower risk at low levels of exposure when DPX data are used as input to the LMS in place of inhaled concentration. Taking into account all the above considerations and data, the nasopharyngeal cancer risk probability was estimated by the formula:

$$P = 1 - e^{\left(-0.0239 \cdot y^2 + 0.0245 y\right)}$$

Where ***P*** is the nasopharyngeal cancer probability for a lifetime exposure (70 years) and ***y*** is the average lifetime internal dose of DPX.

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) model

PBPK model

When NNK enters human body via inhalation, through alveoral enters in the blood circulation and is distributed to the body to a concentration equal to this in blood. Metabolism of NNK mainly occurs in the liver, where it is transformed to NNAL (4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanol), which is excreted by the urine. The PBPK model for NNK and its metabolite NNAL consists by five compartments (kidneys, liver, fat, rapidly perfused tissues RTP, and slowly perfused tissues PPT. Considering that the metabolic pathways are common for smokers and people exposed to ETS, data obtained by biomonitoring of smokers, can be generally used for models optimization. It is observed that NNAL and NNAL-Gluc are excreted in the urine more slowly than would be expected, based on their structures (Hecht et al. 1999; Hecht et al. 2002). One week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding values for the structurally related compounds cotinine and nicotine were 1.1 and 0.5%, respectively. Even 6 weeks after cessation, 7.6% of the original levels of NNAL plus NNAL-Gluc remained. The distribution half-life of NNAL and NNAL-Gluc was 3–4 days, while the elimination half-life was 40–45 days (IARC 2007). Total body clearance of NNAL was estimated to be 61.4± 35.4 mL/min, and the volume of distribution in the â-phase was estimated to be 3800 ± 2100 L, which indicates substantial distribution into tissues (Hecht et al. 1999). After cessation of smokeless tobacco use, the distribution half-lives of NNAL (1.32 ± 0.85 versus 3.35 ± 1.86 days) and NNAL-Gluc (1.53 ± 1.22 versus 3.89 ± 2.43 days) were significantly shorter than those in smokers. There were no significant differences in the terminal half-lives. Ratios of (S)-NNAL:(R)-NNAL and (S)-NNAL-Gluc:(R)-NNAL-Gluc in urine were significantly (3.1–5.7 times) higher 7 days after cessation than at baseline in smokers, which indicates stereo-selective retention of (S)-NNAL in humans. From these results, the authors suggest that there is a receptor in the body, possibly in the lung, for (S)-NNAL (Hecht et al. 2002).

BBDR model

In the case of lung cancer, the major metabolic activation pathways of NNK and its main metabolite NNAL involve hydroxylation of the carbons adjacent to the *N*-nitroso group (α-hydroxylation) which leads, via

diazonium ions, to the formation of two types of DNA adduct: methyl adducts such as 7-methylguanine and *O*^{6}-methylguanine, and pyridyloxobutyl adducts. Glucuronidation of NNAL and pyridine-*N*-oxidation of NNK and NNAL are detoxification pathways. The metabolic activation of NDMA occurs by α-hydroxylation leading, via methyl diazonium ions, to the formation of 7-methylguanine and O^6-methylguanine. Denitrosation, producing nitrite and methylamine, is considered to be a detoxification pathway. Aldehydes are also formed in the metabolism of NNK and NDMA.α-Hydroxylation of NNN can lead to the formation of pyridyloxobutyl adducts whereas detoxification occurs by β-hydroxylation, pyridine-N-oxidation and denitrosation/oxidation to produce norcotinine. Ethylene oxide reacts directly with DNA to form 7-(2-hydroxyethyl) guanine and other adducts. There are competing detoxification pathways involving glutathione conjugation. 4-ABP is metabolically activated by N-hydroxylation. Conjugation of the resulting hydroxylamine with acetate or other groups such as sulfate ultimately produces nitrenium ions that react with DNA to produce adducts mainly at C-8 of guanine. Acetylation of 4-ABP can be a detoxification pathway if it is not followed by N-hydroxylation. Ring hydroxylation and conjugation of the phenols result in detoxification. The balance between metabolic activation and detoxification varies between individuals exposed to these genotoxic components of tobacco smoke and is likely to affect cancer risk because DNA adducts are absolutely central to the carcinogenic process induced by these agents. DNA adducts, if unrepaired, can cause miscoding during replication resulting in permanent mutation (IARC 2007). In order to estimate the risk from exposure to NNK, the main input will be the average lifetime internal dose of NNK and NNAL. The final dose response relation was refined by the interpretation of epidemiological data (Schöllnberger et al. 2006). Having in mind that cancer risk associated to NNK is related also to its metabolites and their internal dose, a biology-based approach that takes into account the internal dose of metabolites produced is an improvement compared to traditional risk assessment that provides a more robust biological basis. Based on the above assumptions and taking into consideration that lung cancer seem to be in a non-linear relationship to the years of exposure, the NNK internal dose - response function is given by the formula:

$$P = 1 - e^{\left(-0.0239 \cdot y \cdot N^{1.3}\right)}$$

where ***P*** is the lung cancer probability, ***y*** is the average lifetime internal dose of ***NNK + NNAL*** and ***N*** are the years of exposure to ETS.

Arsenic model

PBPK model

The PBPK model used in our study for the integrated PBPK dose-response model is based on El-Masri and Kenyon's (El-Masri and Kenyon 2008) model formulation. That model is composed of four interconnected PBPK models (Figure 14), each of them simulating the distribution within the human body respectively of the As^{III}, As^{V}, MMA and DMA.

Inter-model processes connect the four models, specifically:

- *the methylation* of iAs^{III} in MMA^{V} and DMA^{V} and the methylation of MMA^{III} in DMA^{V} (sites of methylation: liver, kidney);
- *the reduction and oxidation* of iAs (sites of reduction and oxidation: lung, liver, kidney)
- the inhibitory effects of As^{III} on the methylation of MMA^{III} to DMA and of MMA^{III} on the methylation of As^{III} to MMA (liver, kidney)

Reduction and oxidation of MMA and DMA are simulated within the same model; it is assumed that these processes occur only in lung, liver, kidney; moreover, systemic circulation of MMA^{III} and DMA^{III}, which are formed by the reduction of the pentavalent form, is not considered. Kidney is the site of urinary excretion for AS^{III}, AS^{V}, MMA^{V}, DMA^{V}, MMA^{III}, DMA^{III}; due to the absence of circulation of MMA^{III} and DMA^{III}, urinary

excretion of these compounds occurs also from lung and liver, considering this as an overall estimate of clearance of the chemicals from tissues where they are formed. The distribution of the four forms of arsenic within the different organs is described through ordinary differential equations of the general form shown in the following Equation:

$$\frac{d\{A(t)\}}{dt} = [K]\{A(t)\} + [B]\{q(t)\}$$

Where:

$\{A(t)\}$ = state variable vector which describes the chemical amount in each assigned target organ

$\{q(t)\}$ = input vector expressing the dose rate of chemical entering the organisms

$[K]$ = state matrix which describes the diffusion exchange rate between target organs

$[B] =$ constant input matrix with describes the exchange rate into target organs.

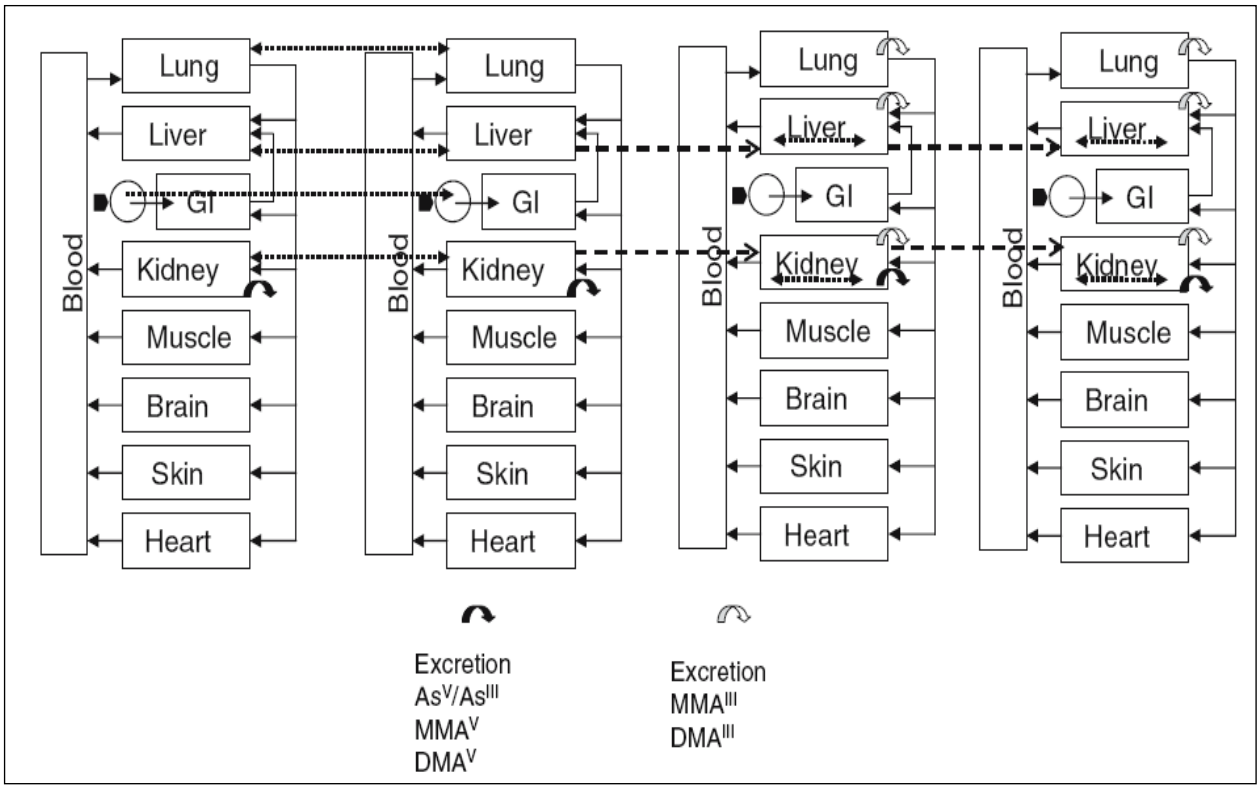

Figure 14. Schematic of the overall PBPK model for inorganic arsenic and methylated metabolites. Source: El-Masri and Kenyon, 2008. Legend: • block arrows to the GI tract lumen (circle): oral exposure to AsIII, AsV, MMAV and DMAV; • small dashed line: reduction of

The reader may refer to El-Masri and Kenyon (El-Masri and Kenyon 2008) for a complete overview of the equations, for each organ and each arsenic form.

Table 10. Physiological and biochemical parameters used within the Arsenic PBPK model

TISSUE	TISSUE VOLUME (L)	BLOOD FLOW (l/min)	TISSUE/BLOOD PARTITION COEFFICIENTS			
			AS^{V}	AS^{III}	MMA	DMA
GI	1.2	1	2.7	8.3	2.2	2.1
SKIN	2.6	0.26	7.9	7.4	2.61	2.4
BRAIN	1.4	0.63	2.4	2.4	2.2	3.3
HEART	0.35	0.2	7.9	7.4	2.61	2.4
KIDNEY	0.28	1	8.3	11.7	4.4	3.8
LIVER	1.82	0.31	15.8	16.5	3.3	3.3
MUSCLE/OTHER	55.5	1.8	2.1	6.7	1.3	1.3
LUNG	0.56	5.2	7.9	7.4	2.61	2.4
BLOOD	5.53	-	-	-	-	-

Table 11. Metabolic parameters used within the Arsenic PBPK model

PARAMETER	DESCRIPTION	VALUE	UNITS
DMA			
Ka	Oral absorption	0.007	min^{-1}
Kred	Reduction of DMA	0.004	min^{-1}
Kox	Oxidation of DMA III	0.65	unitless
Kurine/DMA	Urine Excretion Const	0.13	min^{-1}
MMA			
Ka	Oral Absorption	0.007	min^{-1}
Kred	Reduction of MMA	0.008	min^{-1}
Kox	Oxidation of MMA III	0.63	unitless
Vmax (MMAIII to DMA)	Methylation of MMA III	6.6×10^{-7}	mole/min
Km (MMAIII to DMA)		3×10^{-6}	M
Kinh	noncompetitive	4×10^{-5}	M
Kurine/MMA	Urine Excretion Const	0.3	min^{-1}
INORGANIC AS			
Ka (Asv)	Oral absorption	0.003	min^{-1}
Ka (AsIII)		0.004	min^{-1}
Kred	Reduction of AsV	0.003	min^{-1}

Kox	Oxidation of As III	0.25	unitless
Vmax (AsIII to MMA)	Methylation of As	5.3×10^{-7}	mole/min
Km (AsIII to MMA)		3×10^{-6}	M
Vmax (AsIII to DMA)		2×10^{-6}	mole/min
Km (AsIII to DMA)		3×10^{-6}	M
Kinh	Non-competitive	4×10^{-5}	M
Kurine/As	Urine Excretion Const	0.07	min^{-1}

The PBPK model was validated against the experimental results from Lee (Lee 1999), giving satisfactory matching between those data and the model outputs, as shown in Figure 15.

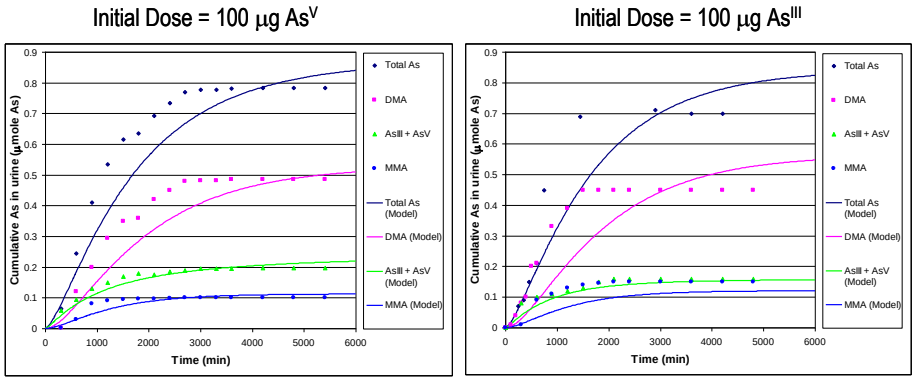

Figure 15. Validation of the As PBPK model

BBDR model

Once the internal dose of the pollutant in target organs have been calculated via the PBPK model, the next step is to link the internal dose with the health point considered to assess the quantitative risk associated with a given exposure. The procedure that we used for the development of the dose-response model for arsenic was the following:

1. selection of a robust experimental data set on health endpoints associated with arsenic exposure;
2. calculation, through the application of the PBPK model, of the internal amount in selected organs given the experimental dataset exposure;
3. linking of the calculated internal amount to the probability of the health risks reported in the experimental dataset
4. development of a dose-response model based on the relationship between pollutant internal amounts and final health points.

Step 4 allows us to develop and parameterise a three-stage model for cancer growth that links internal doses to health risk probability. The general curve, which better describes such a relationship, is in the form of equation 2:

$$P(y) = 1 - e^{(-b\cdot y + c\cdot y^2 + d\cdot y^3)} \quad (2)$$

where:

P[y] = lifetime probability of the health effect

y = biologically effective dose of the toxicant at the target organ

b, c, d = parameters to be calculated fitting a multistage model to the experimental dataset.

Lung, liver kidney and skin fatal cancer has been taken as the health endpoints. We used as independent experimental dataset needed for the development of dose-response models the data reported in the work of Chen et al. (Chen et al. 1992).

In this study the authors investigated the mortality rates due to arsenic exposure amongst residents in an endemic area of chronic arsenicism on the southwest coast of Taiwan. The whole dataset includes 898,806 people, for which a significant dose-response relationship was observed between arsenic levels in drinking water and mortality due to cancer. Analysis of the differences between males and females was also reported.

The study population was stratified into four groups according to the median arsenic level of well water in each village, i.e. < 10 ppm, 0.10-0.29 ppm, 0.30-0.59 ppm, and 0.60 ppm or higher. The study refers to the period 1973-1986, and analysis of people who died from cancers during that period was performed.

