Cross-Mediterranean Environment and Health Network

CROME-LIFE

Deliverable B.1.2

Methodology report outlining the ways to link environmental, human biomonitoring and health status data to derive environment-wide association and integrated health impact assessment

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Understanding the requirements of developing a methodology for linking environmental, human biomonitoring and health status data

General considerations

Describing the links between environmental contamination and human disease (Figure 1) requires a mechanistic understanding of the associations connecting the series of processes linking the several steps within the source to health outcomes continuum.

CROME-LIFE aims to capture and describe the "key events" of this process, focusing on:

- Environmental contamination
- Human exposure through biomonitoring
- Associating the above with clinical data

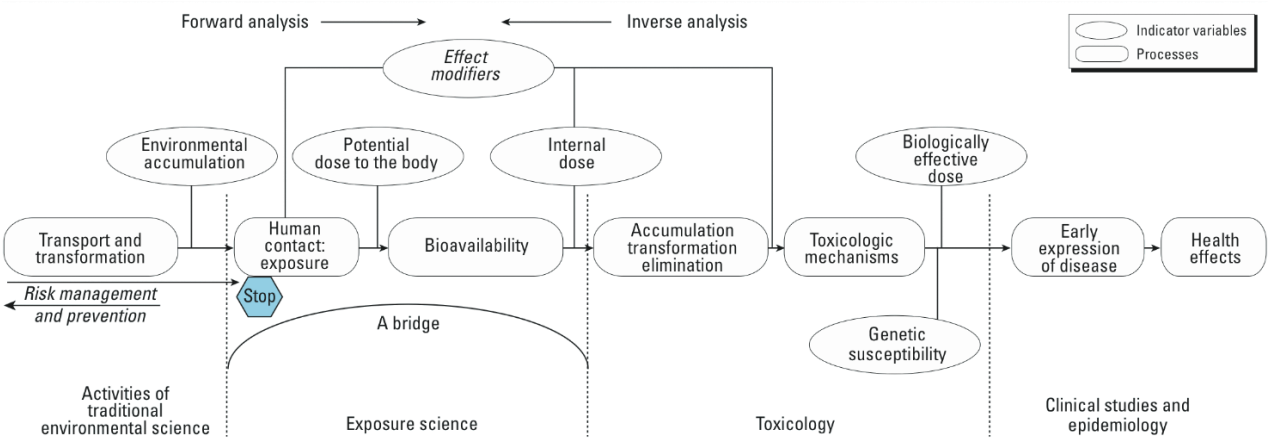


Figure 1: Process continuum from contaminant emissions to a health effect and application to risk reduction and prevention strategies (figure adapted from Lioy (2010))

Linking environmental data to external exposure

The environmental fate and toxicity of the pollutants depends on their physicochemical properties, and consequently on their chemical structure. Although this structure facilitates their intended use, it also affects their mobility and distribution within the environment and consequently the exposure mechanisms (pathways and routes) employed. The way the pollutants come into contact with man affects their bioavailability and, consequently, the potential for manifestation of adverse health effects, adding thus complexity on the linkage between environment and health. The picture outlined above explains why a quantitative assessment of the impact on human health is a valuable aid toward an efficient management of the environment; this becomes even more important when attempting to assess the burden of human disease that can be associated to environmental pressure. In order to identify the potential pathways and routes of exposure, we need to describe the processes governing transportation across several environmental media (Figure 2).

Modelers set up their equations in several formats depending on the objective. Most common in this context are compartment, box or Eulerian models in which the environment is divided or segmented into a number of volumes or boxes, which are fixed in space and are usually

treated as being homogeneous, i.e. well-mixed, in chemical composition. This has the advantage that only one concentration needs be defined per box. Another approach (i.e. Lagrangian) that is widely used in atmospheric and river modelling, is to define a parcel of air or water and follow it, and the chemical in it, in time as the parcel moves from place to place. There are also situations where there is marked heterogeneity in concentration, and it is preferable to set up diffusion/advection/reaction differential equations and solve them either numerically or analytically. This is often done when describing chemical migration in sediments and soils, but it can also be applied to atmospheric dispersion, aquatic and oceanic systems (Mackay 2001). In principle, all approaches should give the same, or similar results. Here we focus primarily on compartmental models because it is likely that they will be most commonly applied in the regulatory context. For some purposes Lagrangian models may be used when evaluating Long Range Transport (LRT) in air or water. Diffusion models can be valuable when seeking a general picture of chemical fate in the global atmosphere or oceans, or when estimating the near-source dispersion of emitted chemicals.

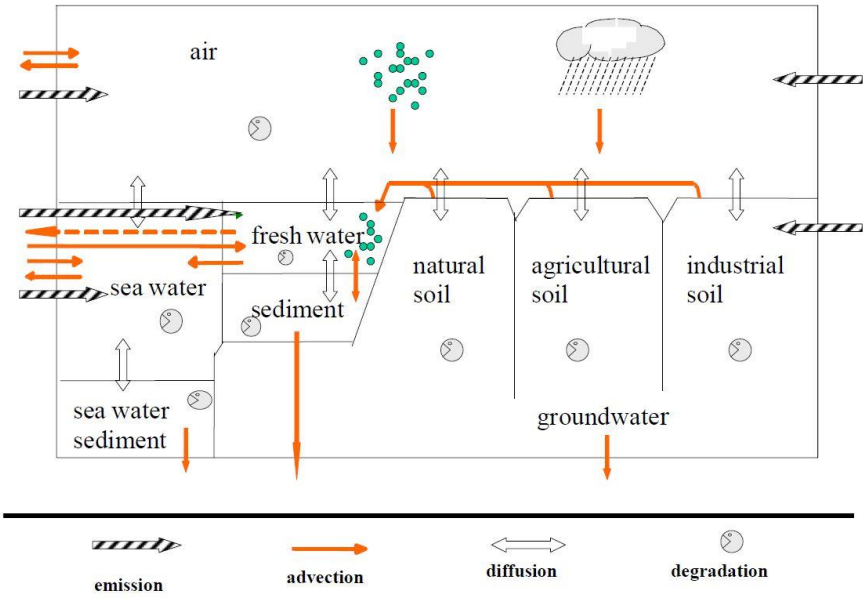


Figure 2: Processes describing transfer across several environmental media

When there are data on chemical properties, inputs and concentrations in a well-identified region, it is possible to set up a model describing this site-specific situation. Models are routinely applied in rivers, lakes, soils, biota and air pollution studies (Mackay 2001). Validation is possible by comparing the model output with observations. MMMs have been set up for regions, nations, continents and even the global system. These can be referred to as models of "real" systems. Another family of models is the "evaluative" models in which the environment is fictitious i.e. it does not correspond to a particular area, but it is realistic. The fate of a variety of chemicals can be evaluated in such models. The same equations are used in real and evaluative models; only the environmental parameters are different. This approach is particularly attractive for international regulation purposes because the assessment is not in a specific region; it is general. Examples are the EQC model of Mackay (Mackay et al. 1996), CalTox (McKone 1993), the SimpleBox model included in the European Union System for

the Evaluation of Substances (EUSES) model used in the European Union (Lijzen 2004) and the Mentor 4M (Georgopoulos et al. 2008).