PCB mixtures

General information

PCBs (polychlorinated biphenyls) are mixtures of aromatic chemicals, manufactured by the chlorination of biphenyl in the presence of a suitable catalyst. The chemical formula of PCBs can be presented as $C_{12}H_{10-n}Cl_n$, where n is a number of chlorine atoms within the range of 1-10. PCBs generally occur as mixtures, where n can vary from 1 to 10. The 10 sites available for possible chlorine substitution result in 209 possible PCB congeners, but only about 130 congeners are likely to occur in commercial products (WHO/IPCS 1993; Erickon 1997; Basel Convention 2003). The general formula of polychlorinated biphenyls shown in Figure 16.

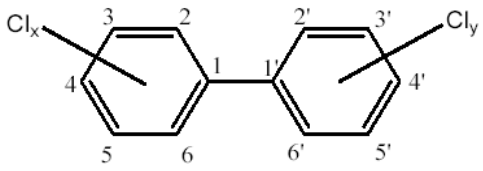

Figure 16. Chemical structure of polychlorinated biphenyls.

Because of their insulating and non-flammable properties, PCBs have been widely used as coolants and lubricants in transformers, capacitors, and other electrical equipment. Out of the 209 PCB congeners, a dozen are now considered by many toxicologists to be "dioxin-like" because of their toxicity and certain features of their structure, which make them similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (2378-TCDD). Under the auspices of the World Health Organization (WHO), 2378-TCDD Toxicity Equivalency Factors (TEFs) have been assigned to the dioxin-like PCB congeners, indicating their toxicity relative to 2378-TCDD, to which itself has been assigned a TEF of 1.0. For example, a PCB congener with a TEF of 0.01 is considered to be one hundred times less toxic than 2378-TCDD. The results of the first WHO determination of TEFs were published in 1994 and were applicable only to humans and mammals (Ahlborg et al. 1994). The second determination, in 1997 (Van den Berg et al. 1998), provided slightly revised TEFs for humans and mammals but also added separate values applicable to fish and birds. Both sets of TEFs are summarized in Table 11.

In June 2005, a WHO-IPCS expert meeting was held in Geneva during which the TEFs for dioxin like compounds, including some PCBs were re-evaluated. For this re-evaluation process, the refined TEF database recently published by Haws and coworkers (Haws et al 2006) was used as a starting point. Decisions about a TEF value were made based on a combination of unweighted relative effect potency (REP) distributions from this database, expert judgment and point estimates. Previous TEFs were assigned in increments of 0.01, 0.05, 0.1, etc., but for this re-evaluation it was decided to use half order of magnitude increments on a logarithmic scale of 0.03, 0.1, 0.3 etc. Changes were decided by the expert panel for 2,3,4,7,8- pentachlorodibenzofuran (PeCDF): TEF=0.3;, 1,2,3,7,8-pentachlorodibenzofuran (PeCDF): TEF=0.03; octachlorodibenzo-p-dioxin (OCDD) and octachlorodibenzofuran (OCDF): TEFs=0.0003; 3,4,4',5-tetrachlorbiphenyl (PCB-81): TEF=0.0003; 3,3',4,4',5,5'-hexachlorobiphenyl (PCB-169): TEF=0.03 and a single TEF value (0.00003) for all relevant mono-ortho substituted PCBs. Additivity, an important prerequisite of the TEF concept was again confirmed by results from recent *in-vivo* mixture studies (Van den Berg et al 2006).

Matrix

Invasive methods: Polychlorinated biphenyls are generally analyzed in blood, mostly in plasma or serum, or in the samples of adipose tissue (Robinson et al. 1990; Choi et al. 2002; Eskenazi et al. 2002; Johnson-Restrepo et al. 2005; Koizumi et al. 2005; Schaeffer et al. 2006).

Non-invasive methods: Polychlorinated biphenyls have been frequently assessed in breast milk (Rogan et al 1986, Clench-Aas et al. 1988; Johansen et al 1994; Becher et al 1995; Korrick and Alsthul 1998; ILCA 2001; Yu et al. 2006). Also cord blood in relation to mother blood was often monitored (Akiyama et al. 1975; Janousek et al. 1994). Another matrix of interest is placental tissue (Pereg et al. 2002; Wang et al. 2005). Besides these matrices, polychlorinated biphenyls were assessed in a large scale of biological species, e.g. fish, mussels, blubber, birds, racoons, bears, etc. (Letcher et al. 1996; Minier et al. 2000; Champoux et al. 2001; Anderson et al. 2003; Smith et al. 2003; Bodin et al. 2004; Devier et al. 2005; Borrell and Aguilar 2006; Minier et al. 2006).

Table 12. Toxicity equivalence factors for several PCB congeners.

Congener Number	IUPAC Chlorobiphenyl Prefix	1994 WHO TEFs[1]	1997 WHO TEFs[2]		
			Humans/ Mammals	Fish	Birds
PCB-77	3,3',4,4'-Tetra-	0.0005	0.0001	0.0001	0.05
PCB-81	3,4,4',5-Tetra-	–	0.0001	0.0005	0.1
PCB-105	2,3,3',4,4'-Penta-	0.0001	0.0001	<0.000005	0.0001
PCB-114	2,3,4,4',5-Penta-	0.0005	0.0005	<0.000005	0.0001
PCB-118	2,3',4,4',5-Penta-	0.0001	0.0001	<0.000005	0.00001
PCB-123	2,3',4,4',5'-Penta-	0.0001	0.0001	<0.000005	0.00001
PCB-126	3,3',4,4',5-Penta-	0.1	0.1	0.005	0.1
PCB-156	2,3,3',4,4',5-Hexa-	0.0005	0.0005	<0.000005	0.0001
PCB-157	2,3,3',4,4',5'-Hexa-	0.0005	0.0005	<0.000005	0.0001
PCB-167	2,3',4,4',5,5'-Hexa-	0.00001	0.00001	<0.000005	0.00001
PCB-169	3,3',4,4',5,5'-Hexa-	0.01	0.01	0.00005	0.001
PCB-170	2,2',3,3',4,4',5-Hepta-	0.0001	–	–	–
PCB-180	2,2',3,4,4',5,5'-Hepta-	0.00001	–	–	–
PCB-189	2,3,3',4,4',5,5'-Hepta-	0.0001	0.0001	<0.000005	0.00001

[1] Ahlborg et al. 1994; [2] Van den Berg et al. 1998.

Kinetics

Despite the enormous number of reports on PCB toxicology, both the causal interpretation of epidemiological studies and the risk assessment of human exposures have been hampered by the lack of information on the pharmacokinetics of various PCB isomers and congeners. Thus, the assessment of exposure by means of measuring either total PCBs or individual congeners in the blood has so far been unsatisfactory (Lotti 2003).

Uptake (by different routes)

The lipid solubility of PCBs increases with chlorination and promotes absorption across lipophilic cell membranes of the skin, the lung and the gastrointestinal tract (Van den Berg et al.1998; Van Birgelen and Van den Berg 2000). Adults in the general population are mainly exposed to PCBs and related compounds by consuming dairy products, meat and fish. In newborns, PCBs are transferred from exposed mothers to their babies both prenatally (in utero) and postnatally via breast feeding (Yakushiji et al.1984; Longnecker et al.1999; Covaci et al.2002). Passive absorption of these compounds is thought to occur via the aqueous environment of the intestine across the more lipophilic cell membranes of the intestine wall. The concentration gradient favours partition across the cells into the blood (Matthews and Dedrick 1984). Food is the main source of human intake of PCBs; intake through drinking water is negligible. Daily intake of total PCBs in Sweden was estimated at 0.05 μg kg^{-1} body weight (bw), with a 50% contribution from fish (Darnerud et al.1995). This is markedly lower than an earlier Finnish estimate of 0.24 μg kg^{-1} bw (Moilanen et al.1986), and might reflect the decreasing trends in PCB levels in Nordic food. The decline is similar in Germany, where daily intake at that time was estimated to be somewhat below 1 μg kg^{-1} bw (Beck 1994).

Several studies confirm that individual congeners and their mixtures are readily absorbed from the gastrointestinal tract of rodents and monkeys. Gastrointestinal absorption of individual congeners in rats has been reported to vary between 66% and 96%. The degree of absorption decreases with increasing chlorination (Bergman et al.1982). Rapid absorption and distribution, comparable to that after oral exposure, has been observed in rats exposed to an aerosol of a PCB mixture (Pydraul A200) via inhalation (Benthe et al. 1972). The effect of vehicles on gastrointestinal absorption of PCBs has not been systematically evaluated.

Several studies with PCB congeners or mixtures have demonstrated effective dermal absorption. In guinea pigs absorption of mixtures was at least 33–56% during 16 days of exposure, whilst monkeys absorbed at least 20% during 28 days of exposure (Wester et al. 1983). In rats, up to 60% of 3,3',4,4'-tetrachlorobiphenyl was absorbed after 3 days of exposure (Jackson et al. 1993).

Metabolism

For the purpose of this project an excellent source of information on PCB metabolism can be found in several published reviews treating this topic (WHO 2000; Lotti 2003; WHO 2003). The rate limiting step in the elimination of PCBs is metabolism, which primarily occurs via the hepatic cytochrome P-450-dependent monooxygenase system, and which varies depending on the chlorination pattern of the congener. Hydroxylated products are major metabolites, with hydroxylation occurring primarily at the para or meta positions if these sites are unsubstituted. Arene oxides occur as intermediate metabolites in the oxidation of some PCBs. They are reactive and can be converted both spontaneously and enzymatically to detoxified products (phenols, dihydrodiols, glutathione conjugates), which are excreted. Alternatively, they can form other potentially toxic (cytotoxic, mutagenic, carcinogenic), covalently-bound substrate macromolecular adducts. Besides hydroxylation and subsequent conjugation, sulfur-containing metabolites (e.g. methyl sulfones) and partially dechlorinated metabolites have also been identified. The

methyl sulfonyl and hydroxylated PCB metabolites have been detected in human milk and plasma as well as in other biological samples from the environment. Both types of PCB metabolites can preferentially accumulate in specific tissues such as the lung and the fetus. The rate of metabolism of PCB and the resultant metabolite pattern vary between different species. The excretion of PCB congeners is, to a large extent, dependent on their rate of metabolism to more polar compounds. Most congeners show biphasic elimination, where the initial half-life is relatively short for all congeners, but the later half-life is much longer and clearly structure-dependent. There is a large variation in half-lives between different PCB congeners depending on the number and position of the chlorine atoms; the range is from a few days to 450 days depending on the congener.

Metabolites of all the congeners studied so far are eliminated primarily via the bile and the faeces. However, those congeners chlorinated to a lower degree are excreted to a greater extent (although less than 5%) via the urine than those chlorinated to a higher degree. Several experiments in both rodents and monkeys demonstrated that PCBs crossed the placental barrier and were distributed to fetal tissues. At birth, approximately equal or lower levels of PCBs were found in the young as compared to the dam. In contrast to this, transfer of PCBs through suckling accounts for much higher exposure of the young than does placental transfer. In studies with PCB mixtures, postnatal exposure generally resulted in higher concentrations in the weaning young than in the mother (WHO 2000).

Most of the toxic coplanar and mono-ortho coplanar PCB is not readily metabolised. Lower chlorinated PCBs and those congeners that have two adjacent unsubstituted carbon atoms and an unsubstituted para-position are thought to form arene oxide intermediates by cytochrome P450 isoenzymes and subsequently to rapidly produce more polar metabolites. As with polychlorinated dibenzofurans and polychlorinated dibenzodioxins, the cytochrome P4501A isoenzymes seem to play an important role in the metabolism of those PCB congeners that are more or less isosteric with 2,3,7,8-tetrachlorodibenzodioxin. Whereas several studies have investigated the metabolism of PCB in different species and shown the involvement of various P450s, much less is known about the isoenzymes involved in their metabolism in humans. Metabolism of PCBs in humans results in the formation of the hydroxylated metabolites. Whereas many hydroxylated PCBs are further converted to either glucuronic acid or sulfate conjugates by phase II enzymes, which facilitates their excretion, some OH-PCBs persist in the body. Glucuronidation of OH-PCBs seems to be correlated with surface area and surface volume of the molecule, thus explaining, in part, why some OH-PCBs may persist in the body. The dominating OH-PCBs retained in human plasma, accounting for up to 20% of total plasma PCBs, are 4-OH-PCBs derived by 1-ortho-PCBs through a 1,2-shift of a chlorine in the para position (Lotti 2003).

Biological half life

It is impossible to offer reliable figures of PCB half-life, because results may vary substantially depending on exposure circumstances and the pharmacokinetic model used for calculation. For instance, PCB clearance rates from serum were different in a group of formerly exposed capacitor workers from those of Yusho patients. Mono-ortho chlorinated congeners were cleared 3–7 times as fast in Yusho patients as in the capacitor workers, while the di-ortho chlorinated congeners were cleared 3–7 times more slowly. These differences have been attributed to possible changes of liver cytochromes induced by dibenzofurans in Yusho patients. A great inter-individual variability in the rates of elimination of PCBs has also been observed. For instance, the half-life of dioxins and PCBs has been shown to increase with age, probably because of a concurrent increase of adipose tissue and reduced metabolism. Variability in the rates of elimination of some more persistent congeners was also observed among Yu-Cheng patients. Whole blood half-lives of 2,3',4,4',5-pentachlorobiphenyl, when calculated 9–18 months after exposure in the two time points, varied from 4.1–24 months (Lotti 2003). Several reviews on this topic are available at http://www.atsdr.cdc.gov/toxprofiles/tp17.html).

Operational aspects of sampling, sample preparation and storage

The sampling procedures vary greatly according to the type of sample matrix. *Adipose tissue* should be frozen after sampling; then the sample can be freeze-dried or homogenised using liquid N_2 and ground with anhydrous sodium sulfate. *Breast milk* (or other milk samples) can also be frozen and, after sampling and transporting to the laboratory, freeze-dried or chemically extracted. If the milk samples are not frozen immediately after sampling they should be treated by potassium dichromate. *Foodstuffs* represent a very broad matrix category. Meat and fish samples should be frozen right after the sampling and the handling with samples after transport is similar to adipose tissue. Butter, fats and oils are generally assumed to be homogenous and usually do not require sample pre-treatment before clean-up procedure (no more than drying with anhydrous sodium sulfate). *Blood* should be clotted and centrifuged to isolate serum. The serum samples should be frozen and stored at -20°C until analytical procedure. Finally, dried samples should be stored at –20°C until they are treated for analyses, preferably in glass containers.

Analytical aspects

Chemical-analytical techniques

Many of the analytical methods used for biological and environmental samples are the methods approved by various agencies and organizations such as EPA and the National Institute for Occupational Safety and Health (NIOSH), Association of Official Analytical Chemists (AOAC) and the American Public Health Association (APHA). Additionally, analytical methods were developed that modify previously used methods to obtain lower detection limits, and/or to improve accuracy and precision (Turci et al. 2004). Methodology for PCB analysis includes several steps: sample collection and storage, extraction, cleanup, and determination (Hess et al. 1995; EPA 1995c; EPA 1999k). The trend is toward congener-specific analysis by high-resolution gas chromatography (HRGC).

PCBs are routinely analysed by one-dimensional capillary gas chromatography equipped with electron capture detector (ECD) or micro-ECD in biological and food samples (DS DS/EN 1528-1

1997). The analysis of non-ortho, mono-ortho and some non-dioxin like PCB congeners requires pre-separation column liquid chromatography and the use of high-resolution mass spectrometry (HRMS) is inevitable. Recent advances include analytical methods that are able to quantify individual PCB congeners to enable TEQ calculations (Patterson et al. 1994; EPA 1999k; Frame 1999). EPA Method 1668 (Revision A) is the most efficient and precise current methodology used to measure individual PCB congeners in water, soil, sediment, and tissue by HRGC/ HRMS (EPA 1999k).

PCBs can also be separated by multi-dimensional GC heart-cutting methods (de Boer and Dao 1991a, 1991b). These methods enhance the separation power of capillary GC, but are limited to the analysis of only a few discrete, critical regions of the chromatogram, and time for analysis may become very long.

Comprehensive two-dimensional gas chromatography (GCxGC) combined with micro-ECD is a method which can be used to analyse complex mixtures of PCBs. The advantages of GCxGC are the large peak capacity and an increase of signal/noise ratios as a result of the focusing effect (Korytár et al. 2002; Korytár 2006).

In a sample containing PCBs, there are often several dozens of different congeners. For practical reasons, all of them are not always measured, but the most important congeners are used as indicators. Σ7PCB, a selection of PCB congeners denotes the sum of the seven marker PCBs. In Belgian chicken incident, only seven abundant congeners were usually measured: congeners with IUPAC numbers 28, 52, 101, 118, 138, 153, and 180 (2,4,4'-TriCB, 2,2',5,5'-TCB, 2,2',4,5,5'-PeCB, 2,3',4,4',5-PeCB, 2,2',3,4,4',5'-HxCB, 2,2',4,4',5,5'-HxCB, 2,2',3,4,4',5,5'-HpCB, respectively). The seven congeners are estimated to constitute about one third of all PCBs in the contaminated feed. 2,2´,4,4´,5,5´-hexachlorobiphenyl (CB-153) is also frequently used as a biomarker for POP exposure, because it correlates very well with both total PCB concentration in plasma and serum (Grimvall et al,. 1997; Glynn

et al,. 2000) and with the PCB derived dioxin-like effect as well as the total POP derived dioxin-like effect (Gladen et al. 1999).

Bio-analytical techniques

U.S. Environmental Protection Agency Method 4425[1] utilizes a reporter gene system (RGS) based on cytochrome P450 to screen samples for a range of organic compounds including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), coplanar polychlorinated biphenyl congeners (PCBs), and high molecular weight polynuclear aromatic hydrocarbons (PAHs). The method is a screening procedure that will detect the total amount of planar compounds in solvent extracts of environmental samples of soil, sediment, tissue, and water. Method 4425 serves as both, a biomarker simulating the response of an organism (with CYP1A) exposed to inducing compounds, and as a bioanalytical technique measuring the levels of these chemicals in the samples. Methods based on similar principles are commercially available[2] (CALUX , CAFLUX assay).

The ELISA (Enzyme Linked Immunosorbent Assay) is commonly used for the analysis of environmental samples and has recently been used for food samples as well (Kočan 2004). The enzyme immunoassay method uses an immobilized antibody to facilitate the separation of targeted analytes (antibody-bound components) from non-target substances using a washing step and an enzyme conjugate to generate the signal used for the interpretation of results. While GC-ECD and/or GC/MS is able to quantify individual PCB congeners of different reactivity and toxicity, the data reported indicate that immunoassay offers a rapid and inexpensive alternative method for estimating "total" PCBs (Zajicek et al. 2000; Fillmann et al. 2002).

Sensitivity and specificity

EPA Method 1668 (Revision A) is the current methodology used to measure individual PCB congeners in water, soil, sediment, and tissue by HRGC/HRMS. Estimated detection limits (EDL) of selected PCB congeners range from 109 to 193 pg L^{-1} for water and 11–19 ng kg^{-1} for soil, tissue, and mixed-phase samples. EDLs are listed in this report for EPA Method 1668 (Revision A; EPA 1999k).

Units

Results on polychlorinated biphenyls are expressed in several ways. The most common is to express concentrations of the analyte as weight per wet matrix weight either as ppb or microgramm per g. Since polychlorinated biphenyls are highly lipophilic, the concentrations of individual congeners are generally expressed as a weight unit per gram of lipid (usually ng g^{-1} lipid).

PCBs are present in biological matrices as complex mixtures and in order to facilitate the comparison of analytical and exposure data, risk assessment, and regulatory control, it has proveduseful to convert the analytical results into toxic equivalents (TEQ). The differences in toxicity are expressed in the toxic equivalency factors (TEFs), estimated from the weaker toxicity of the respective congener in relation to the most toxic compound (2,3,7,8-TCDD) and by summing up the contributions from each congener the total TEQ value of a sample can be obtained. Several different TEF schemes have been proposed. Until recently, the most widely used scheme has been the WHO scheme for PCBs (PCB-TEFs) (Ahlborg et al. 1994). In 1997, WHO and the International Programme on Chemical Safety (IPCS) arranged an international meeting that resulted in a consensus on WHO-TEFs for PCDDs, PCDFs, and dioxin-like PCBs for both human and fish and wildlife risk assessment (Van den Berg et al. 1998; Van den Berg et al. 2006).

1 US EPA http://www.epa.gov/epaoswer/hazwaste/test/pdfs/4425.pdf

2 For more references see http://www.biodetectionsystems.com/lit_ldr.html

Performance characteristics

Analytical reproducibility: The reproducibility of analytical measurements of PCBs should be assured by good laboratory practise, quality assurance and quality control (QA/QC) applied in analytical laboratories and using reliably verified and/or validated methods and techniques (e.g. isotope dilution method using HRMS detection EPA 1668).

Inter- and intra-laboratory variability:Inter-laboratory comparison studies in PCB analysis in different matrices are organised by many accredited/certified institutions worldwide. Inter-laboratory assessment studies on PCDDs/Fs and PCBs in human milk and blood serum have been organized by WHO/EURO in 1989 1992, and 1997. Laboratories further have the yearly opportunity to assess their quality in inter-laboratory quality assessments organised by G-EQUAS (German External Quality Assessment Scheme) – Intercomparison programme for toxicological analyses in biological materials - occupational and environmental medical field (blood serum). Variability of analytical results is reported by Youden-pairs and if the results of the laboratory on two concentration levels (in two samples) of each compound lie within statistically appointed intervals (mean of results ±3 SD, standard deviation), the laboratory gains the certification. The variation coefficients for PCBs ranged from 7 to 14% in Intercomparison programme 35 in 2005. Other proficiency testing or inter-calibration studies are organized by the Food Analysis Performance Assessment Scheme (FAPAS), the Ministry of Agriculture, Fisheries and Food, United Kingdom, by the Norwegian Institute of Public Health, Oslo (Dioxins in Food, dioxin-like PCBs analysed), and by the University Consortium Environmental Chemistry, Marghera.

Validation

There are internationally accepted validated methods for PCB analysis in different matrices: the EPA Method 1668 (soil, sediment, tissue), and the EN 1528 method (fat containing food samples). Because of the lipophilic character of PCBs, these internationally validated methods can be used for breast milk, adipose tissue and human serum as well. Each laboratory that uses modified or non-validated methods should establish performance characteristics of the method such as accuracy, precision, reportable range of the results (another detailed characteristics concerning the result reportable range are: limits of detection, limits of quantification, calibration range, etc.) and prove the reliability of the method by analysing certified reference materials and by inter- and intra-laboratory testing.

Confounding factors

Several factors were reported to influence the levels of PCBs in biological samples:

Age: PCBs bioaccumulate in the fatty tissues of humans and animals during the lifetime, hence higher age is associated with higher body burden of PCBs (Bjerregaard et al. 2001; Shadel et al. 2001; Chao et al. 2004; Hovander et al. 2006; Uehara et al. 2006).

Gender: The complex role of gender should be taken into consideration when PCB exposure in human population is being assessed. Results do not show clear trends in exposure between genders among studies. Higher PCB levels in men, if compared to women, were often reported (Falk et al. 1999; Hanrahan et al. 1999; Petrik et al. 2006), but other authors did not confirm this association (Apostoli et al. 2005; de Saeger et al. 2005; Dirtu et al. 2006).

Diet: The major source of PCBs in the human population represents food, mainly food of animal origin - fish, meat, milk and diary products (Feeley 1995; WHO 2003). PCB exposure was strongly influenced by fish intake in populations characterized by high fish consumption (Grimvall et al. 1997; Dewailly et al. 1993; Sjodin et al. 2000).

Socio-economic status: Borell et al. (2004 reported higher PCB serum concentrations associated with family income, but not with maternal education in Afro-American women. The authors concluded that maternal socioeconomic indicators may influence the effects of exposure to PCBs among African-American pregnant women. Vartiainen et al. (1998) and Chao et al. (2004) found positive associations

between PCB levels in breast milk and maternal education. Race differences in exposure to several PCB congeners were reported with higher exposure in non-white participants (James et al. 2002).

Body Mass Index (BMI): James et al. (2002) has shown the decline in total PCBs to be 11% per 5-unit increase in BMI. The inverse relationship between PCB body burden and BMI was confirmed by other authors (Vartiainen et al. 1998; Wolff et al. 2000; Hagmar et al. 2006). In contrast, other studies reported positive association between PCBs and BMI (Furberg et al. 2002; Tee et al. 2003).

Parity and lactation: In general, higher levels of PCBs in breast milk were found in primiparas and decreasing PCB levels were associated with longer lactation (Furst et al. 1989; Vartiainen et al. 1998; Grimvall et al. 1997; Needham and Wang 2002).

Concentrations reported in literature

PCB congener levels in human milk collected for related WHO (WHO 2005) surveys in 2005/2006, 2001/2002 and 1992/1993 the are listed in Table 13, Table 14 and Table 14, respectively. Table 16 reports PCB concentrations in human blood plasma, whereas Tables 17 and 18 give PCB-153 congener levels in human blood serum, where Table 18 provides, in addition, data on the sum of PCBs.

Dose-response/effect relationships

Despite an enormous number of reports on PCBs, both the causal interpretation of epidemiological studies (Wolff et al. 1997; DeVoto et al. 1997) and the risk assessment of human exposures (Safe 1994; Safe 1990; Safe 1998) have so far been hampered by the different toxicological properties of various congeners and isomers, their large variability in kinetics and dynamics, and the relative paucity of key data regarding humans. In particular, numerous factors including isomer and congener composition, various degrees of contamination with other chemicals (e.g. chlorinated benzofurans), and quantitatively inconsistent data have made it difficult, up to now, to interpret PCB levels in human blood. Risk assessment consists of four steps: hazard identification, exposure assessment, dose-response assessment, and risk characterization. By definition concentration-effect relationship means a link between the exposure of a given system to a substance over time and the magnitude of a specific, continuously graded change to that system. As outlined in the previous section, such PCB exposure-response functions for humans are rather largely unknown. This is why in risk assessment in connection to human PCB exposures the procedure relies mainly on data obtained with experimental animals. This requires introduction of various uncertainty factors when applying to human population.Epidemiological studies are crucial to risk assessment, but offer only incomplete insight due to the limitations of observational studies, which depend, e.g., on study opportunities. Often the follow-up is too short, or the exposure estimates do not reflect the long induction time of the health outcomes under study. These problems tend to cause an underestimation of the true effects caused by PCBs (Grandjean P, 2003).

Health effects of PCBs

Because of their ubiquitous occurrence in all kinds of environmental media, persistent organic pollutants (POPs) have become some of the most important global environmental contaminants (Jones and Voogt 1999). Laboratory and field observations of animals, clinical and epidemiological studies and studies with cell cultures demonstrated that exposure to certain POPs may be associated with a wide range of biological effects including biochemical deviations, carcinogenesis, neurological changes, reproductive and behavioral abnormalities and dysfunctions of the immunological network (Ritter et al. 1995). Among POPs, the polychlorinated biphenyls (PCBs) represent one of the most important groups of chemicals. Aroclor-1254 is one of the many technical mixtures of PCBs that were widely used in industrial insulation materials, plasticizers and heat transfer fluids. Like DDT various PCBs have been detected in human tissues, especially in fat tissues including breast milk (Kodama and Ota 1980; Mersch-Sundermann et al. 1996). The use and production of PCBs have been banned for decades, but PCBs are still an environmental problem. The concentrations in food have declined since the pioneering reports by Sören Jensen and others in the 1970s and 80s. However, it was recently reported that in fish from some parts

of the Baltic Sea, the decline has not continued during the 90s (Atuma et al.1996; Bignert 2002; Kiviranta et al. 2003).

PCBs are classified by EPA as carcinogens, particularly with regard to the liver. Reproductive and developmental effects may also be related to occupational exposure to PCBs and eating contaminated fish. Studies indicate that PCBs concentrate in human breast milk. PCBs can be passed easily into the bloodstream from a pregnant woman to a fetus, and from a breastfeeding mother to a nursing infant. Slight effects on birth weight, head circumference, gestational age and/or neonatal behavior have been reported in infants of mothers who were consumers of PCB-contaminated fish, or were otherwise exposed to PCBs (Patandin et al.1999; Grandjean et al. 2001; Karmaus and Zhu 2004; Hertz-Picciotto et al. 2005).

Table 13. Median PCB-153 congener levels in human milk reported in the 4th WHO-Coordinated Survey of Human Milk for Persistent Organic Pollutants - Report of status and results as available on August 11, 2006 by Dr. Reiner Malisch.

Country	Matrix	Year of sampling	N° of samples in pool	PCB-153 (ng g^{-1} fat, median)
Cyprus	Human milk	2005/2006	(>50)	11.67
Norway	Human milk	2005/2006	(>50)	30.54
Slovak Republic	Human milk	2005/2006	51	107.17
Luxemburg	Human milk	2005/2006	(>50)	52.89
Hungary	Human milk	2005/2006	(>50)	7.98
Czech Republic	Human milk	2005/2006	(>50)	155.54
Sudan	Human milk	2005/2006	(>50)	21.49

Table 14. PCB levels in human milk reported in the 3rd round of the WHO-coordinated exposure study 2001 – 2002 (Van Leeuwen and Malisch, 2002).

Country	N° of pools	PCBs (WHO-TEQ pg g^{-1} fat, median)		Σ PCBs (ng g^{-1} fat, median)	
		median	range	median	Range
Australia	2	2.89	2.52 – 3.26	30	25 – 36
Belgium	2	12.6	11.22 – 13.98	191	169 – 213
Brazil	11	1.77	1.3 – 12.28	16	10 – 97
Bulgaria	3	4.21	3.74 – 4.70	42	32 – 52
Croatia	2	7.17	6.82 – 7.52	135	121 – 150
Czech Republic	3	15.24	14.32 – 28.48	502	496 – 1009
Egypt	9	5.48	4.41 – 8.26	106	12 – 140
Fiji	2	1.75	1.70 – 1.80	17	16 – 19
Finland	2	5.85	5.66 – 6.03	91	84 – 98
Germany	4	13.67	12.80 – 14.311	220	188 – 238
Hong Kong SAR	11	4.73	2.80 – 6.58	45	16 – 80
Hungary	3	2.87	2.38 – 4.24	34	29 – 59
Ireland	4	4.57	2.72 – 5.19	60	41 – 65
Italy	4	16.29	11.02 – 19.33	253	195 – 323
Luxembourg	2	13.67	12.98 – 14.36	217	196 – 237
New Zealand	3	3.92	3.50 – 4.71	37	30 – 41
Norway	2	8.08	6.56 – 9.61	119	106 – 132

Philippines	2	2.38	2.22 – 2.54	26	26 – 26
Romania	3	8.06	8.05 – 8.11	173	165 – 198
Russia	7	13.45	12.92 – 22.95	126	84 – 311
Slovak Republic	4	12.60	10.72 – 19.49	443	331 – 621
Spain	6	9.42	6.93 – 17.94	241	162 – 467
Sweden	1	9.71	-	146	-
The Netherlands	3	11.57	10.90 – 13.08	192	178 – 210
Ukraine	3	19.95	14.10 – 22.00	136	103 – 148
USA	2	4.61	3.69 – 5.52	54	43 – 64

Table 15. Median PCB-153 congener levels and other PCB levels in human milk reported in the 2nd round of the WHO-coordinated exposure study 1992 - 1993 (WHO European Centre for Environment and Health. Levels of PCBs, PCDDs and PCDFs in human milk. Environmental Health in Europe No.3).