The single compartmental mass balance

The first step in model development is to divide the environment into a number of compartments of defined volume that are fixed in space. Considering first a single compartment, it is possible to set out the input and output processes. Included can be discharge or emission, advective inflow in air or water (and the corresponding advective outflow), diffusion to and from adjacent compartments, formation from other chemical species and degrading reactions to form other chemical species. A simple mass balance foresees that the rate of inventory change of the mass of chemical in the volume must equal the total rate of chemical input minus the total rate of output. Mathematically this is expressed by the differential equation:

$$\frac{dm}{dt} = \mathrm{T}_{IN} + E + F_{IN} + S - \left(T_{OUT} + F_{OUT} + R\right)$$

(with units such as g/h) where ***m*** is mass in the compartment (e.g. g), ***V*** is volume (e.g. m^3) and ***C*** is concentration (e.g. g/m^3). This is the same equation as applies to cash in a bank account, i.e. change in funds per month equals monthly income less monthly expenditure. A particularly useful and simple version applies when the inventory is fairly constant with time, and thus the derivative on the left side is small or zero. Input rates then equal output rates under steady-state conditions. The advantage of making this steady-state assumption is that the mathematics becomes algebra rather than calculus.

The next task is to predict the various output process rates as a function of the chemical concentration. If the input rates are known and all output rates can be expressed as a function of concentration, then the mass balance equation can be used to calculate the chemical concentration and hence the mass of chemical in the box and the rates of the various loss processes.

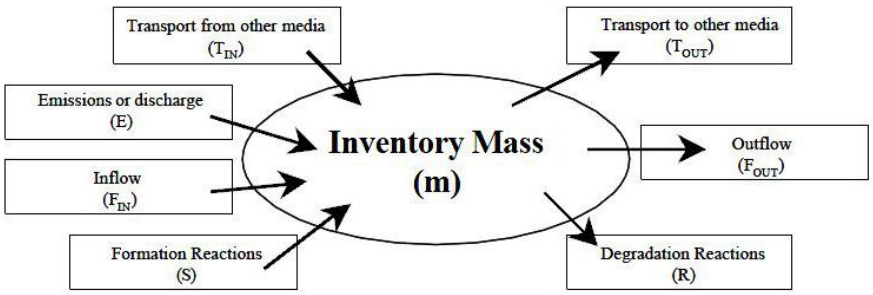


Figure 3. Derivation of expressions for compartmental concentrations at steady-state using rate constants (Mackay 2001)

An important quantity is the persistence of the chemical. This can be expressed as the residence time of the chemical in the box, which is best calculated at steady-state. This is the mass of chemical in the box divided by the total rate of output (or input when steady-state applies). Under non steady-state, or dynamic conditions, a characteristic time can be calculated similarly as the mass divided by the output rate. This is the average time that the chemical spends in the single compartment or box and is a first indication of persistence. It is possible to calculate a residence time attributable to reaction and other loss processes such as outflow both individually and collectively. Here we use the word persistence as generally

expressing the longevity of the chemical in the environment. Residence time, characteristic time and half-life have specific mathematical definitions.
When calculating persistence, not all loss processes are relevant. Outflow by advection is not a permanent environmental loss process. It only transports a chemical from one environmental location to another. On the other hand, reaction eliminates a chemical from the environment permanently and completely. If the only loss is by reaction with a half-life $t_{1/2}$, then the rate of reaction is VCk_R where k_R is $0.693/t_{1/2}$ and is the rate constant. The residence time t is then $(VC)/(VCk_R)$ or $1/k_R$ and equals $t_{1/2}/0.693$. In this case $t_{1/2}$ is 69% of t. Some models consider loss processes other than reactions as irreversible losses, e.g. sediment burial or transport to the stratosphere.

Extension to multiple compartments

If the model consists of two connected boxes, the same approach can be applied twice, once to each box, and to the combination of the two boxes, i.e. the system as a whole. The residence time in each box or in the system of two boxes is a simple extension of the single box approach. Overall persistence in a multimedia system can be expressed using the residence time in the system without considering advective losses:

$$\frac{dm}{dt} = T_{IN} + E + F_{IN} + S - (T_{OUT} + F_{OUT} + R)$$

where $f_i = m_i/m_{tot}$=mass fraction in compartment i and R_{total} is the total rate of reaction.
Other compartments can be added. Kleka et al. (2001) have suggested a minimum of three compartments but there is a general consensus that four (air, water and soil plus sediment) are required to adequately represent the environmental fate of a chemical and by extension, its overall persistence.
A key conclusion is that persistence is best expressed as a residence time attributable to reaction only. For a single compartment this is the half-life divided by 0.693. For multiple compartments the overall residence time is a weighted average of the individual residence times, and the weighting depends on the mode-of-entry and the partitioning characteristics of the chemical (Mackay 2001).

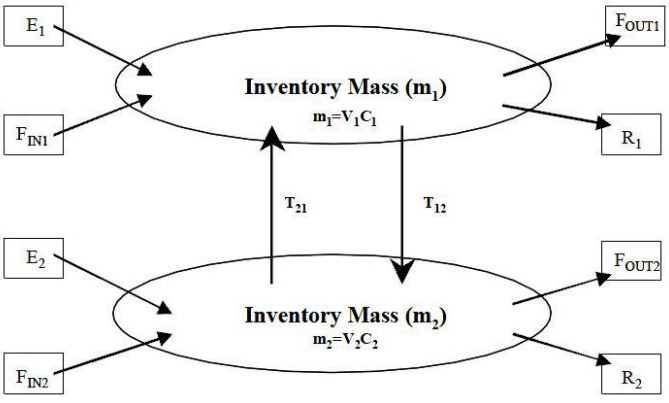


Figure 4: A multiple compartment system using steady state rate constants (Mackay 2001)

In the frame of CROME-LIFE project, multimedia environmental concentrations are estimated by the TAGS computational platform (Sarigiannis et al. 2012), which implements detailed analysis of different environmental scales (e.g. regional, local) and the final

interaction with the living micro-environments, providing input for multi-pathway and multi-route exposure.

Linking biomonitoring data to external exposure

Introduction

It is generally agreed that risk assessment in understanding potential toxicity of chemicals can be greatly improved by the development of biological indicators of exposure, early toxicological effect, and genetic susceptibility. These biological indicators have been called biomarkers. Biological markers are measurements conducted in biological samples that evaluate an exposure or biological effect of that exposure. Biomarkers are generally classified into three groups: biomarkers of exposure[1], biomarkers of effect, and biomarkers of susceptibility. Typically, biomarkers of exposure include measurements of the actual toxin or its metabolite. An interaction between a xenobiotic or endogenous component can also represent a biomarker of exposure. Frequently, biomarkers of exposure are those that involve DNA or protein adducts. Biomarkers of effect measure the biological response that is mechanistically involved in a pathway leading to injury and disease. Gene mutations and chromosomal rearrangements induced by carcinogen exposure are examples of biomarkers of effect. Most DNA adducts are biomarkers that assess exposure and effect. Biomarkers of susceptibility can be defined as indicators signaling unusually high sensitivity to a toxic exposure. This may include measurements of the activity of enzymes or the ability of the cell to efficiently repair DNA damage.

One particularly well-suited source of information on aggregate exposure to environmental agents is human biomonitoring (HBM). Human biomonitoring can be defined as "the method for assessing human exposure or their effect to chemicals by measuring these chemicals, their metabolites or reaction products in human specimens, such as blood or urine" (CDC 2005).