Country	Town	N° of individual samples in pool	PCB-153 (ng g^{-1} fat, median)	dl PCBs (WHO-TEQ pg g^{-1} fat, median)	Σ marker PCBs (ng g^{-1} fat, median)
Albania	Tirana	10	32.2	2.3	63.4
	Librazhd	10	22.2	1.7	42.5
Austria	Vienna	13	146.0	11.7	380.6
	Tulln	21	119.0	12.4	302.5
	Brixlegg	13	174.0	19.0	449.4
Belgium	Brabant Wallou	8	128.6	7.4	275.5
	Liege	20	142.6	4.7	306.2
	Brussels	6	122.1	7.8	260.5
Canada	Maritimes 92	20	37.7	4.1	86.1
	Quebec 92	20	59.5	6.8	137.3
	Ontario 92	20	49.6	7.7	128.0
	Prairies 92	20	21.6	3.2	57.9
	British Columbia 92	20	25.5	3.5	70.2
	All provinces 92	100	44.5	5.3	111.7
	Gaspe	12	94.7	12.7	219.7
	Canada all provinces 81	200	87.0	12.1	212.1
	Basse Cote-Nord	4	283.9	25.4	558.6

	Ungave Bay	4	282.8	14.1	576.0
	Hudson Bay	5	716.7	21.3	1361.4
Croatia	Krk	10	103.0	6.1	218.4
	Zagreb	13	99.8	8.0	219.5
Czech Rep.	Kladno	11	215.0	6.0	531.6
	Uherske Hradiste	11	424.8	9.8	1068.5
Denmark	7 different cities	48	99.6	4.5	209.4
Finland	Helsinki	10	88.8	4.6	188.9
	Kuopio	24	65.1	2.4	133.5
Germany	Berlin	10	165.0	11.7	375.0
Hungary	Budapest	20	31.6	1.7	61.3
	Scentes	10	23.2	1.4	45.0
Lithuania	Palanga	12	163.5	20.4	362.0
	Anykshchiai	12	127.4	20.7	287.0
	Vilnius city	12	143.3	20.5	321.7
Netherlands	mean of 17 individual samples	17	113.3	11.0	253.1
Norway	Tromso	10	127.5	19.5	272.6
	Hamar	10	127.9	10.4	264.5
	Skien/Porsgrun n	10	140.8	9.5	301.5
Pakistan	Lahore	14	5.7	2.3	18.9
Russian Fed.	Arkhankelsk	1	94.4	8.6	196.8
	Karhopol	1	50.5	4.9	102.3
Slovak Rep.	Michalovce	10	434.9	13.3	1015.1
	Nitra	10	207.7	6.1	489.0
Spain	Bizkaia	19	185.7	10.6	461.3
	Gipuzkoa	10	187.6	8.2	451.7
Ukraine	Kiev nr.1	5	122.7	15.0	263.5
	Kiev nr.2	5	91.2	11.5	191.3
United Kingdom	Birmingham	20	56.9	4.3	129.5
	Glasgow	23	57.8	4.0	130.8

Exposure to PCBs can also be by inhalation or skin contact. Studies show that irritations such as lesions, rashes, and burning eyes and skin can occur in PCB-exposed workers (Fischbein et al. 1982; Smith et al. 1982; Tsai et al. 2006).

Populations at high risk of exposure to PCBs include nursing infants whose mothers consume large amounts of contaminated fish; embryos, fetuses, and neonates; and people who work or live in buildings that have high concentrations of PCBs in the indoor air supply.

Table 16. Median PCB-153 congener levels in plasma from humans reported by Lotta Hofvander (Polychlorinated biphenyls and their metabolites in human blood, Method development, identification and quantification, Doctoral thesis in environmental chemistry, Faculty of Science, Stockholm University 2006)

Country	District	Sampling year	sex/matrix	N° of samples	PCB-153 (ng g^{-1} fat, median)
Slovakia	Michalovce	2001	female/male	175	240
	Svidnik/Stropkov	2001	female/male	122	570
Netherlands		1998/2000	maternal	51	100
		1998/2000	cord plasma	51	115
		2001/2002	maternal	90	63
		2001/2002	cord plasma	9	62
Faroe Islands		1994/1995	maternal	57	430
		2000/2001	children	42	310
Sweden		1995	female	16	50
		1995	female	16	290
		1991	male	20	220
		1991	male	12	450
		2000/2001	maternal	15	56
		2000/2001	cord plasma	15	44
Latvia		1993	male	19	160
		1993	male	26	920
Nicaragua		2002	female	4	14

		2002	female	4	100
Canada Quebec	Nunavik	1993/1996	cord plasma	10	131
	Lower N.Shore	1993/1996	cord plasma	10	215
	Southern Quebec	1993/1996	cord plasma	10	52

Table 17. Median PCB-153 congener levels in blood serum presented by Dr. Kočan at UC Davis, CA, July 10-13, 2006

Country	District	Sampling year	Specimen from	N° of samples	PCB-153 (ng g^{-1} fat, median)
Slovakia	Michalovce, Svidnik	2001/2002	mothers	1094	138
			mothers	141	224
			6-month-old babies	141	168
			16-month-old babies	141	152

Biomarker Systems for PCB exposure, effects and susceptibility

Regarding the use of biomarkers, an important document published by EHP (Bennett and Waters 2000) should be taken into account. The following general commentary fully applies to biomarkers targeting at PCB risk. The majority of diseases are the consequence of both environmental exposures and genetic factors. To understand the relationship between exposure and adverse health effects, scientists are working to identify biomarkers - key molecular or cellular events that link a specific environmental exposure to a health outcome. Biomarkers are indicators of molecular and cellular events in biological systems, and may allow epidemiologists and clinicians to better examine relationships between environmental hazards and human health effects. The identification, validation and use of biomarkers in environmental medicine and biology will depend fundamentally on an increased understanding of the mechanism of action and the role of molecular and biochemical functions in disease processes. For environmentally-induced diseases, molecular

Table 18. Median PCB-153 congener levels and sum of PCBs in blood serum reported in literature

Country	Note	N° of samples	PCB-153	Σ PCBs
Germany[1]	Infants breast-fed/bottle-fed (serum)		0.38/0.10 ng ml^{-1} serum	1.19/0.29 ng ml^{-1} serum
Japan[2]	Maternal serum	32		61.5 ng g^{-1} lipid (0.467 ng g^{-1} serum)
	Umbilical cord serum	32		63.8 ng g^{-1} lipid (0.136 g^{-1} serum)
Japan[3]		24	0.171 ng g^{-1} whole blood	0.771 ng g^{-1} whole blood
Alaska, St. Lawrence	Human blood serum	60		1500 ng g^{-1} lipid

Island[4]				
Mohawk women[5]	Human blood serum	111		1.2 ng g^{-1} serum

[1]Lackmann, et al. 2004; [2]Fukata et al. 2005; [3]Hirai et al. 2005; [4]ACAT 2003; [5]Fitzgerald et al. 2004.

biomarkers will play a key role in understanding the relationships between exposure to toxic environmental chemicals, the development of chronic human diseases and identifying those individuals at increased risk for disease. Although much progress has been made to identify potential biomarkers, the challenge still remains to validate, in a robust manner, the accuracy, reproducibility, specificity, and sensitivity of biomarkers, and to assess the feasibility and cost-effectiveness of applying biomarkers in large population-based studies. Such validated biomarkers will be invaluable in the prevention, early detection and early treatment of disease. It can be stated that few scientists apply stringent criteria to biological end points before proclaiming them biomarkers. While established guidelines for biomarker validation exist, methods for their implementation and case studies testing the methods are rare. Molecular epidemiology relies on molecular genetics and biochemical techniques to assess the risks of disease at the individual level, as opposed to classical epidemiology which is based on the determination of disease risks in populations. Molecular epidemiology tools include: markers of exposure (blood or urine levels of exogenous compounds and/or their metabolites), markers of biologically effective doses (DNA or blood protein adducts); markers of biological effects (chromosomal aberrations, sister chromatid exchange, micronuclei); markers of cancer susceptibility (genotyping and phenotyping for polymorphism in xenobiotic metabolism).

Sentinel aquatic organisms, with regard to biomarkers for monitoring of PCB human exposure

The aquatic environment is particularly sensitive to the toxic effects of contaminants since a considerable amount of the chemicals, PCBs included, used in industry, urbanization, and in agriculture enter marine and other aquatic environments. The aquatic environment is a sink for endocrine disrupting chemicals (EDCs) and other organic chemicals; therefore it is not surprising that there exist many examples of endocrine disruption in fish (Kime 1998, Kime 1999). Molecular and cellular biomarkers measured in aquatic organisms respond rapidly to the stress caused by environmental contaminants, and can be used to assess the health status of organisms and to obtain early-warning signals before irreversible damage occurs at a higher level of biological organization (Huggett et al. 1992). This property makes aquatic biomarkers indispensable for human environmental health. In a multi-pollution context, it is now recognized that the use of a series of biomarkers is necessary to provide a good understanding of the actual impact of contaminants on organisms. A critical aspect of the multi-biomarker approach is the selection of complementary biomarkers in order to obtain the most complete and reliable information (Cajaravillem et al. 2000; De Lafontaine et al. 2000). The choice of biomarkers to be assessed is most often determined a priori, by considering their physiological role and the (eco)toxicological significance of their responses as characterized in information concerning the numerous potential interactions between contaminants involved in the mechanisms responsible for biochemical, cellular or physiological responses. Providing evidence of the mechanisms involved for each chemical element alone constitutes the first step in understanding how they can interfere when they are present in mixtures (Ait-Aissa et al. 2003). As sentinel aquatic organisms the following species occur in available literature: blue mussels (*Mytilus sp.,* Devier et al. 2005), zebra mussels (*Dreissena polymorpha,* Minier et al. 2006), smallmouth bass (*Micropterus dolomieu* , Anderson et al. 2003), demersal fish (*Solea ovata,* Au and Wu 2001) and others.

Cytochrome P450 1A subfamily induction, potential biomarker of PCB exposure

The cytochrome P450 1A subfamily has been frequently used as biomarker of contaminant

exposure as a result of their induction in wildlife (fish, birds, mammals) that inhabit industrialized areas (Arinç et al. 2000; Široká and Drastichová 2004). In particular, carps from the Kalamazoo River Superfund Site contaminated with polychlorinated biphenyls (PCBs) were responding to PCB exposure via

upregulation of CYP1A independent of the activation of the oxidative stress response genes normally thought to be co-regulated with CYP1A (Fisher et al. 2006).

Hepatic ethoxyresorufin-O-deethylase (EROD) activity as an indicator of PCB exposure

The review published by Whyte et al. (2000) compiles and evaluates existing scientific information on the use, limitations, and procedural considerations for EROD activity (a catalytic measurement of cytochrome P4501A induction) as a biomarker in fish. A multitude of chemicals induce EROD activity in a variety of fish species, the most potent inducers being structural analogs of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Although certain chemicals may inhibit EROD induction/activity, this interference is generally not a drawback to the use of EROD induction as a biomarker. The various methods of EROD analysis currently in use yield comparable results, particularly when data are expressed as relative rates of EROD activity. EROD induction in fish is well characterized, the most important modifying factors being fish species, reproductive status and age, all of which can be controlled through proper study design. Good candidate species for biomonitoring should have a wide range between basal and induced EROD activity (e.g., common carp, channel catfish, and mummichog). EROD activity has proven value as a biomarker in a number of field investigations of bleached kraft mill and industrial effluents, contaminated sediments, and chemical spills. Research on mechanisms of CYP1A-induced toxicity suggests that EROD activity may not only indicate chemical exposure, but also may precede effects at various levels of biological organization. A current research need is the development of chemical exposure-response relationships for EROD activity in fish. In addition, routine reporting in the literature of EROD activity in standard positive and negative control material will enhance confidence in comparing results from different studies using this biomarker.

Exposure of rainbow trout to 3,3‘,4,4‘-tetrachlorobiphenyl (PCB-77) (1 mg kg^{-1}) for 21 days strongly induced EROD activity (Ait-Aissa et al. 2003). In newly fertilised rainbow trout eggs the order of EROD induction potential was: total extract > polycyclic aromatic compounds fraction > dicyclic aromatic compounds-fraction including PCBs > aliphatic and monocyclic aromatic compounds fraction in all matrices (Sundberg et al. 2005b). The lack of a clear relationship between toxicopathic effects and EROD induction in the same test system underlines the need for a battery of biomarkers for estimating environmental risk (Sundberg et al. 2005a; Sundberg et al. 2006).

Molecular chaperons heat shock proteins HSP70 and HSP60 as non-specific biomarkers of proteotoxicity in relation to PCB exposure

Heat-shock proteins (HSPs) are a family of diverse proteins, of molecular size ranging from 10 to 150 kDa, involved in the transport, folding and assembly of newly synthesised proteins. These proteins have been found in organisms as diverse as bacteria, molluscs, fish, insects and humans (Lindquist and Craig 1988), showing their important cellular function and relevance for living organisms. Under adverse environmental conditions, the heatshock proteins act to repair and protect cellular proteins in order to minimise protein aggregation (Cheng et al. 1989; Chiang et al. 1989; Martin et al. 1992; Kim et al. 1993; Ryan and Hightower 1996; David and Grongnet 2001). Thus, besides constitutively expressed members, there are some stress induced proteins that can be monitored and related to environmental stressors (Hightower 1991; Becker and Craig 1994). Consequently, over the past decade, an increasing number of studies have explored the possibility of using HSPs as biomarkers of adverse effects at the cellular level in both laboratory and field experiments (for reviews see Sanders (1993) and de Pomerai (1996)). Some authors have proposed that the induction and subsequent accumulation of HSPs, specifically HSP70, may be useful in environmental monitoring (Sanders 1990; Kohler et al. 1992; Sanders and Martin 1993). Alteration in cellular proteins, by either activated PCB adducts and/or ROS attack, can trigger the induction of HSP70. In rainbow trouts exposed to coplanar 3,3‘,4,4‘-tetrachlorobiphenyl (PCB77) (1 mg kg^{-1}) for 21 days an induction of heat shock proteins HSP70 was observed (Ait-Aissa et al. 2003).

Expression of multi-xenobiotic resistance (MXR) proteins

The expression of multi-xenobiotic resistance (MXR) proteins is a biomarker of human exposure to PCBs. The multi-xenobiotic resistance phenotype is derived from the expression of membrane-bound proteins

(MXR-proteins or P-glycoproteins) that can actively transport toxic compounds out of the cell, thus decreasing their toxicity (Endicott and Ling 1989). These proteins belong to a superfamily of transporters termed ABC-proteins or traffic-ATPases (Ames et al. 1990; Higgins 1992). Members of this family have been identified in nearly every organism in which they have been looked for: these include micro-organisms, yeast, plants and animals (Higgins 1992). The importance of this system for aquatic organisms has been shown by its potential to protect them from nuclear damage (Waldmann et al. 1995) and deleterious effects on cell division during embryonic development (Toomey and Epel 1993). The expression of MXR proteins (or P-glycoproteins, Pgp) is inducible by exposure to toxic compounds (Minier and Moore 1996) and the amount of Pgp varies significantly between differentially polluted sites (Minier et al. 1993). Accordingly, the use of the MXR phenotype could be an index of exposure to toxic compounds (reviewed by Minier et al. 1999). In zebra mussels (*Dreissena polymorpha*) from Seine estuary with PCB toxic equivalent quantities varying between 20 to 40 pg dioxin equivalents g^{-1} dry weight high levels of multi-xenobiotic resistance proteins were detected (Minier et al. 2006).

Antioxidant defence biomarkers in relation to PCB exposure

In mammals and fish liver, PCBs are metabolized by the P450 enzymes to mono- and hydroxyl metabolites, which can be oxidized to the highly reactive corresponding (semi)quinones. These compounds generate ROS and form adducts with and alter macromolecules such as DNA and proteins (Safe, 1994; Srinivasan et al. 2001). PCB77 strongly induced EROD activity, HSP70 and TEAC, but no effect on antioxidant defence biomarkers of rainbow trout exposed to coplanar 3,3‘,4,4‘-tetrachlorobiphenyl (PCB-77) for 21 days (1 mg kg^{-1}) was observed (Ait-Aissa et al. 2003) and no significant mRNA upregulation occurred in the specific oxidative stress genes (gamma-glutamylcysteine synthetase and magnesium superoxide dismutase) and metabolic genes (phosphoenolpyruvate carboxykinase and nucleolin) examined in carp fish population resident in the Kalamazoo River Superfund site in Michigan contaminated with polychlorinated biphenyls (PCBs) (Fisher et al. 2006).

Oxidative damage indicators seem to be more suitable biomarkers for PCB effects. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and PCBs have been shown to elicit oxidative stress as indicated by decreased antioxidant levels and increased superoxide anion production, lipid peroxidation, GSSG:GSH ratio, and DNA damage in mammals and fish (Hassan et al. 1983 and 1985a,b; Stohs et al. 1983; Wahba et al. 1989; Pohjanvirta et al. 1990; Stohs et al. 1990; Alsharif et al. 1994; Palace et al. 1996; Hori et al. 1997; Hassoun et al. 1997; 2000; Shertzer et al. 1998). After incubation of calf thymus DNA with either higher- or lower-chlorinated PCB congeners, no significantly increased levels of oxidative DNA damage above background levels, measured as 8-oxo-7,8-dihydro-2′deoxyguanosine.could be detected (Schilderman et al. 2000).

Hormones thyroxin, testosterone and gonadotropins as biomarkers of PCB exposure

No associations between the POP-markers (CB-153) and thyroxin, testosterone, and gonadotropins, respectively, were found in middle-aged and elderly men (fishermen) with CB-153 concentrations of 370 ng g^{-1} lipid (median) and 5th and 95th %-ile values of 110 and 1010 ng g^{-1} lipid (Rylander et al. 2006).