HBM is frequently included as part of observational studies of exposure. The largest biomonitoring studies in EUROPE include the German Environmental Survey on Children (Schulz et al. 2007; Schulz et al. 2012) and COPHES (Becker et al. 2013; Joas et al. 2012), while from USA examples include NHANES (Needham et al. 2005; Woodruff et al. 2011; Yorita Christensen et al. 2013) NHEXAS (Georgopoulos et al. 2006), TEAM (Lyons et al. 2008), the National Children's Study (NCS) (Lioy et al. 2009), to mention a few. However, in the absence of other information, the results are not easy to interpret for the study participants, and they cannot lead directly to the implementation of an intervention strategy. As a result, one concern about biomonitoring is its utility in epidemiology and risk assessment beyond stating that one has been exposed and that the levels may be associated with a health outcome. The goal is to eventually use many of these biomarkers as public health standards, which are coupled with other exposure information to achieve successful interventions, the lead case being a notifiable example (Dixon et al. 2009). A current and future challenge will be how the combination of biomonitoring, in-home measurements, and environmental data can be linked (Lioy et al. 2009). Probably the main achievement of HBM data is that it provides an integrated overview of the pollutant load any participant is exposed to, and hence serves as an

[1] Within the context of this paper, most attention is given to biomarkers of exposure. Throughout the text, HBM will therefore refer strictly to biomarkers of exposure, except when explicitly stated differently.

excellent approximation of aggregate exposure. The internal dose of a chemical, following aggregate exposure has a much greater value for environmental health impact assessment as the internal body concentration is much more relevant than mere exposure data (direct EDR-relationship in Figure 5).
However, it needs to be stressed that HBM in itself cannot replace environmental monitoring and modeling data. Most often, environmental monitoring data for different environmental compartments (air, water, food, soil) provide better insight into potential sources, hence allowing the development of more informed and appropriate risk reduction strategies. At the same time, mathematical approaches to describe the pharmacokinetic and toxicokinetic behavior of environmental agents (generally referred to as PBTK models) offer a more mechanistic insight into the behavior and fate of environmental agents following aggregate exposure (Indirect EDR-relationship in figure 5). As biomarker data also reflect individual accumulation, distribution, metabolism and excretion (ADME) characteristics of chemicals, HBM data offer an excellent opportunity for the validation of these PBTK models. Ultimately, combining both lines of evidence to assess exposure prove to be optimal for relating complex exposure to environmental agents to potential adverse health effects assessment.

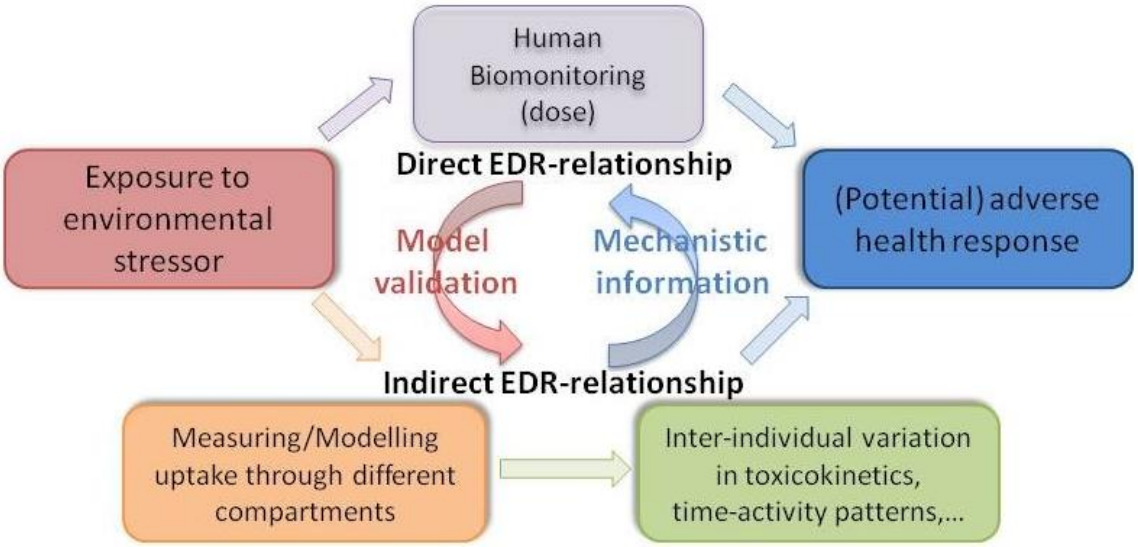


Figure 5: The Exposure-Dose-Response Triad to evaluate the potential adverse health effects of exposure to environmental agents (adapted from Smolders and Schoeters (2007))

In the next chapter a brief overview on the state-of-the-art of human biomonitoring is given with a focus on the practical application of biomarkers. The main stress is given to biomarkers of exposure, as these are currently best described, and have direct applicability is assessing aggregate exposure. Baseline information is provided on three main items that govern the validation and interpretation of HBM data for 15 classes of environmentally relevant chemicals reported in Table 2 (Smolders et al. 2009):

1. Sample collection and storage
    - Matrix
    - Kinetics
    - Sampling and storage
2. Sample measurements

- Analytical aspects
- Performance
- Validation
- Confounding factors

3. Data interpretation
   - Concentrations in literature
   - Dose-response relationships

Biomarkers of exposure

Sample collection and storage

Matrix

Generally, there are two broad categories of matrices for human biomonitoring, invasive and non-invasive ones. As a rule of thumb, non-invasive biomarkers should be promoted as a superior option, when they offer the same information as invasive biomarkers (Table 1).

Invasive matrices

Blood is the most frequently used invasive matrix to determine biomarkers as blood is a universal link between all tissues of the organism (Paustenbach and Galbraith 2006b). The invasive character of sampling however often negatively affects participation rates, and there are ethical issues involved in using blood. Often special consent needs to be obtained from both participants and local and national ethical oversight committees to use blood as a biomonitoring matrix. Additionally, the blood volume that can be collected normally is limited. This makes the use of blood for biomonitoring in children suboptimal. Blood analysis is often carried out for substances that are slowly excreted from the organism (Polkowska et al. 2004).

An important advantage of using blood as a matrix is that concomitant with exposure markers, also many relevant biomarkers of effect can be determined in blood. Combining both exposure and effect markers in a biomarker battery, makes relating exposure, dose, effect and health impact much more relevant.

Non-invasive matrices

Urine probably is the most used matrix in which biomarkers are measured. The collection and analysis of urine carries no associated risks, sample volumes can be large and samples are obtained for different age classes, including little children with minimal impact (Bradman and Whyatt 2005; Kozlowska et al. 2003; Polkowska et al. 2004). Unfortunately, for many biomarkers, urine is not the most reliable indicator of exposure because it often contains excreted metabolites instead of parent compounds (Paustenbach and Galbraith 2006a). Because chemicals are often slowly excreted over the course of hours or days after exposure, also toxicokinetic factors may hamper the usability of urine as a matrix. Although this can be reduced by collecting 24-hr samples rather than single spot samples, the timing of sample collection remains an essential aspect of biomonitoring using urine as a matrix (Barr et al. 2005; Kissel et al. 2005). Urinary creatinine concentrations, specific gravity and osmolarity are common methods for adjusting dilution of urine samples. The most commonly used method is creatinine adjustment that involves dividing the analyte concentration by the creatinine concentration. Guidelines for creatinine adjustment and proper data interpretation are available in literature (Barr et al. 2005; WHO 1996).

Cord blood, amniotic fluid and breast milk provide an overview of the pollutant load of mothers, and at the same time provide relevant information on the *in utero* or early life exposure of babies (Shen et al. 2007).With the current interest in *in utero* and childhood exposure reflecting windows of extreme vulnerability, these matrices deserve extensive attention when preparing HBM programs. Potentially problematic issues using cord blood or amniotic fluid may arise from the fact that sampling is not always straightforward as obviously collection of HBM samples is not the first priority at time of delivery. Breast milk is a reliable matrix to monitor the presence of fat-soluble contaminants such as polychlorinated biphenyls (PCB), brominated flame retardants (BFR) or dioxins ((Shen et al. 2007; Uehara et al. 2006; Yu et al. 2007)) and may be the most important route of exposure to contaminants. Finally, Iyengar and Rapp (2001), provided an extensive overview on the applicability of the placenta for biomonitoring purposes, although the use of this matrix has been questioned several times because of the difficulty to collect comparable and representative samples.
Also other, less frequently used, matrices have been used for biomarker quantification. Hair and finger/toe nails generally are used as long term accumulators of metals such as arsenic or methylmercury (Sera et al. 2002). Exhaled breath on the other hand can be used for the very short-term exposure of volatile components, such as the disinfection byproduct trihalomethane (Gordon et al. 2006; Lindstrom and Pleil 2002).
The use of non-invasively collected matrices can be a valuable alternative to, or addition for, invasive matrices for most contaminants discussed. Generally, there is good agreement between invasive and non-invasive biomarker values (Esteban and Castaño 2009). However, the applicability of non-invasively collected matrices is sometimes hampered by incomplete knowledge of, for example, toxicokinetics and validated sampling, sample treatment and analysis procedures. On the other hand, non-invasively collected matrices can offer substantial advantages for practical and routine implementation, such as increased participation rates, repetitive sampling, more efficient inclusion of susceptible and vulnerable populations, and improved cost-efficiency (Smolders et al. 2009).

Kinetics

As already mentioned earlier, there is an interaction between the matrix used and the kinetics of biomarkers. Different matrices reflect exposure over different time periods. Probably the best known examples of this phenomenon is Pb, where half-lives change from 35 days in blood, to about a year in soft tissues and 20 years in bones (Table 1).
Aggregate exposure combined uptake over multiple routes, and kinetics depend highly on the uptake route (Godschalk et al. 2003), (Heinrich-Ramm et al. 2000). Therefore, it is difficult to derive general rules on kinetics of environmental toxicants. Some chemical classes such as organophosphate (OP) pesticides and phthalates, are characterized by rapid metabolism and short half-lives, while others like dioxins, Pb or Cd can remain present in the body for many years. For a variety of chemicals, physiology-based pharmacokinetic (PBTK) models were developed, which offer great flexibility in modeling exposure scenarios for which there are limited data, and relating this to internal dose. Because of the need for assumptions regarding extrapolations across exposure or uptake routes, the development, validity and sensitivity of these PBTK models is particularly relevant to assess human exposure to environmental toxicants (Chiu et al. 2007).

Table 1: relevant parameters for "sample collection and storage" for a selected number of environmental contaminants (Smolders et al. 2009)

Compound	**Matrix**		**Kinetics**			**Sampling**	
	Invasive	*Non-invasive*	*Uptake routes*	*Metabolism*	*Half-life*	*Procedures*	*Storage*
Alkylphenols	Blood serum	Breast milk, cord blood	Food	Rapid	10-15 h (in fish)	Standard procedures	-20°C
Arsenic	Blood (only high-level)	Urine (recent), finger nails + hair (chronic)	Ingestion	Not relevant	40-72 h	Standard procedures	-80°C
Bisphenol-A	Serum blood, red blood cells	Cord blood, breast milk, urine	Food	Rapid	< 24 h	Standard procedures	-20°C
Brominated flame retardants	Blood, adipose tissue	Breast milk	Food, dust particles	Rapid elimination	11-18 d for DeBDE, 2 days for TBBPA	Standard procedures, beware of contamination	-20°C
Cadmium	Blood	Urine	Inhalation, food	Not relevant	100 days in blood, > 10 years in urine	Metal free containers	4°C or frozen
Dioxins	Blood, adipose tissue	Breast milk	Food	Very resistant	Several years	Standard procedures	-20°C
Disinfection byproducts	Blood (THMs)	Breath (THMs), urine (HAAs)	Ingestion, inhalation, dermal absorption		Minutes (THMs), days (HAAs)	Standard procedures (Blood, urine), Tedlar bags (exhaled air)	
Fluorinated surfactants	Blood (plasma albumin)	Cord blood, breast milk	Food, inhalation of particles	Very resistant	8.7 years	Standard procedures	-20°C
Lead	Blood		Inhalation, ingestion	Not relevant	35 days (blood), 1 year (soft tissues), 20	Metal free containers	4°C or frozen

					years (bones)		
Organochlorine insecticides	Blood or adipose tissue	Urine	Inhalation, dermal uptake, ingestion			Standard procedures	
Organophosphate pesticides	Blood	Amniotic fluid, urine	Ingestion, inhalation, dermal uptake	Rapid	< 48 hours	Standard procedures, 24h urine samples	
Parabens	Breast tissue	Urine, skin, hair	Dermal uptake			Standard procedures	
Phthalates	Blood	Urine, saliva, amnionic fluid	Food, inhalation, dermal uptake	Rapid (DEHP)	6-12 h	Standard procedures, saliva in glass tubes	-20°C -40°C (saliva)
Polycyclic aromatic hydrocarbons	White blood cells (adducts)	Placenta (adducts), urine (1-OH)	Food, inhalation		18-20 h (1-OH), 20-120 days (protein adducts) months (DNA adducts)	Standard procedures	-20°C
Polychlorinated biphenyls	Blood, adipose tissue	Breast milk, cord blood	Food	Congener dependent	5-450 days	Standard procedures	-20°C

Sampling and storage

Generally, standard procedures are available for the most frequently used matrices such as blood and urine. Many national and international guidelines exist, outlining the proper methodology to adequately sample and store different tissues (TNO 2005; WHI 1997). Samples are generally stored frozen between -20°C and -80°C, though matrices containing metals may be stored at 4°C. However, caution needs to be taken so that storage does not alter the concentration of contaminants under study. Since sampling and storage always carry a risk for cross-contamination of samples, appropriate choice of containers and cleaning procedures may be important to take into account. For example, Reid et al. (2007) showed that common lab equipment and components such as syringes, pipette tips or parafilm may leach out significant amounts of phthalates, thus potentially producing cross-contamination of blood samples.

Sample measurement

Analytical aspects

Generally, three groups of analytical techniques may be considered for the 15 chemical classes or chemicals that are included in Table 2. Every group has its own measurement specificities and pre-treatment methodologies.

Metals such as As, Cd, or Pb are generally measured using techniques such as Atomic Absorption Spectroscopy (AAS) or Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Generally, the determination of trace amounts of metals in urine, blood, breast milk or other biological matrices requires the conversion into their ionic forms, by microwave-, ultraviolet- or ultrasound-assisted sample decomposition.

A second group of techniques uses traditional chemical-analytical techniques to measure organic contaminants in different tissues. Generally, these techniques are a combination of Gas or Liquid Chromatography (GC of LC) and Mass Spectrometry (MS), or High Performance (or Pressure) Liquid Chromatography (HPLC). For many new or emerging chemicals such as phthalates or parabens, analytical techniques are still often the subject of rapid technological developments, and no "gold-standard" methods are available yet.

Finally, a third group includes the bioanalytical techniques such as Enzymelinked immunosorbant assays (ELISA) and receptor-based assays. These techniques do not apply traditional chemical qualification or quantification methods, but rely on the use of biochemical mechanisms such as antigen or receptor binding processes to quantify the presence of specific compounds in human matrices. Although many of these techniques are not able to replace chemical-analytical techniques, they offer a rapid and cost-efficient additional tool for the detection and (semi-)quantification of various groups of environmental contaminants such as alkylphenols (Zeravik et al. 2004), dioxins and dioxin-like compounds (Behnisch et al. 2001), or polycyclic aromatic hydrocarbons (Shantakumar et al. 2005).