Prostate-specific antigen (PSA) in sperm, sperm DNA integrity and sperm motility as a potential biomarker of PCB exposure

There was a significant linear association between CB-153 and the total amount of PSA (slope [b] 522.5, 95% confidence interval [CI] 24.0, 20.9; P 5 0.02). With age, abstinence time and smoking included in the model the association became non-significant (b 521.4, 95% CI–3.0, 0.1; P 5 0.07) (Rignell-Hydbom et al. 2005a). In the same population group a significantly lower DNA fragmentation index (%DFI), a measure of sperm chromatin structure, was found in the lowest CB-153 quintile (< 113 ng g^{-1} lipid) compared with the other quintiles (Rignell-Hydbom et al. 2005b). When CB-153 was categorized into quintiles, the subjects in the quintile with the highest concentration (>328 ng g^{-1} lipid), tended to have decreased sperm motility compared with the subjects in the lowest quintile (<113 ng g^{-1} lipid) (Rignell-Hydbom et al. 2004).

Biomarkers of DNA damage, mutagenesis and carcinogenesis

Among the adverse effects caused by POPs, DNA damage, mutagenesis and carcinogenesis are crucial. For example, POPs, such as chlordane, toxaphene, DDT, PCBs, heptachlor, hexachlorobenzene and benzofuran, have been classified as probable carcinogens. Numerous toxicological studies have been published, which showed negative results for PCBs in the Salmonella assay in the absence and presence of an exogenous metabolizing system S9 (Wyndham et al. 1976). Aroclor-1254 was not able to induce micronuclei *in-vivo* using polychromatic erythrocytes obtained from mammalian bone marrow (Heddle et al. 1983), supplying evidence that the PCB-mixture does not act as a direct genotoxicant. Also no treatment-related effects were seen on bone marrow micronuclei following subchronic exposure at the minimum risk level to a complex mixture of persistent contaminants, including polychlorinated biphenyls (Wade et al. 2002) in male rats. Administration of Aroclor-1254 was per se devoid of any influence on cytotoxic and cytogenetic effects in bone marrow polychromatic erythrocytes and pulmonary alveolar macrophages but tended to attenuate these changes produced by cigarette smoke or ethanol in both types of cell (Balansky et al. 1993). Both PCB-126 and non dioxin-like PCB-153 were not genotoxic to porcine follicles independently of the stage of their development. (Gregoraszczuk et al. 2004). The teachers in a school polluted by polychlorinated biphenyls did not manifest an increased level of micronucleated cells or sister chromatid exchanges (Wiesner et al. 2000). Several other studies are in agreement with these data (van Pelt et al. 1991). Concerning PCB metabolites, five methylsulphonyl polychlorinated biphenyl congeners failed to induce the formation of micronucleated cells at doses of 5.2 to 9.6 ppm, which were about 35,000 times higher than the concentrations in the lungs and adipose tissue of healthy Japanese people (Nagayama et al. 1995). On the other hand, a significant increase in micronuclei was recorded in lymphocytes from newborns born 12 months after contamination (9.36 +/- 5.60), in comparison to controls (5.53 +/- 3.02) and newborns born 18 months after contamination (6.14 +/- 3.57) due to a leakage of several tons of polychlorinated biphenyls into the environment and groundwater (Milosevic-Djordjevic et al. 2005). Also in workers exposed to polychlorinated biphenyls the incidence of micronuclei as well as the exchange of sister chromatids were elevated as compared to controls (Joksic and Markovic 1992). The PCB congener, 3,4,5,3',4'-pentachlorobiphenyl, was found to be a very potent inducer of micronuclei what can be considered as indicator of biological and genetic damage due to exposure to carcinogens or mutagens, in cultured human lymphocytes (Nagayama et al. 1995).

However, Aroclor-1254 is a well-known inducer of xenobiotic-metabolizing enzymes (XME), especially of cytochrome-dependent oxigenases, e.g. aryl hydrocarbon hydroxylases. CYP1A1 play a key role in the activation of Benzo-a-pyrene (BaP) and other polycyclic aromatic hydrocarbons (PAHs). Therefore, from the mechanistical point of view, Aroclor-1254 should be able to enhance the genotoxicity of PAHs, especially of BaP in metabolically complement cells such as HepG2. This assumption was supported by studies (Mendoza-Figueroa 1985) in which Aroclor-1254 enhanced the genotoxicity of BaP in primary liver cells of rats. Additionally, according to Mersch-Sundermann et al. 1996 the in vivo treatment of rats with PCB-mixtures enhanced the toxifying potency and the CYP1A content of S9 derived from the livers of the treated animals. As shown in another study (Wu et al. 2003), Aroclor-1254 alone did not affect the MN frequencies in HepG2 cells, but when the cells were pretreated with 23-181 microM Aroclor-1254, BaP exposure caused significantly more MN than BaP alone.

Genetic susceptibility to environmental PCB - impact for human health

The most important chronic diseases of modern industrialised societies, including heart disease and cancer, have a multifactorial origin, and a great number of environmental and genetic risk factors have been identified. Gene-environment interactions are of primary importance in the area of individual susceptibility to the development of specific diseases in their response to environmental hazards. Many scientific studies demonstrate that certain genetic polymorphisms can serve as biomarkers of susceptibility to specific environmentally-initiated diseases (Au et al. 1999). Biomarkers of susceptibility, indicates the inter-individual variation in mechanistic processes on the continuum between exposure and effect. An individual's susceptibility to environmentally-mediated diseases may arise from genetic causes

or from non-genetic factors such as age, gender, disease state, or dietary intake. Genetic polymorphisms may function as biomarkers of susceptibility, but it is important to keep in mind that it is actually the phenotype that is of importance for the final response to the hazardous insult. With regard to the multifaceted biological effects of polychlorinated biphenyls the spectrum of clinical endpoints to which these biomarkers should target is very broad: development of Parkinson's Disease, susceptibility to allergic responses, association between glutathione S-transferase pi polymorphism and susceptibility to a variety of diseases including cancers of the lung, oesophagus and stomach, genetic polymorphisms associated with susceptibility to breast cancer, endothelial cell activation and the pathology of atherosclerosis.

DNA adducts as biomarkers of PCB exposure

Biphenyls are metabolically activated to electrophilic quinoid species capable of binding to DNA. Rodents as well as human hepatic enzymes metabolize lower chlorinated biphenyl congeners to reactive intermediates that form DNA adducts *in-vitro.* The para-quinone metabolites of PCBs are, in part, involved in direct DNA adduction (Pereg et al. 2002). *In-vivo* PCB-DNA adducts could not be detected in rats orally treated with a mixture of PCBs (Aroclor-1242) by either the butanol- or by the NP1-enrichment procedure in rat target tissue DNA (Schilderman et al. 2000). In another quantitative study using HPLC/ESI-MS/MS and ^{32}P-postlabeling-HPLC the adduct levels were in the range of 3-1200 adducts per 10^8 nucleotides. These results demonstrate that increasing chlorine substitution is associated with lower yields of DNA adduct (Zhao et al. 2004).

Pesticides

There is a large body of literature reporting health effects of pesticides[3] and it is out of the scope of this document to summarize the evidence for impacts of pesticides - which are toxic by definition - on organisms. Less information is available on health effects of mixtures of pesticides. Mixture or combination effects are particularly relevant for health effects of plant protection products, as food frequently contains multiple residues, pesticide products usually are mixtures of several active substances[4] and humans usually are exposed to pesticides via multiple exposure routes (Table 19).

Table 19. Human exposure to pesticides

Uptake route	source of exposure
ingestion – food	- insecticides used for the protection of food and feed - herbicides residuals in food - residues in food originating from food contact material such as packaging
ingestion – water	- residuals of plant protection products in drinking water originating from agricultural activities - insecticides used for combating Malaria and other diseases through drinking water treatment with larvicides
inhalation	- application of pesticides - pesticides used for avoiding the transport related spread of species (container treatment) - products used for the disinfection of furniture, waste, air-conditioning systems and swimming pool water
dermal contact	- application of pesticides incl. veterinary use such as combating pet's parasites - pharmaceuticals e.g. used to combat lice or fungi - insecticides and fungicides used to protect consumer products - pesticides in water

Epidemiological studies report increased health risks due to self reported or assumed pesticides exposure, for example differences in the probability of certain health cases between farmers and less exposed persons (see section C-1.3.2.2), but information on the pesticides mixture is lacking and substance specific exposure has not been characterised precisely. Exposure to pesticides is complex and difficult to quantify so that dose-response-relationships for effects of defined pesticide mixtures on human health are not available yet. More is known about effects on targets like enzymes or cells tested *in-vitro* and impacts on test organisms taken as an indicator or biomarker for human health risks. A review of the 1985-1998 literature on the health risk of low-dose pesticides mixtures has been published by Carpy et al. (2000). The authors report studies on acute toxicity, *in-vitro* cell toxicity, sub-chronic toxicity, carcinogenesis, mutagenicity, neurotoxicity, and immunological effects. Anyway, despite their relevance for

[3] datasheets reporting toxicological data and health effects of pesticides can be downloaded using ESIS, the European Chemical Substances Information System (http://ecb.jrc.ec.europa.eu/esis/). The United States Environmental Protection Agency provides respective information via the page http://www.epa.gov/pesticides/reregistration/status_page_d.htm. Another source of information is the pesticides data sheets page of http://www.inchem.org/pages/pds.html.

[4] for example, chlorophenoxy herbicides are mixtures of similar active compounds (e.g. 2,4-D and esters, 2,4,5-T and esters, and others) with a similar mechanism of action present in various combinations in marketed products (see http://www.inchem.org/pages/pds.html for products constituents). The term active substance is defined in Part G.

risk assessment these systems do not allow to conclude on the number of cases of impaired health to be expected at a given exposure situation. Mixture risk assessment for pesticides thus focused on predicting the complex human exposure and comparing the outcome to known exposure levels of no concern.

The footprint database[5] provides information on health issues for active substances of pesticides by classifying the available state of knowledge using the categories "yes, known to cause a problem", "no, known not to cause a problem", "possibly, status not identified" and "no data" for the health issues "carcinogen", "endocrine disrupter", "reproduction / development effects", "acetyl cholinesterase inhibitor", "neurotoxicant", "Respiratory tract irritant", "Skin irritant" and "Eye irritant". For some endpoints a limited number of studies on health effects of pesticides mixtures which might be of interest in the context of full chain assessment are shortly mentioned in the following where emphasis is laid on endpoint specific biomarker systems indicating effects of pesticides mixtures. Anyway, health endpoint specific biomarkers for effects of pesticides are, in many cases, not specific for pesticides but reflect effects of all substances and other stressors affecting the respective health endpoint.

Toxicity to humans of nearly all types of pesticides is linked to neurotoxic effects. Since they act via different mechanisms it is unlikely that integrating bioassays or marker systems exist reflecting effects of all substance classes on neuronal functioning and development. Another study using cell lesions as indicator for combined pesticides effects has been performed by Ito et al. (1995). The authors investigated the effects of pesticides mixtures (19 organophosphorous and one organochlorine pesticide) on induced liver lesions in rats and used the intensity of preneoplastic lesion development initiated by diethylnitrosamine as an indicator for adverse effects.

Genotoxicity

Genotoxicity of pesticides has been investigated in numerous studies some of which were based on *in-vitro* tests of effects on human cells. Usually, significant effects, relative to the control, are taken as an indicator for genotoxic effects on humans, but they do not allow calculating the number of cases of impaired health from pesticides exposure and are therefore for limited significance for the full chain assessment of policy impacts. Anyway, in many cases linear dose-effect relationships were observed, for example between DNA damage scores and exposure levels for the neonicotinoid imidacloprid, and another herbicide, RH-5849[6], using the comet assay (see below) with human peripheral blood lymphocytes *in-vitro* (Feng et al. 2005). Such quantitative dose-effect algorithms might allow calculating the probability of genotoxic effects from the pesticide concentration to which cells are exposed, which, in turn, might be calculated using PBPK modelling using pesticide intake rates as data input. Anyway, integrated policy assessment requires additional information such as the relationship between genotoxicity biomarker and human health cases.

Biomarker systems for genotoxicity of pesticides have been used in several publications. Das et al. investigated genotoxicity induced by pesticide mixtures using specific effects on human blood lymphocytes as an indicator for DNA damage. In the study mentioned above (Feng et al. 2005) the mutagenicity risk due to human exposure to the neonicotinoid insecticide imidacloprid was tested using the comet assay, the micronucleus test (MN) and the sister chromatid exchange (SCE) test with blood lymphocytes from healthy volunteers incubated with pesticides. All methods are described in detail, references are given and the publication further compares the significance of the genotoxicity biomarkers used and their interpretation. Generic information on the micronucleus test is provided by Decordier and Kirsch-Volders (2006). A further study on genotoxic effects of imidacloprid on human peripheral blood lymphocytes has been performed by Costa et al. (2009). The authors used the comet assay and the MN test mentioned above. Whether reactive oxygen species were involved in the process leading to genotoxicity was

[5] http://sitem.herts.ac.uk/aeru/footprint/en/index.htm

[6] RH-5849 (2'-benzoyl-l'tert-butylbenzoylhydrazine) is an entomological growth-regulating agent.

investigated by the use of the 2',7'-dichlorfluorescein diacetate (DCFH-DA) assay in human T lymphocytes as described in Engelmann et al. (2005). A further unspecific biomarker for pesticides effects is using an enhanced detoxification demand as a biomarker for adverse effects on cells. The indicator is the increase in the activity of glutathion-s-transferase, an enzyme involved in the detoxification of various xenobiotic chemicals including pesticides.

Endocrine disruption

Regarding endocrine disruptors several specific problems should be noted. Firstly, for many active substances of pesticides it is not known whether they have endocrine disrupting effects. Testing is difficult as effects occur at concentrations substantially below the concentrations applied for other endpoints, exposure during certain developmental stages of the embryo has stronger effects, and a wide spectrum of endpoints is affected. The second problem results from the missing information on the composition of the mixture. Sewage slugde has been found to have endocrine disrupting activity, but it seems to be difficult to assign this activity to a certain set of compounds. The question arises whether biomarkers for endocrine disrupting effects are available. Hayes et al. (2006) used larval growth and development of frogs, their sex differentiation, immune function, and plasma corticosterone levels as indicator systems for endocrine disrupting effects of pesticides and found that mixtures had much greater effects on larval growth and development of frogs than single compounds. Frogs were used because endrocrine disruption is assumed to be responsible for Amphibian declines. Interactions may play a more important role for endocrine disruptors as compared to other chemical/endpoint groups. This assumption is supported by a study on the cancer risk attributable to mixtures of chemicals: Even if the exposure to certain pesticides or phthalates alone may not cause breast cancer, there is concern about the cocktail effect as exposure to a combination of these chemicals may interact with pharmaceuticals, in this case hormones, to trigger cancer (Health and Environment Alliance Report 2008).

Groups of pesticides evaluated together

Common mechanism groups

For chemicals having a common mechanism of action[7] concentration or dose addition as desribed in Part A, section A-4.3 of this document is the appropriate method for assessing combined pesticide toxicity. Extensive guidance[8] published by the EFSA and US EPA exists on how to deal with cumulative exposure to pesticides having the same mechanism of action. The available methodology is shortly described for organophosphates, N-methyl-cabamates, triazines and chloroacetoanilides in the sections C-1.2.1.1 to C-1.2.1.4.

Organophosphates

Organic phosphate type insecticides[9] inhibit acetylcholinesterase (AChE), an enzyme catalyzing the hydrolysis of the neurotransmitter acetylcholin into cholin and acetic acid, a reaction necessary to allow a

[7] for a definition of the term "common mechanism" see Part G.

[8] a guidance on cumulative risk assessment (EPA 2002a) of pesticide chemicals that have a common mechanism of toxicity is available at http://www.epa.gov/pesticides/cumulative/methods_tools.htm. The summary report of the EFSA scientific colloquium on cumulative risk assessment (EFSA 2006) and other EFSA documents (EFSA 2008, EFSA 2009, EFSA 2010) gives further information on methods used to decide whether a combined exposure to several pesticides should be regarded as a risk to human health, even if the concentrations of the single compounds are below the level of no concern set as maximum residues levels (MRLs, defined in Part G). EC regulation 149/2008 provides a list of the allowed pesticides and MRLs for each pesticide for a wide variety of food types (EU legislation is summarised in section C-4.3).