Table 2: Overview of relevant parameters for "sample measurement" for a selected number of environmental contaminants (Smolders et al. 2009)

Compound	**Analytical aspects**		**Performance**		**Validation**	**Confounding factors**
	Techniques	*Sensitivity*	*Analytical reproducibility*	*Inter/Intra-laboratory variability*		
Alkylphenols	GC-LC/MS, HPLC, ELISA	5-500 ppb (ELISA), LC/MS <0.5 mg/ml ;RP :HPLX 0.5-1 ng/ml	2.5-5 % (GC/MS, breast milk) 10 % (LC/MS, blood)			None described so far
Arsenic	AAS, ICP/MS	0.1-1 µg/l (total As)	3-15 %	5-30 %	Certified standards, reference materials	Diuresis, age, gender, smoking, area of living
Bisphenol-A	GC-LC/MS, HPLC	<1 ng/l (breast milk)	15 %	<15%	Available various matrices	Food consumption patterns
Brominated flame retardants	GC/MS	LOD 0.3-0.7 pg/g fat (PBDE) LOQ < 10 pg/g fat (TBBPA) LOQ 30-1500 pg/g fat (HBCD and DeBDE)	25% (DeBDE, HBCD)	Large variation	None	Occupation, diet
Cadmium	AAS, ICP/MS	0.01-0.1 µg/l	1-2 %	5-10%	Widely available, Certified standards, reference materials	Diuresis, smoking
Dioxins	HR GC/MS	0.05 pg/g fat	13 %		Widely	Age, diet, BMI,

	Bioanalytical				validated	gender
Disinfection byproducts	GC/MS	<1 pg/l-0.1 mg/l (THMs, urine) 0.3-0.5 μg/l (TCAA, urine) 0.1-0.5 mg/m³ (THMs, exhaled air)	2.8-8.5 %		Available from USEPA	Age, gender, occupation
Fluorinated surfactants	GC-LC/MS	Depends on compound measured	Large differences		None	Gender
Lead	AAS, ICP/MS	0.05-0.1 μg/l	1-2 %	5-10 %	Widely available	Smoking, menopause
Organochlorine insecticides	GC/ECD-MS	5-10 ng/l (urine, SPE + GC/MS/MS)	No data available	No data available	No data available	No data available
Organophosphate pesticides	GC/MS	No data available	Generally good		Accepted by ACGIH	Diuresis
Parabens	HPLC/MS, GC/MS	No data available	Recovery 50%	No data available	None	Cosmetics user patterns
Phthalates	HPLC, LC/MS	0.01-0.5 μg/l (blood, breast milk) 1-30 ng/l (urine)	No data available	No data available	None	Age
Polycyclic aromatic hydrocarbons	HPLC/fluorescence GC/MS ELISA ^{32}P-postlabelling	0.05 μg/l (HPLC, 1-OH) 0.1 μg/l (GC/MS, 1-OH)	No data available	No data available	^{32}P-postlabeling standardized by IARC	Ethnicity, vitamin C uptake
Polychlorinated biphenyls	GC/ECD-MS Bioanalytical	11-19 ng/kg tissue	7-14 %	No data available	Widely available	Age, diet, BMI

Performance and validation

There are important differences to be considered among the different chemicals with respect to the performance characteristics of the measurement techniques. Generally, metal analyses have a high analytical reproducibility and low inter- and intra-laboratory variability, while for some of the new or emerging chemicals, reproducibility is much lower and variability may be large (Van Leeuwen et al. 2006). Very often performance is considerably improved with the availability of appropriate reference materials and round-robin tests, such as the QUASIMEME or QualiMed testing schemes (QUASIMEME 2006; Schaller et al. 2002).

Confounding factors

Confounders (or confounding factors), in the simplest way, are defined as extraneous variables that are associated with the exposure under investigation and are risk factors for the disease. Especially in the context of using HBM for integrated environmental health impact assessments, factors such as occupation, dietary habits or smoking may exert a confounding effect. Confounders can be broadly divided in two classes: lifestyle-related and non-lifestyle confounders:

- Lifestyle-related confounders are factors that are related to the behavior or life-style of the constituent. Smoking and diet are frequently observed examples of lifestyle confounders.
- Non-lifestyle confounders are factors that are totally or partially outside of the constituents power of alteration, such as gender, age, or ethnicity as well as the presence of other chemicals or occupational/environmental exposures.

It is important that these confounding factors are considered when calculating and reporting biomarker data for aggregate exposure assessment, since they may exert an important impact on the identification of high-exposure groups. For example, smoking behavior, not only of the constituent itself but also of their immediate surroundings, exerts a well-documented effect on the Cd or Pb dose (Ikeda et al. 2005; Mortada et al. 2002; Mortada et al. 2004). In addition, the relationship between age of participants and presence of bioaccumulating compounds such as PCB and pesticides have extensively been illustrated (Bjerregaard et al. 2001; DeCaprio et al. 2005; Wong et al. 2002).

Data interpretation

Properly interpreting, reporting, and putting into context of human biomonitoring data are key aspects of any HBM project considering the potentially sensitive nature of HBM data (Paustenbach and Galbraith 2006a, b). HBM data provide direct information on the link between concentrations of toxicants from aggregate exposure and associated adverse health effects, without including sometimes uncertain physiological aspects such as bioaccumulation, transformation or excretion processes. Within the context of aggregate exposure, HBM may greatly increase the ability to provide more detailed information on the link between internal toxicant concentrations (dose) and the related health effects.

Without going into extensive detail on the complex matters concerning the interpretation of HBM data, it is highlighted that basic data interpretation needs to include three initial steps (Table 3):

- How do measured HBM data compare to concentrations already reported (in literature)?
- Are there, based on known dose-response relationships, health effects to be expected?
- Can time trends, spatial patterns, or susceptible subgroups be identified?

Table 3: Overview of relevant parameters for "data interpretation" for a selected number of environmental contaminants (Smolders et al. 2009)

Compound	**Concentrations in literature**	**Dose- response relationships**
Alkylphenols	Nonylphenol: 0.3 mg/kg fat (breast milk) < 15.17 ng/ml (cord blood) 0.6-16 ng/g serum (The Netherlands) 14-222 ng/g serum (Japan)	In rats: relationships with reproductive parameters
Arsenic	0.08-0.25 µg/g (hair) 0.34 µg/g nail 5-40 µg/day (24h urine sample) <10µg/g creatinin (As + metabolites	Chromosomal mutations, skin cancer, reproductive, neurological, developmental effects
Bisphenol-A	0.8-1.1 ng/ml (breast milk) 1.28 ng/ml (urine) < 4.05 ng/ml (cord blood) 0.41 ng/ml (blood)	Increased prostate, decreased epididymal weight in mice
Brominated flame retardants	20.5 ng/g lipid (BDE-47 in serum) 0.6 ng/g lipid (HBCD in breast milk)	No clear effects
Cadmium	< 2µg/l (blood, adult non-smoker) <5 µg/l (blood, adult smoker) <0.5 µg/l (blood, children) <2 µg/g creatinin (urine adult) <0.5 µg/g creatinin (urine children)	Tubular proteinuria
Dioxins	3.3-22.3 pg/g fat (breast milk) 20-60 pg TEQ/g fat (blood) Critical TCI 1-4 pg TEQ/kg/bw/day	Carcinogenic, (neuro)developmental delays, chloracne, decreased lung function, thyroid hormone status
Disinfection byproducts	4-54 µg/m³ (exhaled air) 0.5-5.2 µg/l (plasma) 0-30 ng/min (urine followed in time)	No data available
Fluorinated surfactants	1-81 ng/ml (PFOS, blood) 2-40 ng/ml (PFOA, blood) 0.7-5.8 ng/ml (PFOSA, blood)	Toxicological data for rodents Little human data NOAEL 0.03-0.15 mg/kg/day

Compound	Concentrations in literature	Dose- response relationships
Lead	30-50 μg/l (adults, blood) 10-30 μg/l (children, blood)	Cognitive impairment, reduced haemoglobin synthesis, anaemia, encephalopathy
Organochlorine insecticides	0.1-5.1 μg/l (DDE in urine of children) 0.06-0.74 μg/l (HCB in urine of children)	Non-Hodgkin's lymphoma, leukemia, reproduction
Organophosphate pesticides	4.9-908 μg/l (DMP in urine of children) 1.6-526 μg/l (DEP in urine of children)	Reproductive effects
Phthalates	165 μg/g creatinine (MEP in urine) 4 μg/g creatinine (MEHP in urine)	NOAEL 3.7-8000 mg/kg bw/day (urine, DEHP)
Polycyclic aromatic hydrocarbons	0.05-4 μg/l (1-OHpyrene in children's urine) 147 ng/g creatinine (1-OHpyrene in urine)	Oxidative stress, genotoxicity
Polychlorinated biphenyls	8-155 ng/g fat (PCB-153, breast milk)	(liver) Carcinogen, reproduction, neurodevelopment

Concentrations in literature

There is a constantly increasing amount of HBM data available for a wide variety of compounds. As many papers in literature report HBM data from case-control research projects however, the concentrations reported may focus on potentially high-exposure or particularly susceptible groups there may not necessarily reflect the average exposure characteristics of the general population. Hence, caution needs to be taken when interpreting data based on literature concentrations. Of more immediate use are HBM data generated through large scale survey programs, that aim at periodical measurements in order to produce information on the prevalence of exposure to a wide variety of toxicants in the general population. An excellent source of information on biomarkers to assess aggregate exposure in the general population is found in publications from the HBM survey projects executed by USA's Centers for Disease Control and Prevention (CDC 2005), the German Environmental Survey (GerES) (Becker et al. 2002; Becker et al. 2003) or the Flemish Environment and Health Survey (Koppen et al. 2009; Schroijen et al. 2008). These projects provide an excellent source of information on biomarker values in the general population, to some extent also breaking up the available HBM data into different subpopulations. As these national survey data provide an overview of the average aggregate exposure of the general population, they have been used to develop reference values for 23 substances (i.e. the 95th percentile of the distribution of concentrations of a specific compound in a body fluid of a reference population) and for a limited number of chemical substances also health based HBM-values, based on toxicological and epidemiological data (HBM-Kommission 1996; Umweltbundesamt 2007). These 23 reference values and four HBM-values can be used by policy makers and risk assessors to directly evaluate aggregate exposure of the general population, but require a careful and thorough evaluation of the available scientific evidence.

Dose-response relationships

One of the main aims of HBM is to provide data on the internal toxicant dose in constituents, based on the partial uptake from all different environmental compartments. Although these internal dose data are generally much closer to the potential adverse health effects than mere exposure data in different compartments, the availability of dose-response relationships is essential for a clear linkage of HBM and health data. Unfortunately for many chemical classes, there is only limited data on dose-response relationships, which often are developed from either animal testing, occupational or accidental exposure, or epidemiological data.

At the same time, these relationships generally only relate exposure through one specific environmental compartment to health effects, and hence only provide incomplete information on the relationship between aggregate exposure and health effects. Hence, in order to maximize the usefulness for environmental health impact assessment, exposure-response relationships should be replaced by dose-response relationships. Including internal dose as a measure of aggregate exposure will greatly enhance interpretability the relationship between environmental agents and associated health effects (Connell et al. 1999; Escher and Hermens 2004).

Data availability

With respect to the availability of human biomonitoring data, a distinction needs to be made between two different types of biomarker data: (1) those emerging from research projects, and (2) those collected from broad-based population surveys. Research projects often are aimed at gathering information to address a priori defined research questions, and are set up in such a way that the hypothesizes relationship between environmental exposure, biomarker, and health outcome is optimally considered. Broad population survey projects however, aimed at providing an overview of contaminant loads in the general population without necessarily addressing specific research questions, benefit more from measurements in matrices that provide a long-term overview of pollutant exposure, integrating exposure along all possible pathways without necessarily discriminating among sources. A number of parameters are of main importance when describing sample collection and storage for a wide variety of environmental pollutants (Table 4).

Table 4: Different aspects of research and survey projects generating biomarker data

	Research project	**Survey project**
# participants	Low	High
Exposure classification	High	Low
Aim	Hypothesis testing	Background values
Inter-individual variability	Low	High
Study control	High	Low
Source identification	Individual level	Population level

Because often several thousands of individuals are included in population survey studies, it would be both financially and logistically impossible to describe environmental exposure and health effects in the same detail that is generally the case in specific and detailed research studies. In large HBM survey projects, biomarker data are often gathered and reported without corresponding detailed external exposure data, leaving the relationship between internal and external exposure as one to be determined (Clewell et al. 2008; Smolders et al. 2009). The

typical left-to-right progression from Figure 5 is not necessarily followed in a human biomonitoring survey project, and retracing source identification and identifying early warning signs of adverse health effects becomes a difficult task. Hence, for improved understanding of human biomonitoring data originating from survey projects, additional approaches for "exposure reconstruction", or "inverse dosimetry" are needed.

In the last decade or so, several large-scale population surveys on human biomonitoring have emerged across Europe. Broad population surveys, such as GerES in Germany, the Environmental Health Monitoring Studies in the Czech Republic or the Flemish Human Biomonitoring Program in Belgium have focused on quantifying background information on the exposure in the general population, without necessarily addressing immediate research questions (Table 5). The most extensive human biomonitoring survey, NHANES in the USA, covered 212 different chemicals in a representative subsample of the American population.

Table 5: A brief overview of some large scale HBM population surveys. URLs indicate where results for the different surveys can be obtained

Country	Survey name	URL for Results
Belgium	Flemish CEH	http://www.milieu-en-gezondheid.be/English/index.html
Canada	CHMS	http://www.hc-sc.gc.ca/ewh-semt/contaminants/human-humaine/index-eng.php
Czech Republic	Environmental Health Monitoring	http://www.szu.cz/topics/environmental-health/environmental-health-monitoring
Germany	GerES	http://www.umweltbundesamt.de/gesundheit-e/survey/index.htm
International	COPHES-project	http://www.eu-hbm.info/
International	ESBIO-inventory	http://www.hbm-inventory.org/scid/e-formv2/default.asp
International	WHO-human milk	http://www.who.int/foodsafety/chem/pops/en/index.html
USA	NHANES	http://www.cdc.gov/exposurereport/

Research projects on the other hand have developed and tested biomarker detection and quantification methods for a much wider range of chemicals and human matrices that those included in the survey programs in Table 5, although these methods may not always be fully validated. In a recent effort, the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) and the German Chemical Industry Association joined hands to increase the knowledge on the internal exposure to chemicals of the general German population. Emphasis of the cooperation is placed on substances with either a potential health relevance or to which the general population might potentially be exposed to a considerable extent. Over a period of 10 years, new analytical biomonitoring methods will be developed to measure exposure for 50 chemicals. The Chemical Industry Association and Federal Ministry share responsibility in the project, with VCI taking up the overall responsibility and the funding of the HBM method development, while BMU will apply the developed methods in population-based HBM surveys.

To conclude, there certainly is no lack of human biomonitoring methods, data, or studies to limit their use for aggregate exposure assessment. Even more, the National Research Council highlighted that "*The ability to generate new biomonitoring data often exceeds the ability to evaluate whether and how a chemical measured in an individual or population may cause a*

health risk or to evaluate its sources and pathways of exposure" (NRC 2006). Hence, the challenge of including biomarker data should not about collecting data, but about proper interpretation of the data for aggregate exposure assessment and environmental health impact assessment.