[9] e.g. acephate, azinphos-methyl, bensulide, chlorethoxyfos, chlorpyrifos, chlorpyrifos-methyl, diazinon, dichlorvos (DDVP), dicrotophos, dimethoate, disulfoton, ethoprop, fenamiphos, fenthion, malathion, methamidophos, methidathion, methyl parathion, mevinphos, naled, oxydemeton-methyl, phorate, phosalone, phosmet, phostebupirim, pirimiphos-methyl, profenofos, terbufos, tetrachlorvinphos, tribufos and trichlorfon.

cholinergic neuron to return to its resting state after activation[10]. Health effects of chronic exposure occur if the inhibition exceeds the rate of acetylcholinesterase regeneration (Hazleton 1955). Organophosphates degrade faster than organochlorides and are therefore widely used for combating insects. As they affect a specific metabolic process the concentration or dose addition concept (A-4.3) is recommended in several publications. A specific EPA publication (EPA 2002b) on cumulative exposure and risk assessment for mixtures of organophosphates is available. The described procedure is based on the following principle: The ability of each compound of the group to inhibit AChE is related to the respective activity of the index chemical, methamidophos[11]. The resulting relative potency factors which are specific for oral, dermal and inhalative uptake are given for several organophosphate insecticides on pp 17 and 18 of EPA (2002b). Other index compounds have been selected in a study on the cumulative exposure to AChE inhibiting compounds in the Dutch population (Boon and van Klaveren, 2003). The authors show that the overall outcome of the cumulative risk assessment depends on the choice of the index chemical.

The EU pesticides database[12] reports an acceptable daily intake (ADI) of 0.001 mg per kg body weight per day, an Acute Reference Dose (ARfD) of 0.003 mg per kg body weight per day and the classification of methamidophos as "R26/28: Very toxic by inhalation and if swallowed", "R24: Toxic in contact with skin" and "R50: Very toxic to aquatic organisms". Anyway, there is no algorithm available directly relating long-term uptake of this insecticide to human health. ADIs and ARfDs were gained through extrapolation from animal testing data and *in-vivo* and *in-vitro* assays such as those reported in the methamidophos IUCLID dataset [13] published by the European Chemicals Bureau.

Biomarker for organophosphate exposure:

A reduced Acetylcholinesterase (AChE) activity in blood, relative to the control group, is a biomarker for human exposure to organophosphates (Mutch et al. 2008). The study further reports a higher total urinary dialkylphosphate (DAP) concentration as a biomarker integrating over organophosphates. The determination of 8OHdG[14] allowed identifying increased formation of reactive oxygen species in the exposed group. But, increased oxidative stress is not specific for effects of organophosphates and can not be regarded as a biomarker for this substance group. Applying the Comet method revealed higher DNA damage associated with pesticides exposure. Anyway, DNA damage is not a suitable marker for organophosphates exposure, as a multiplicity of factors affects genotoxicity.

The net document[15] "Biomarkers for exposure: Organophosphates" gives details on biomarkers for chlorpyrifos exposure: The organophopsphate insecticide chlorpyrifos and its more toxic metabolite, chlorpyrifos-oxon, undergo hydrolysis. One hydrolysis product of both substances, 3,5,6-trichloro-2-pyridinol and its degradation product, (TCP), is a specific biomarker for chlorpyrifos exposure. TCP does not inhibit cholinestrase enzymes. Hydrolysis of the insecticide occurs in nature as well and can be taken up by humans what complicates the interpretation of biomarker levels. Diethylphosphate (DEP), a chlorpyrifos-oxon hydrolysis product which is not specific for chlorpyrifos, is used as a biomarker for general organophosphate exposure (see above). Anyway, the authors state that more research is needed to derive biomarker-health relationships for these marker substances.

[10] For detailed informationon the mode of action and symptoms of exposure to organophosphates see http://www.npic.orst.edu/RMPP/rmpp_ch4.pdf.

[11] CAS No. 10265-92-6.

[12] http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.detail

[13] available via http://ecb.jrc.ec.europa.eu/esis/index.php?PGM=ein

[14] 8-hydroxy-2'-deoxyguanosine

[15] National Pesticide Information Center, (NPIC) located at the Oregon State University, USA, http://npic.orst.edu/mcapro/OPBIOMARKERs.pdf

Several dialkylphosphate metabolites were used as group specific biomarkers (Becker et al. 2007, Schulz et al. 2009) for exposure of children to organophosphates: dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP). These metabolites are degradation products of nearly all organophosphates such as chlorpyrifos, diazinon, malathion and parathion and are dertermined in urine. A human bio-monitoring program[16] revealed reference values for organophosphate metabolites reflecting the exposure of the german population to this pesticides type (Becker et al. 2007, Schulz et al. 2009).

N-methyl-carbamates

N-methyl-carbamates[17] (NMCs) are insecticides poisoning organisms by reversibly inhibiting AChE. Whereas AChE inhibition by organophosphate insecticides is irreversible, the enzyme activity recovers within minutes to hours after inactivation by carbamates (EPA 2007). The NMCs share the ability to inhibit AChE by carbamylation of serine hydroxyl groups located in the active site of the enzyme and therefore cumulative risk assessment[18] has been performed using the dose addition approach (EPA 2007) with oxamyl[19] as the index or reference chemical. The ability to inhibit AChE in the brain of rats was used to relate the toxicity of the NMCs to the toxicity of the index chemical.

Oxamyl is on the positive list of allowed pesticides in the EU and widely used to protect potatoes from aphid infestations of cyst nematodes (EC SANCO 2006). Both the acceptable daily intake (ADI) and the Acute Reference Dose (ARfD) of oxamyl are set to 0.001 mg per kg body weight per day[20]. Oxamyl is classified as "R26/28: Very toxic by inhalation and if swallowed", and "R21: Harmful in contact with skin" and "R51/53: Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment". According to WHO (2008) oxamyl is unlikely to occur in drinking water, but for other carbamates, aldicarb[21] and carbofuran[22] drinking water threshold concentrations of 10 and 7 µg l^{-1} were set, respectively.

Biomarker for *N*-methyl-carbamates exposure:

A reduced AChE activity is an indicator for both, exposure to organophosphate insecticides and NMCs. The fast reversibility is typical for carbamates, so that measurements with a high time resolution might be suitable to distinguish between effects of these pesticides groups. Brain AChE inhibition measurements show less variability than determinations in blood (EPA 2007).

Triazine herbicides

The triazine pesticides and their metabolites are a group of closely related herbicides. They are banned in the EU but used widely in agriculture and non-agricultural sites in other parts of the world. Triazines block the electron transport in photosystem II and are applied for combating weed (mono- and dicotyledons). Triazines are relatively stable, so that residuals of former use can be still present in the environment. Simazine, atrazine and terbutylazine and their degradation products have been frequently found in

[16] http://www.umweltbundesamt-umwelt-deutschland.de/umweltdaten/public/theme.do?nodeIdent=3186

[17] e.g. Aldicarb, Carbaryl, Carbofuran, Formetanate*, Methiocarb*, Methomyl*, Oxamyl*, Pirimicarb*, Propoxur* and Thiodicarb. The substances marked with "*" are included in the list of substances approved for use in the EU.

[18] Detailed information on cumulative risk assessment for carbamates is available at http://www.epa.gov/pesticides/cumulative/common_mech_groups.htm#carbamate

[19] CAS No. 23135-22-0

[20] http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.detail

[21] CAS No. 116-06-3

[22] CAS 1563-66-2

European river waters (Loos et al. 2009). Anyway, their concentration levels were distinctly below the level above which risk to human health can be expected and much lower than drinking water threshold[23] values set by the WHO (WHO 2008).

Certain triazines[24] have been identified by U.S. EPA as a common mechanism group because of their ability to cause neuroendocrine and endocrine-related developmental, reproductive and carcinogenic effects resulting from altered levels of hypothalamic gonadotrophin-releasing hormone (GnRH) and catecholamine levels, which affect via several steps the development of the reproductive system and its maintenance and functioning in adulthood. Reproductive hormones further modulate numerous other metabolic processes. For cumulative risk assessment performed by U.S. EPA[25] (EPA 2006a) the examined triazines were considered to be toxicologically equivalent to atrazine.

Biomarker for Triazine exposure:

Lucas et al. (1993) proposed to develop an ELISA[26] for the mercapturic acid conjugate of atrazine as a biomarker for human atrazine exposure. This compound has been found to be the major urinary metabolite of this herbicide. According to Buchholz et al. (1999) mercapturate metabolites and dealkylated atrazine dominate the early metabolites in the urine. Several days after the dose was delivered, other metabolites of atrazine were prevailing. Deethyl atrazine and atrazin mercapturate were found in urine of workers collected 8 hours after exposure due to herbicide application, underlining the suitability of these metabolites as indicators for exposure (Perry et al. 2000). But, only 37 samples out of 99 samples taken from person who reported atrazine exposure contained deethylatrazine. Only 50 samples were tested positively on atrazine mercapturate, suggesting that a lack of atrazine biomarker does not indicate zero exposure. Furthermore, it remains questionable, whether the methods can be used to screen for low level chronic exposure of humans via drinking water.

ELISA assays for the metabolites of atrazine and cyanazine are now available and have been used in a study published by Hines et al. (2003) for monitoring of herbicide biomarkers in urine of exposed herbicide applicators. Levels of other atrazine metabolites, deethyl atrazine and deisopropyl atrazine, were measured using gas chromatography with mass selective detection.

Chloroacetanilides

Chloroacetoanilides are systemic herbicides inhibiting growth of plants through blocking an enzymatic step of the gibberellin pathway. With respect to toxicology, the chloroacetanilides acetochlor[27], alachlor[28] and butachlor are considered as a common mechanism group due to their ability to cause nasal turbinate tumors via a metabolite that leads to cytotoxicity and regenerative proliferation in the nasal epithelium. They have a further common end-point – acetochlor, alachlor and butachlor affect T3/T4

[23] the drinking water threshold values (WHO 2008) for atrazine (CAS No1912-24-9), simazine (CAS No. 122-34-9) and terbutylazine (CAS No. 5915-41-3) are 2, 2 and 7 μg l^{-1}, respectively. The maximum and most frequently detected concentrations in European river water were 0.046 and 0.001 μg l^{-1} for atrazine, 0.169 and 0 μg l^{-1} for simazine, and 0.124 and 2 μg l^{-1} for terbutylazine, respectively (Loos et al. 2009).

[24] atrazine, simazine, propazine, diaminochlorotriazine (DACT), desethyl-s-atrazine (DEA) and desisopropyl-s-atrazine (DIA); DACT, DEA and DIA are metabolites.

[25] the document "Triazine cumulative risk assessment" is avaialble at http://www.epa.gov/oppsrrd1/REDs/triazine_cumulative_risk.pdf.

[26] ELISA abbreviates "enzyme linked immunosorbent assay".

[27] CAS No. 34256-82-1

[28] CAS No. 15972-60-8

hormone production in the thyroid of rats. Cumulative risk assessment has been performed by the U.S. EPA[29] for acetochlor and alachlor (EPA 2006b) with alachlor as the index chemical. Butachlor has been excluded because it has no registered uses in the U.S. In the European Union acetochlor, alachlor and butachlor are not on the list of allowed pesticides.

Biomarker for exposure to chloroacetanilides:

Several publications are available on chloroacetanilides effects on earthworms and respective unspecific bioindicators. Exposure of male rats to butachlor lead to increased activities of enzymes involved in the metabolisation of xenobiotics[30] (Hisato 1998). Anyway, biomarkers for these processes located in liver cells are not specific for chloroacetanilides. Specific ELISA kits are available for the determination of alachlor mercapturate and metolachlor mercapturate, the metabolites of alachlor and metolachlor, respectively, in urine. Hines at al. (2003) applied these test kits for measuring biomarker concentrations in urine of workers occupationally exposed to chloroacetanilides herbicides during application of these substances.

Similar mode of action groups

Organophosphates and *N*-methyl-carbamates might be grouped as they have a similar mode of action – they inhibit AChE, whereas the mechanism of action differs. A precondition for grouping is that the toxicity, relative to the index chemical, can be tested. Since the fast recovering inhibition by *N*-methyl-carbamates can not be directly compared to the more stable AChE inhibition by organophosphates both pesticide classes are treated separately in risk assessment.

Other pesticides classes

For the above mentioned classes of pesticides, i.e. organophosphates, N-methyl-carbamates, trianzines and chloroacetoanilides, cumulative risk assessment has been performed as mentioned above. Other classes of chemicals are widely used, but not yet tested for mixture effcts. EFSA (2006) lists organophosphorous substances, carbamates, conazoles, pyrethroids and dicarboximides as groups of compounds that most likely need to be prioritised for cumulative risk assessment on the basis of hazard, and, partly, exposure considerations. Triazins are excluded from this list as they are banned in the European Union. In the following information is given on pyrethroid and neonicotinoid insecticides because of their high market share and on bipyridinium herbicides because of their high toxicity to humans as the latter are responsible for rare cases of acute pesticides toxicity.

Pyrethroids

Pyrethroids[31] are widely used insecticides with increasing share of the market. They are not only applied as plant protection products but also for combating anopheles mosquitos transmitting malaria, against human ectoparasites (head lice, *Pediculus humanus;* scabies, *Sarcoptes scabiei*) and against moths (*Tineidae*). Some are used as repellents. Because of their rapid degradation, accumulation in the food chain does not play a role for toxicity to humans. Pyrethroids block Na^+ channels in membranes of nerve cells and thus block the de-excitation of nerves. They are less toxic to mammals than organophosphates (see above) and organochlorides (e.g. DDT) but cause neurological disorders at high doses. Tremor and ataxia are typical for type 1 substances whereas type II pyrethroids induce slow movements among other symptoms. Some pyrethroids are assumed to have endocrine disrupting effects (e.g. Kim et al. 2005). Studies on developmental neurotoxicity of pyrethroids performed with mice and rats as test organisms

[29] the document "Cumulative risk from chloroacetanilide pesticides " is avaialble at http://www.epa.gov/pesticides/cumulative/chloro_cumulative_risk.pdf

[30] glutathione-S-transferase (GST), UDP-glucuronosyltransferase (UDPGT), cytochrome P4502B (P450), NAD(P)H-qinone oxidoreductase 1 (NQO1).

[31] permetrin, cypermethrin, cyfluthrin, deltamethrin, transfluthrin.

have been reviewed by Shafer et al. (2005). Indoor application seems to be the major source of pyrethroid exposure.

Bioassays for pyrethroid exposure:

Degradation products of pyrethroids analysed using GC-MS in urine are biomarker substances for pyrethroid exposure. Degradation of most pyrethroids (with the exception of cyfluthrin) leads to 3-PBA (3-phenoxybenzoic acid). Other products are more specific metabolites: cis- and trans-Cl_2CA[32] for permethrin, cypermethrin and cyfluthrin, and Br_2CA[33] for deltamethrin. Results of a monitoring initiative reporting average pyrethroid metabolite concentrations in urine indicating the background exposure of children in Germany are available in Becker et al. (2007) and Schulz et al. (2009). According to Shan et al. (2003) the major pyrethroids such as permethrin, cypermethrin and cyfluthrin contain a phenoxy-benzyl group, so that phenoxy-benzoic acid is a common metabolite of these substances. The authors developed an immunoassay to PBA, tested this assay with exposed workers[34] and concluded that the PBA assay might be suitable as a monitoring tool for human exposure to pyrethroids. Nichkova et al. (2007) developed microarray immunoassays allowing to detect both the biomarker for pyrethroid insecticide exposure (3-PBA) and the biomarker for exposure to the herbicide atrazine (atrazine-mercapturate) simultaneously.

Neonicotinoid insecticides

Clothianidin[35], Imidacloprid[36] and other related compounds[37] are chlorinated analogs of nicotine. They are widely used systemic insecticides applied via irrigation, taken up by the roots and applied to combat beetles and aphids. They are further used for flea (*Pulicidae*) treatments of pets and for seed coatings of maize and rapeseed. Clothianidin is assumed to be responsible for the honeybee decline observed in the vicinity maize fields after sowing of coated maize. Neonicotinoid insecticides act by inhibiting the degradation of the neurotransmitter acetylcholin by AChE. They have a relatively low toxicity to mammals.

Biomarker for neonicotinoid exposure:

No evidence for specific biomarkers indicating exposure of humans to neonicotinoid insecticides was found. Of course, unspecific tests on endpoints such as genotoxicity have been performed, and results of toxicological studies on common effects of neonicotinoid exposure are available via substance specific review reports[38]. Earthworms react with a change in behaviour when they are exposed to imidacloprid – even at relatively low concentrations (Capowiez et al. 2003). The authors propose to use earthworm behaviour as a biomarker for pollutant effects.