Physiology Based ToxicoKinetic (PBTK) models

The modelling/computational tool for linking external exposure from multiple sources to biomonitoring data is the Physiology Based ToxicoKinetic (PBTK) models. The latter are continuously gaining ground in regulatory toxicology, describing in quantitative terms the absorption, metabolism, distribution and elimination (ADME) processes in the human body, with a focus on the effective dose at the expected target site (Bois et al. 2010). This trend is further amplified by the continuously increasing scientific and regulatory interest about aggregate and cumulative exposure; PBTK models translate external exposures from multiple routes (Yang et al. 2010) into internal exposure metrics, addressing the effects of exposure route in the overall bioavailability (Sarigiannis and Karakitsios 2011; Valcke and Krishnan 2011) or the dependence on critical developmental windows of susceptibility, such as pregnancy (Beaudouin et al. 2010), lactation (Verner et al. 2008) and infancy (Edginton and Ritter 2009).

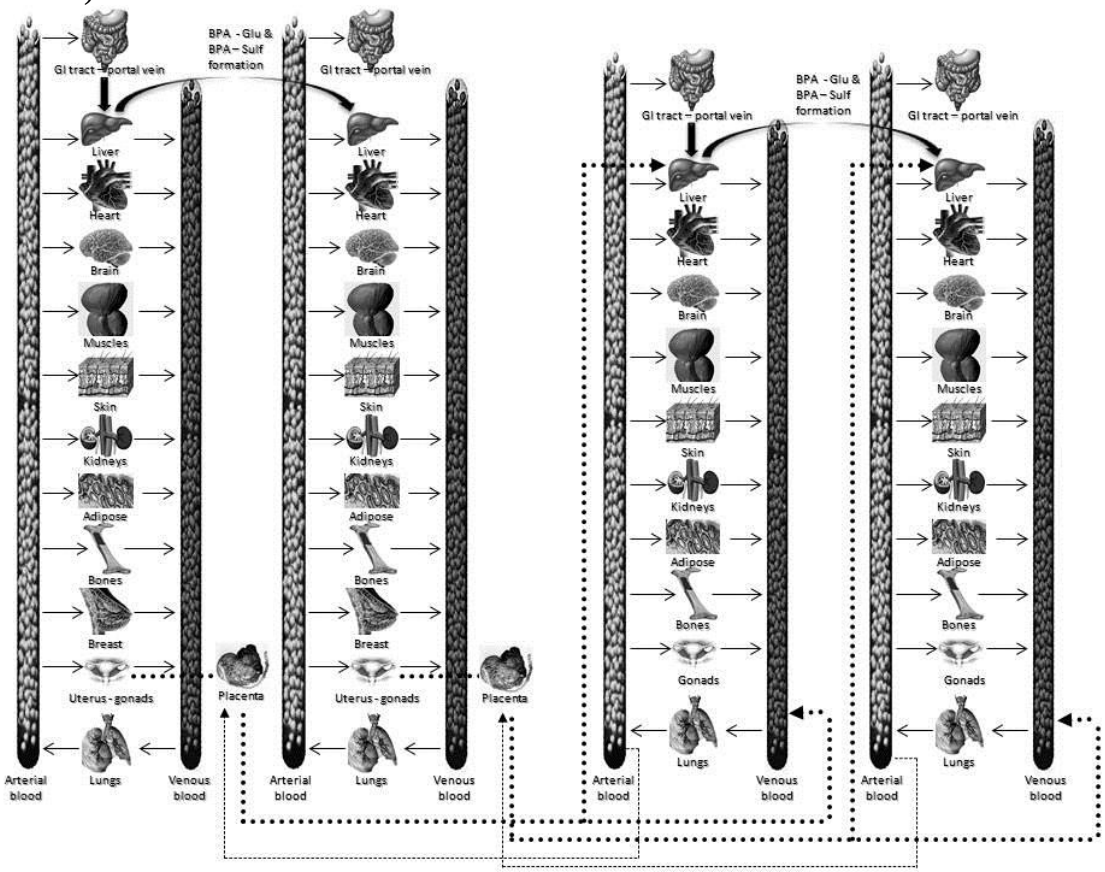


Figure 6: Conceptual representation of the generic PBTK model including Mother-Fetus interaction

With regard to cumulative exposure, PBTK models offer the advantage of calculating the effect of the interactions among the mixture compounds at the level of metabolism, however due to the inherent difficulties arising, the existing applications are limited to VOCs (Cheng and Bois 2011; Haddad et al. 2000; Sarigiannis and Gotti 2008) and metals (Sasso et al.

2010). Recently, efforts have shifted also towards the integration of whole-body physiology, disease biology, and molecular reaction networks (Eissing et al. 2011), as well as integration of cellular metabolism into multi-scale whole-body models (Krauss et al. 2012). Considering the opportunities offered by the use of PBTK models in exposure/risk characterization, several research groups are developing generic PBTK models, either stand-alone models such as PK-Sim (Willmann et al. 2003), Indus-Chem (Jongeneelen and Berge 2011) or incorporated within integrated computational platforms for exposure assessment such as INTERA (Sarigiannis et al. 2011) and MENTOR (Georgopoulos et al. 2008). The development of generic PBTK models is substantiated by the recent advances in quantitative structure–activity relationships (QSARs) and quantitative structure–property relationships (QSPRs) (Peyret and Krishnan 2011; Price and Krishnan 2011), providing the basis for development of relevant PBTK models regarding data-poor or new chemicals.
The formulation of the generic PBTK model that will be used in the frame of CROME-LIFE (a conceptual representation is given in Figure 6) is described in Annex 1.

Biomonitoring data assimilation

One of the main options of combining PBTK models and HBM data is to make inferences about environmental exposure scenarios for biomarker data collected in population-based studies (Mosquin et al 2009). As previously outlined, biomarker data from large-scale HBM surveys often adequately reflects population variability, yet frequently lack the necessary activity information to take into account biomarker kinetics, or to develop aggregate exposure profiles. Exposure scenarios can be variable, with multiple possible routes and time variability of exposure. PBTK models may allow disentangling the contribution of different environmental compartments through exposure reconstruction. For example, Gosselin et al (2006) used the biologically based toxicokinetic model developed by Carrier et al (2001) to estimate the dynamic profile of MeHg in both blood and hair. While many PBTK models used a simple one compartmental model to describe the fate of MeHg in the human body (assuming a steady-state in MeHg uptake and excretion), Carrier's model accounts for fluctuating MeHg levels in fish, but also for fluctuating fish consumption patterns. Through the combined use of measuring and modeling, Gosselin et al (2006) was able to describe the bilateral links between the MeHg daily intake and the mercury concentration in blood and hair samples collected at any given time following the onset of any MeHg ingestion period. It was clearly illustrated that the steady-state is not valid for MeHg toxicokinetic modeling, and the probable time duration of MeHg exposure is essential when back-calculating MeHg intakes. Particularly, it was concluded that biomarker studies would be better off focusing more on gathering data on the period during which the individual might have consumed contaminated food, rather than the need to quantify the daily MeHg intake.
Also Sohn et al (2004) provided a good example of the synergy that is created when biomonitoring data and PBTK models are integrated for reconstructing population exposure. Combining venous blood concentrations of TCE (trichloroethylene) and PBTK modeling showed that population-scale variability of pharmacokinetics dominate the uncertainty of the predicted TCE concentration in air, and suggested that one should not spent excessive resources obtaining exposure onset and duration data if the primary objective is reducing uncertainty in the predicted concentration of TCE in air. The authors concluded that in order to achieve optimal synergy among biomarker measurements and PBTK modeling, the persistence of the biomarker should be long relative to exposure duration for estimating long-term, or population scale, exposure effects.