Bipyridinium herbicides

Bipyridinium herbicides[39] are non-selective fast herbicides killing weeds via deleterious formation of reactive oxygen species. Paraquat is one of the most common herbicides used in the world. It is banned in the EU since 2007. Diquat, a similar compound, is included in the positive list of PPPs allowed in the European Union. Bipyridinium herbicides are highly toxic to mammals and humans affecting heart, lung, liver and kidney by enhancing the formation of reactive oxygen species and radicals. Excessive ROS formation can induce the degeneration of dopaminergic neurons and may lead to neuro-degenerative disorder.

[32] cis and trans-3-(2,2-dichlorvinyl)-2,2-dimethylcyclopropancarbonic acid
[33] 3-(2,2-dichlorvinyl)-2,2-dimethylcyclopropan-1-carbonic acid
[34] no quantitative exposure-biomarker-relationship
[35] clothianodin (CAS No. 210880-92-5)
[36] imidacloprid (CAS No. 13826-41-3)
[37] acetamiprid, dinotefuran, nitenpyram, thiacloprid, thiamethoxam
[38] http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection&a=1
[39] e.g. paraquat (CAS No. 1910-42-5) and diquat (CAS No. 2764-72-9).

Biomarker for exposure to bipyridinium herbicides:

To our knowledge there are no specific biomarkers for exposure to bipyridinium herbicides available. Tests indicating increased production of reactive oxygen species (ROS) are not specific for bipyridinium herbicides as a wide variety of metabolic processes leads to an increased rate of ROS production. As an example, the 2',7'-dichlorfluorescein diacetate (DCFH-DA) assay (Engelmann et al. 2005) is mentioned here which can be used to detect increased oxydative stress in human T lymphocytes.

Grouping of pesticides by health endpoint

There is no established methodology available allowing to assess the endpoint specific health risk due to a combination of chemicals having different modes of action and affecting the same endpoint. Although the independent action method for response or effect addition described in detail in this document allows to calculate summed effects of mixtures of chemicals acting via independent pathways, the applicability of this method is limited for the following reasons: Dose-response relationships for the respective effect are required for each compound of the mixture which are, in many cases, available for effects on *in-vitro* systems or test organisms but lacking for human health endpoints. A further complication results from the uptake route specifity of the dose response relationship. A further reason for the limited applicability is that the response addition approach can be used under the precondition that interactions can be excluded. Methods used to test whether no-interaction can be assumed usually require dose-response-algorithms which are not available for many substance-endpoint combinations.

The hazard index and the interactions based hazard index method described in Part A, sections 4.4.1 and 4.5.1, respectively, are applied for assessing potential health risks due to mixtures of chemicals affecting a given health endpoint by acting with the same or differing mode of action. Exposure is compared to a reference exposure assumed to be acceptable in terms of risk to human health. Although the hazard index and related indices mentioned in section 4.4 of Part A are not suitable to give information about the number of cases of impaired health to be expected for a certain complex exposure or policy scenario they represent an important tool for risk assessment and help to decide whether exposure of the population or subgroups to a certain mixture of chemicals should give rise to concern.

For pesticides the suitable substance specific limit value, the reference (ref_i) of no concern, usually is the accepted daily intake rate (ADI) relevant to human health but not to certain health endpoints. Calculating the endpoint specific health risk index for a mixture of pesticides by summing up the exposure to limit value ratios, requires health endpoint specific limit values assumed to be protective for the selected endpoint. This kind of reference values, e.g. cancer health risk limit values for humans, are usually not available for pesticides. There are two possibilities to deal with this problem: The first is to identify compounds of the mixture affecting the same health endpoint on the basis of available evaluations (e.g. the pesticides classification[40] given on the web site of the footprint project), to relate exposure to generic ADIs instead of endpoint specific limit values, and then summing up the ratios to derive the index value. The second possibility is to identify compounds of the mixture showing effects when tested with a certain indicator system (e.g. genotoxicity tests), to relate exposure to the substance specific NOAEL derived with this test and summing up these ratios for all components of the mixture.

Grouping of unknown mixtures of unknown substances

Grouping pesticides by effects on indicator systems is of high importance because to date combination toxicology is facing a generic problem: for many potentially toxic substances produced or just present at relevant amounts the mechanism of action is unknown and their toxicity has not been evaluated[41]. For

[40] endpoint categories are carcinogen, endocrine disrupter, reproduction / development effects, acetyl cholinesterase inhibitor, neurotoxicant, respiratory tract irritant, skin irritant, eye irritant and classification is done by assigning "Yes, known to cause a problem", "No, known not to cause a problem", "Possibly, status not identified" or "No data". The database is available at http://sitem.herts.ac.uk/aeru/footprint/en/index.htm.

[41] according to a presentation Ann Richard, US EPA, NCCT, for about 80% of substances produced at significant amounts no toxicity information is available. For pesticides this percentage makes up about 40%.

pesticides the knowledge level is relatively good but far from being sufficient. The U.S. EPA has launched a set of projects on chemicals risk assessment with the aim to develop methods for the prediction of chemical toxicology suitable to deal with a high number of substances, to improve incorporation of molecular toxicology and computational science, to reduce standard rodent toxicology tests and to increase cost efficiency (Collins et al. 2008). Complex data on chemical structures, results of High-Throughput-Screening and rodent test data are evaluated in order to identify groups of chemicals characterised by their effects on cellular pathways and more generic health endpoints.

With respect to mixtures the approach is based on the identification of relationships between the structure of a substance and its toxicity. If structural features of a substance group are correlated with toxicity revealed from cell based or cell free *in-vitro*-assays determined using high throughput screening, and the same structural features are correlated with *in-vivo* toxicity test results[42], *in-vitro* assay results or even structural features might be sufficient to predict the mechanism of action and health effects. The approach aims at predicting the mode of action and/or toxicity of a high number of chemicals within short time period. In the context of mixtures of chemicals with unknown mode of action the methods might be suitable to sort the compounds of a mixture by predicted modes of action in order to define groups of chemicals for which additive combination-toxicology-approaches (Part A, section 4.4), such as concentration or dose addition, or hazard index related methods, can be applied.

Full chain assessment of changes in pesticides related EU policy

Exposure to pesticides is, in general, exposure to a mixture of substances. Therefore pesticides specific information is provided here which might be relevant for the application of the methodology described in Part A of this document. Section C-1.3 reports some aspects which might be helpful for the emission-to-exposure-modelling part of the full chain assessment. Exposure-to-health-effect modelling for pesticides is tackled in section C-1.3.2 and section C-1.4 gives an overview over the current pesticides legislation of the European Union.

From emission to exposure - sources of pesticides related information

Emission data for pesticides for the EU member states are available[43]. The data refer to the period 1992 to 2003. In the meantime new policy came into force (see below) so that the current situation and market share of substance classes can be assumed to deviate considerably. Whether pesticides emission data are reliable has been investigated in a study conducted with the aim to map the pesticides concentration from pesticides emission data which are reported to EUROSTAT by the member states, and, vice versa, to use monitoring data to calculate emissions. According to the study reported pesticides emission data were not plausible[44]. For the calculations a methodology close to the TGD was used[45].

Several models are available suitable to describe complex exposure of humans to pesticides. The model NORMTOX has been applied to quantify multi-intake route, media and multi-compound exposure of humans to pesticides[46]. A comparison of this and four other human pesticides exposure models named

[42] the list of *in-vitro*-assays (more than 500) and *in-vivo* endpoint tests (more than 70) is available to the public

[43] for each EU member state data on the area cultivated with different crops, quantities of PPP used by crop and area, and the top chemical classes and active substances are given in EUROSTAT (2007), download via http://epp.eurostat.ec.europa.eu/cache/ITY_OFFPUB/KS-76-06-669/EN/KS-76-06-669-EN.PDF; see also Gyldenkaene & Sorensen, Proceedings of the 1st open NoMiracle workshop 2006.

[44] Alberto Pistocchi, JRC, personal comunication.

[45] the emission – concentration relationship assumes one emission per year with exponential decrease in soil concentration transformed into an constant average conc over the year, degradation in soils is considered, degradation in water not.

[46] NoMiracle deliverable D.4.1.1 focusing on uncertainty and variability of the modelling.

CARES, LifeLine, SHEDS and TRIM.Expo/APEX has been performed[47] within the NoMiracle project. The tools net page[48] of the US EPAs Office of pesticides Programs mentions several exposure models for pesticides: DEEM, the Dietary Exposure Evaluation Model, the LifeLine™ model and CARES, the Cumulative and Aggregate Risk Evaluation System. Calendex™ was used to integrate various pathways while incorporating the time dimension of pesticides exposure for cumulative risk assessment performed by the U.S. EPA. LifeLine™ was used to evaluate exposures through the food pathway. For details on the results of cumulative exposure assessment see EPA (2002b) and EPA (2007).

For the modelling of combined exposure we can take advantage of the experience gained in the field of mixtures risk assessment during the previous years. Although there is no generally agreed approach yet for combined risk assessment of pesticides at the European or international level (EFSA 2006), substantial improvements have been achieved for certain groups of substances. Mixtures risk assessment consists of exposure/dose estimation followed by the second step, the comparison of the combined dose to a reference level, a level of no concern. For all agreed groups of pesticides characterised by a common mechanism of action for which risk assessment has been performed, the combined exposure and the overall extent of threshold exceedances could be modelled. These groups are organophosphate insecticides, carbamates, triazines and chloroacetanilides. In the following specific advantages and limitations are discussed. Considering further substance groups, particularly neonicotinoid insecticides and pyrethroids, is also discussed as they are among the pesticides with the highest relevance for policy development. Neonicotinoids, N-methyl-carbamates, organophosphate insecticides[49] and pyrethroids are among the substance classes currently used in the European Union at significant amounts.

Regarding *organophosphate insecticides* it should be noted that currently only few organophosphates[50] are on the positive list of substances allowed for use in the European Union and the substance group will be phased out as other insecticides with lower toxicity to mammals are used at increasing amounts. Anyway, there is still a considerable exposure of the population to organophosphates. The main intake route is food. Cumulative exposure to AChE inhibiting compounds in the Dutch Population and young children has been investigated by Boon and van Klaveren (2003). It might be an interesting task to use a similar approach at the European level and by this to tackle the problem of the susceptible sub-group children. The output of the exposure modelling would be a daily intake, which could be compared to the threshold set by policy. The exposure-to-health-modelling would be restricted to the evaluation of the overall percentage inhibition of AChE. To our knowledge, there is no quantitative relationship between the biomarker AChE- inhibition and cases of impaired human health. Monetary evaluation would not make sense. A set of biomarkers for human exposure is available integrating over the most important organophosphate insecticides. Bio-monitoring activities using these biomarkers as indicators for exposure revealed background exposure levels for the german population and specific data for children, a susceptible subgroup with exposures close to the level of concern.

In a similar way cumulative exposure to *N-methyl-carbamates* through food consumption could be evaluated in order to compare the combined exposure to a level of no concern. Respective results obtained by the U.S. EPA are available (see C-1.2.1.2). A decreased AChE activity is a biomarker for effects of carbamates and organophosphate insecticides. But, AChE inhibition can probably not be used

[47] NoMiracle deliverable D.4.2.14 "Receptor oriented approaches in wildlife and human exposure modelling: a comparative study".

[48] http://www.epa.gov/pesticides/cumulative/methods_tools.htm

[49] glyphosate is a widely used fast degradable organophosphate herbicide frequently occurring in groundwater together with its degradation products. Cumulative risk assessment and exposure characterisation activities for organophosphates mentioned above refer to insecticides but not to the herbicide.

[50] out of the list of organophosphates listed in the footnote in section xx currently only for the following substances "included" is reported for the criterium "status under Directive 91/414/EEC" in the EU pesticides database: Chlorpyrifos, Chlorpyrifos-methyl, Dimethoate, Ethoprop, Fenamiphos, Phosmet, Pirimiphos-methyl. This means that only for these substances the EPA factors characterising the toxicity of the compounds relative to each other can be used directly for the dose summation procedure if the exposure assessment is restricted to substances approved for use in the European Union. of course, imported food can contain a wide variety of organophosphate compounds.

as an indicator integrating over both groups of pesticides, as exposure induces differing biomarker responses due to the faster recovery following inhibition by carbamates as compared to inhibition by organophosphates. To our knowledge, there is no comparative study available providing information on the relative effect of long-term chronic exposure to both substance groups. This assumption is supported by the fact that the EPA cumulative risk assessment was performed for NMCs and OPs separately.

Triazines such as atrazin, simazin and terbutylazin are not on the EFSA list of priority groups[51] for which cumulative risk assessment should be urgently performed (EFSA 2006) as they are banned[52] in the European Union and human exposure in Europe is mainly due to imported food. For policy impact assessment in Europe triazines therefore seem to be irrelevant unless food imported to the European Union is considered as a source of exposure. In the U.S. triazines are widely used. The EU pesticides database provides atrazine MRLs for several types of food. Anyway, calculating triazine exposure requires to distinguish between imported and European products, e.g. between Californian citrus fruits and those of European origin. Biomarker metabolites for atrazine exposure have been identified, but it should be also noted that these substances are found during the first days after short term exposure of humans. It is questionable whether the identified metabolites can be used as biomarkers for long term chronic exposure to this herbicide, because the spectrum of metabolites found in urine varies with time after exposure. Biomarkers were tested with workers having applied atrazine prior to sampling. Anyway, health effects of occupational exposure, i.e. persons applying the herbicide atrazine seem to play a more important role in the U.S. where atrazine is not banned.

To model exposure of the European population to *pyrethroids* would be a new and important task. To assess exposure is more complicated for pyrethroids than for organophosphate insecticides as other intake routes than food consumption[53] should be taken into account. Exposure to pyrethroids is also due to home use such as veterinary applications and combating insects. Biomarker systems integrating over several quantitatively important pyrethroids have been found and bio-monitoring data have been collected in order to characterise the background exposure of the german population and children. Although pyrethroids show a relatively low general toxity to mammals a better kowledge on combined exposure would be an important step forward as several pyrethroids are known to act as endocrione disruptors[54], thus influencing human health at very low concentrations distinctly below exposure levels causing other toxic effects.

The same holds true for *neonicotinoids*. Assessing human exposure to neonicotinoid pesticides would be new and important as well as the behaviour of compounds belonging to this relatively new substance class[55] in the environment is not well known and for most neonicotinoids no data are available allowing to decide whether they act as endocrine disruptors[56]. Thus tackling neonicotinoid exposure might be too ambitious.

There are further aspects of mixtures of pesticides which might be of relevance for the description of complex exposure situations:

[51] organophosphates, carbamates, conazoles, pyrethroids, dicarboxymides, microtubule/spindle inhibitors, phthalimides and dithiocarbamates (EFSA 2006)

[52] after the ban of atrazin and simazin the triazin terbutylazine has replaced atrazine for a while and is present in European rivers at slightly higher concentrations than atrazin ansd simazin (Loos et al. 2009).

[53] a GIS model used for european wide mapping of pyrethroid emissions, their fate and the contamination of water and soils by pyrethroids is available (Pistocchi et al. 2009) and might be a basis for modelling uptake via routes other than food ingestion and home use related routes.

[54] pyrethroids known to be endocrine disruptors are, for example, bifenthrin, deltamethrin, fenvalerate, resmethrin and tau-fluvalinate (see footprint database, http://sitem.herts.ac.uk/aeru/footprint/en/index.htm). For most substances respective data are not available.

[55] European neonicotinoid emissions are not reported in the EUROSTAT (2007).

[56] thiamethoxam is a neonicotinoid reported to have endocrine disrupting activity by the footprint database, http://sitem.herts.ac.uk/aeru/footprint/en/index.htm, for others no data are available.

- considering simultaneous exposure and uptake of active substances put on the market as a combination, i.e. as constituents of a certain product, would facilitate exposure modelling. For example, the product named Baygon is a mixture of the pyrethroids beta-Cyfluthrin[57] and Transfluthrin[58].
- it is unlikely that the complexity of exposure will be fully covered by modelling. The complexity of pesticides exposure arises, for example, from the fact that, even if two substances share the uptake route and the mechanism of action, a prediction of combined exposure seems to be difficult if the process leading to exposure differs. The organophosphate Dimethoate[59] can be found in drinking water as a result of incomplete degradation of products used in agriculture. The presence of another pesticide with the same mode of action, Temefos, in drinking water can be due to public health requirements, as this organophosphate insecticide is recommended by the WHO as a larvicide against mosquitos and water fleas to combat malaria and other diseases. The number of sources considered by modelling will probably be limited.
- a further uncertainty in the prediction of exposure will arise from the fact that pesticide products usually are mixtures of active substance and their production byproducts with varying relative contributions of the single compounds and thus varying toxicological properties of products.
- even if the intake rate is known for a certain pesticide for a given uptake route and medium, the real intake may deviate from this assumed rate due to the fact that pesticide products usually consist of active and inert agents. Active agents, the substances with the potential to kill or control harmful organisms, are mixed with inert carrier substances facilitating the uptake or enhancing the bio-availability and thus modulating or determining the exposure characteristics of the active substance. Costa et al. (2009) compared genotoxicity induced by an active substance to genotoxic effects of the commercial pesticide product and found a slightly higher response when co-formulants were present.

Exposure to health effect modelling for pesticides

Comparison of exposure to thresholds of no concern

Acute exposure to high doses of toxic substances can have severe health effects. Full chain assessment of policy impact could aim at calculating the decrease in cases of acute poisoning to be expected from a ban of the respective pesticide. Anyway, other more common health endpoints with higher relevance in terms of number of cases and total costs seem to be more relevant for full chain assessment.

A more reasonable way to assess the impact of policy on the overall risk of acute pesticides exposure to human health is to assess the cumulative acute exposure situation as described above for the respective population or certain susceptible sub-groups and to compare exposure expressed as the daily intake rate to the ARfD value, the acute reference dose which is available for pesticides via the EU pesticides database[60]. If the concentration or dose addition method is applied for exposure assessment, the overall acute exposure level is compared to the ARfD of the index or reference compound. If the mixture consists of substance groups with different modes of action, the hazard index method or related methodology can be applied in order to compare exposure to the acute exposure level of no concern. Both dose and effect summation can be performed under the precondition that interactions are absent. The method usually applied to test whether interactions take place is previously described. Anyway, if this method is not applicable due to a lack of data, it seems to be common practise to disregard interactions at low environmental concentrations as they are assumed to be irrelevant at exposure concentrations distinctly below the LOAEL.

Adverse health effects due to acute pesticides exposure in Europe can be assumed to be rare so that effects of *chronic exposure* may play a more important role. The health risk due to chronic pesticides exposure can be assessed, in a similar way as the acute risk, by comparing chronic exposure of the population to the ADI value, the acceptable daily intake. The result is an indicator for the overall health risk attributable to the pesticides mixture and the extent or frequency of threshold exceedances, but does not

[57] CAS No. 68359-37-5
[58] CAS No. 118712-89-3
[59] The WHO sets a threshold of 6 µg l^{-1} for Dimethoate (CAS 60-51-5) in drinking water.
[60] http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection&a=1

allow calculating the impact of policy changes on the number of cases of impaired health for defined health endpoints. Chronic exposure to pesticides is known to increase the risk to several common health endpoints such as endokrine disruption, cancer, liver lesions and degenerative diseases of the nervous system, but dose-response-relationships specific for active substances or pesticides classes are lacking.

Dose-response relationships for unspecified pesticides exposure

Some *epidemiological studies* report the risk due to the long-term exposure to "pesticides" but not the risk due to chronical exposure to certain active compounds. The quantitative relationship between "pesticides exposure" and Parkinson's disease risk (Priyadarshi et al. 2001) might be used to calculate the number of cases per member state from the number of persons "exposed" to pesticides. The authors report a combined odds ratio of 1.42 (95% CI 1.05-1.91) for farming, exposure to farm animals or living on a farm as the result of a meta-analysis[61]. Changes in the number of farmers as a susceptible sub-group of the population due to policy changes would be the respective data input for the policy impact assessment. To model effects of specific pesticides seems to be inadequate because, first, there is a lack of exposure data specific for active substances, second, many pesticides are supposed to enhance the risk of Parkinson's disease (Dick 2007), and, third, bipyridine herbicides inducing acute Parkinsonism are banned in the EU. Anyway, modelling health effects of sub-acute exposure to pesticides focusing on this neurodegenerative disease would be highly relevant to policy development as the social and economic burden of neurodegenerative diseases is high and still growing. The importance of the topic is underlined by the proposal of the European Commission for a Council recommendation[62] on measures to combat neurodegenerative diseases published on the 22nd July 2009.

In a similar way the number of cases of hypertensive disorders of pregnancy, pregnancy induced hypertension and preeclampsia, attributable to unspecified pesticides exposure might be calculated from the relationships given in Saldana et al. (2009), see Table 20. The authors evaluated self reported pesticides exposure data of wifes of farmers.

Table 20. Association (adjusted odds ratios) between pesticides exposure and hypertensive disorders during pregnancy (Saldana et al. 2009). Self-reported potential exposure due to pesticides-related activities during the first trimester of pregnancy is considered.

	potential exposure to pesticides due to	
	residential activities	agricultural activities
Pregnancy-induced hypertension	1.27 (95%CI: 1.02-1.60)	1.60 (95%CI: 1.05-2.45)
Preeclampsia	1.32 (95%CI: 1.02-1.70)	2.07 (95%CI: 1.34-3.21)

Several publications are available on health effects of occupational exposure due to the production of pesticides. They are not described in detail here as non-environmentally related occupational exposure is outside the scope.

Confounding factors and combination effects of pesticides and other stressors

Most of the diseases relevant for the full chain assessment of policy actions are influenced by a wide variety of stressors of chemical, physical and biological nature, cultural factors and by background stressors. A sound full chain assessment of changes in pesticides related policy requires a methodology suitable to predict not only combination effects of mixtures of chemicals but also combined effects of chemical, physical and other stressors and predisposing factors (Figure 17). That the combination of all factors plays a role arises, for example, from a study on Parkinson's disease published by Bronstein et al.

[61] the respective odds ratio for studies done in the United States was 1.72 (95% CI 1.20-2.46).
[62] COM(2009)379/3 available via http://ec.europa.eu/research/press/2009/pdf/com_2009_0379_en.pdf

(2009). The authors report evidence that people exposed to pesticides have an increased risk of Parkinson's disease[63]. With respect to predisposing factors it has been found that men are at greater risk than women, tobacco smoking reduces the risk.

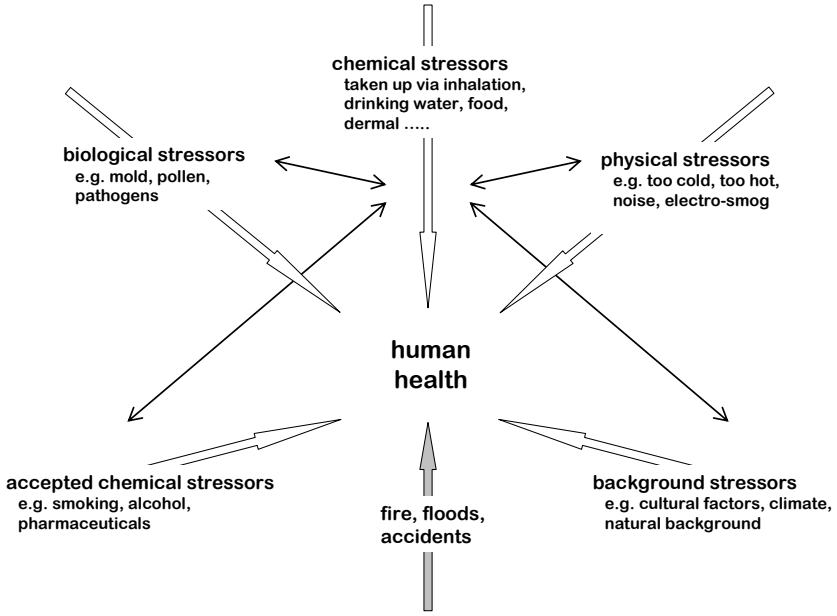

Figure 17. Direct health threat (grey arrow) versus chronic stressors. Effects of a single chronic stressor (open arrows) might not be of risk, but the combination. Combination toxicology aims at evaluating the overall risk by adding the risk attributable to chemical compounds, or even, adding additional effects of biological, physical and other stressors. The case that chemicals can influence the effects of each other, or even more complicated, that other types of stressors show interdependencies is far from being understood and describing such systems is a major challenge of combination risk assessment.

Current EU pesticides legislation – considering mixture effects?

According to the legislation of the European Union on Plant Protection Products[64] (Directive 91/414/EEC)[65] active substances can be used if they have no harmful effects on human health, they do not cause unacceptable effects on the environment[66] and if they are sufficiently effective against pests. Active substances are approved at the EU level, whereas preparations[67], the marketed plant protection products containing certain mixtures of active substances are authorised at the member state level. Substances on the market before 1993[68] and still of interest for the producing industry have been tested during recent years. Those which meet the above mentioned criteria are together with the new substances for which meeting of criteria had to be proved on a list of allowed active substances which is given in the pesticides database provided by the European Commission[69]. The database further provides

[63] in a meta-analysis of 19 studies Priyadarshi et al. (2001) extracted a quantitative relationship between pesticides exposure and Parkinson's disease. Due to the lack of precise exposure data no dose-response relationships are given for single active substances or groups. Bipyridinium herbicides, such as paraquat, diphenyl (fungicide), dithiocarbamates (e.g. maneb), organochlorides (aldrin, lindane etc.), organophosphates (e.g. parathion) and pyrethroids (permethrin) have been reported to play a role in this context (reviewed by Dick, 2006).

[64] for the definition of plant protection products see section 7.

[65] a consolidated version of 1st May 2009 including the list of authorised PPPs is available at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1991L0414:20090501:EN:PDF

[66] whether it could be used safely with respect to groundwater and non-target organisms such as birds, mammals, bees and earthworms.

[67] for the definition of preparations see section 7.

[68] see COM(2001) 444, for a list of these substances see SANCO/2692/2001

[69] http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection&a=1

"Acceptable Daily Intake" levels (ADIs) reflecting the risk due to long term exposure and the "Acute Reference Dose" (ARfD), the dose above which acute effects might occur and other substance specific information, such as risk assessment reports.

EU legislation[70] sets maximum residue levels of permitted pesticides in or on food and feed. For each permitted active substance and each food or feed category specific threshold values are listed. They refer to residues of products used for the protection of plants, substances used in veterinary medicine and biocides[71] and to the active substances and their metabolites and/or breakdown or reaction products.

Recently a new regulation on placing on the market of plant protection products, specifying strict criteria for approval of substances, has been adopted by the European Parliament and the Council. According to this regulation, carcinogens, mutagens, endocrine disruptors, substances toxic for reproduction or which are very persistent will not be approved, unless exposure to humans is negligible.

Current EU pesticides legislation does not consider mixture effects. But the necessity to deal with the problem of multiple residues in food and the need for rules defining how uptake via multiple routes should be combined has been recognized as this topic is widely discussed in the public.

Regulatory bodies will have to deal with several problems:

- Setting a maximum acceptable threshold concentrations or ADIs for certain chemical groups with a common mechanism of action such as organophosphates would be a first step forward, but would disregard most of the mixture constituents as the majority of active substances in a sample usually belong to different chemical groups.
- Additive approaches based on grouping substances by effects (e.g. summing up doses of compounds causing dysfunction of the nervous system in humans but acting via differing mechanisms) requires testing results obtained under comparable conditions and evaluating effects on a common human health endpoint. Such data are not available in most cases, and, again, only a part of the mixture constituents would be considered.
- Using the hazard index approach for assessing the risk to human health is practicable and transparent but the question arises whether HQs of all mixture compounds should be added[72], or, alternatively, HQs of substances grouped on the basis of their relevance for a certain health effect.
- Adding HQs of all compounds found has the advantage that it is transparent and easy, but has the disadvantage that improving the detection limit of the analysis and thus identifying a high number of compounds present at very small amounts might increase the overall hazard index, whereas an analysis of the same sample with higher detection limit would indicate a lower risk.
- Adding HQs of compounds within a common health effect would probably reveal additional information on specific risk of the mixture, but has the disadvantage that for most active substances currently in use the dataset on health effects is incomplete: For three of the 13 compounds in Table 12 it is known that they have no endocrine disrupting effects, for 3 it is known that they might have these effects, but it is not clear, and for 7 others no data on endocrine disruption are available.
- A further problem connected with the use of the hazard index approach for the risk classification of mixtures is that a suitable reference has to be chosen. It has to be decided whether the HQs should be calculated as a residue versus MRL or a predicted daily dose versus the ADI or both, and, in the case of dose versus ADI, whether the doses and ADIs for adults or children should be applied.

[70] Regulation (EC) No. 396/2005 on maximum residue levels (MRLs) and the amending Regulation 149/2008 listing MRLs for each active substance and each type of food and feed. EU wide MRL are valid since September 2008.

[71] for a definition of biocides see Part G.

[72] Krautter et al. (2006) describe an evaluation scheme for pesticide mixtures considering both threshold exceedances (ARfD, ADI, MRLs) and HIs, the latter calculated as the sum of HQs of all substances in the sample: http://www.greenpeace.de/fileadmin/gpd/user_upload/themen/umweltgifte/greenpeace_bewertung_pestizide_neu.pdf

- The lack of knowledge about actual or potential synergetic interactions is a general problem. Legislation can deal with this problem by diminuishing reference concentrations or doses of no concern by a factor assumed to be protective, but whether this factor should be 2, 3 or 10 is a matter of speculation.

Table 21. Example for a cocktail of pesticides found in a grape sample[73] (the grape imported from Italy was purchased in a supermarket in Germany in october 2009). Chemical groups, pesticide types and health effects are listed for the 13 active substances found. For each substance concentrations were below the respective maximum residue level set by legislation and thus the sample was within legal limits. Data marked with *were taken from the pesticides database developed within the footprint project[74]. Carc = carcinogen; end = endocrine disrupter; repr = reproduction/ development effects; AChE = acetylcholinesterase inhibitor; neuro = neurotoxicant; resp = respiratory tract irritant; yes: known to cause a problem; no: known not to cause a problem, ?: possibly causing a problem but not identified; -: no data.

active substance	chemical group*	pesticide type*	target	health issues*					
				carc	end	repr	AChE	neuro	resp
Endosulfan-sulfate	organochlorine	acaricide, insecticide	neurotoxic, affects the transfer of nerve impulses in insects and mammals	?	?	-	no	yes	-
Chlorpyrifos-ethyl	organophosphate	insecticide	AChE inhibitor, causes dysfunction of the nerval system	no	?	yes	yes	no	no
Chlorpyrifos-methyl	organophosphate	acaricide, insecticide	AChE inhibitor, causes dysfunction of the nerval system	no	no	-	yes	?	no
Triadimefon	triazole	fungicide	disrupts membrane function	?	?	yes	no	?	-
Trifloxystrobin	strobilurin	fungicide	inhibits electron transfer and respiration	no	-	yes	no	no	-
Boscalid	carboxamide	fungicide	inhibits sperm germination	?	no	?	no	no	-

[73] Respective data for a total of 34 samples (30 of conventional production and 4 bio-products) are available at http://www.greenpeace.de/themen/chemie/presseerklaerungen/artikel/greenpeace_test_weniger_pestizide_in_trauben. Grapes from conventional agriculture contained, on average, residues of 4.7 different active substances and 10% of the samples contained 10 or more pesticides.

[74] http://sitem.herts.ac.uk/aeru/footprint/en/index.htm.

active substance	chemical group*	pesticide type*	target	health issues*					
				carc	end	repr	AChE	neuro	resp
Cyprodinil	anilinopyrimidine	fungicide	blocks certain synthesis pathways within the cells	no	-	?	no	no	yes
Dimethomorph	morpholine	fungicide	lipid synthesis inhibitor	no	-	?	no	no	no
Fenhexamid	hydroxyanilide	fungicide	Disrupts membrane function, inhibits spore germination	no	-	no	no	no	no
Fludioxonil	phenylpyrrole	fungicide	inhibits phosphorylation of glucose	?	-	?	no	no	no
Fluopicolide	benzamide	fungicide	protectant	?	-	no	no	no	no
Metalaxyl	phenylamide	fungicide	protectant suppressing infections, sporangial formation and mycelial growth	no	-	no	no	no	-
Thiametoxam	neonicotinoid	insecticide	affect the central nervous system by binding to an postsynaptic ACh receptor	?	no	no	no	no	?

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