More recently, Ruiz et al (2010) used PBTK modeling to gain further insight in urinary cadmium biomarker data from the most recent NHANES biomonitoring survey. From these data, the authors predicted urinary Cd concentrations by age at various intake doses from 10 to 100 μg Cd/day, and particularly demonstrated the marked elevated uptake of Cd in 6-11 year old children. The model also predicted a 1.4- to 1.6-fold higher urinary Cd excretion in females compared to males in all age groups. The study demonstrated that computational techniques such as PBTK models can be useful predictors for delineating population subgroups at special risk as a function of age and gender.

Ideally, biomonitoring requires the collection of other exposure information to identify the routes and the pathways of exposure and to eliminate sources. This complex problem has led to research programs that are beginning to develop theoretical reconstructive modeling tools to retrace the biomonitoring results backward to the routes of exposure. Thus, PBTK models are also used for assimilating biomonitoring data, through exposure reconstruction, meaning the quantification of exposure components related to the observed biomarkers concentrations. Considering that biomonitoring data represent an integration of exposure from all sources and routes, they also represent short-term exposures as well as cumulative internal dose due to repeated exposures with the knowledge of toxicokinetics and different biomarkers for chemicals. Biomonitoring data makes it feasible to identify metabolic pathways and target metabolites at very low levels of exposure in humans, which is helpful to understand the human toxicokinetics. There are three approaches for linking biomonitoring data to health outcomes: direct comparison to toxicity values, forward dosimetry and reverse dosimetry (Clewell et al. 2008). Biomonitoring data can be directly compared to toxicity values in the case where the relationship of the biomarker to the health effect of concern has been characterized in the human. In forward dosimetry, pharmacokinetic data in the experimental animal can be used to support a direct comparison of internal exposure in humans, providing an estimate of the margin of safety in humans. It is possible to determine the relationship of biomarker concentration to effects observed in animal studies. Alternatively, reverse dosimetry can be performed to estimate the external exposure that is consistent with the measured biomonitoring data for comparison with an animal-based health standard, such as a Reference Dose (RfD) (Figure 7).

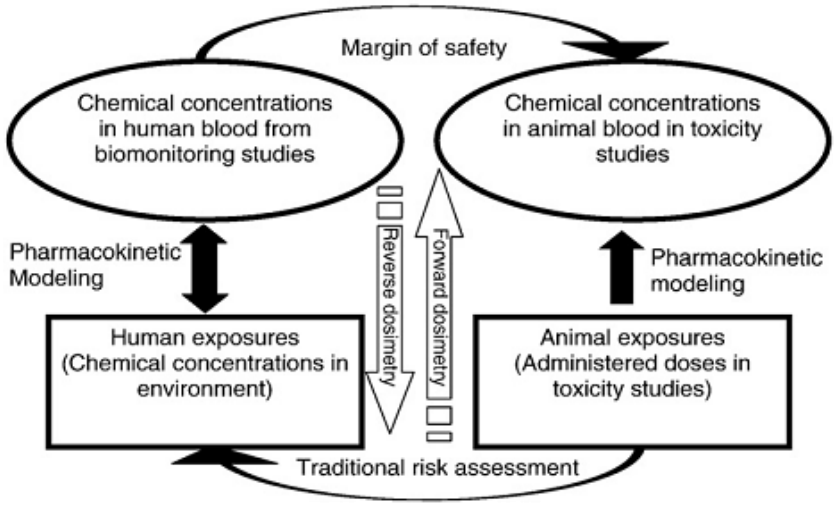


Figure 7: Interpretation of biomonitoring data (Clewell et al. 2008)

A typical reverse dosimetry approach uses a physiologically based pharmacokinetic model to identify exposures consistent with human biomonitoring data, as shown in Figure 8. A PBTK

model and Monte Carlo simulation are used to predict biomarker concentration distribution at the same assumed exposure level and different exposure patterns and pharmacokinetic parameters; the resulting distribution is then inverted to obtain the exposure conversion factor (ECF). Multiplying the ECF distribution by a measured biomarker concentration results in a distribution of exposure (Tan et al. 2006). In risk characterization, forward dosimetry and reverse dosimetry are complementary to each other. Quantitative interpretation of biomonitoring data can best be accomplished by linking PBTK modeling with exposure pathway modeling within a probabilistic framework.

An additional reason for which PBTK modeling is necessary for the interpretation of biomonitoring data is that PBTK models by quantifying the ADME procedure are the only way available to us for defining the quantitative links between the timing of exposure(s) and sampling. Tracked biomarkers (either the parent compound or the metabolites) have a concentration profile varying through the day depending on the exposure profile and the kinetics of elimination-clearance.

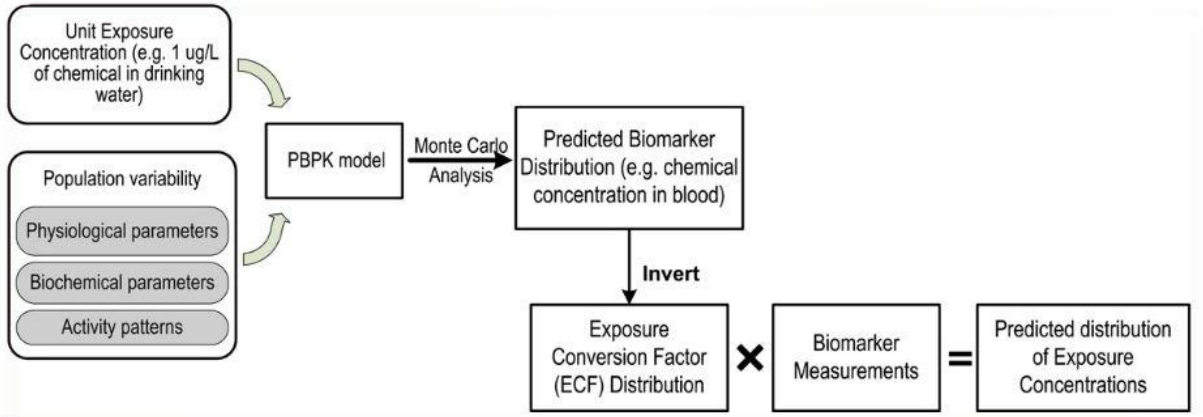


Figure 8: Schematic of the generalized Monte Carlo approach to reconstruct exposure from biomonitoring data (Clewell et al. 2008)

Although forward prediction of biomarker levels due to exposure is a reasonable expectation, the reverse procedure is a very demanding task as much as it is important. The difficulties are not limited only to the specificity and the sensitivity of the biomarker of choice, but also on the numerical frame that it is needed. Nevertheless, some techniques currently under development (Georgopoulos et al. 1994; Georgopoulos et al. 2009) allow the assessment of exposure levels to some extent through "inverse modeling" techniques.

Inverse modeling by reversing a PBTK model is not as easy as it may seem at first sight. PBTK models are compiled by a large set of differential equations, and the more refined the model, the larger the set. Thus, the re-built of a complex PBTK model to work backwards is almost impossible, unless it is a very simple model as the one by Rigas (2001), who used urinary biomarker data and the inverse solution of a simple, two-compartment toxicokinetic (PK) model for chlorpyrifos (CPF) to estimate the magnitude and timing of doses, based on the Minnesota Children's Pesticide Exposure Study (MNCPES, but the compromises made in the detail of the model are reflected in the overall backwards capability.

As a result, attempts in inverse modeling are focused in computational numerical techniques. Georgopoulos et al. (1994) used the maximum likelihood estimation (MLE) method in conjunction with PBTK modeling for reconstructing short-term (30 min) exposure to chloroform, and to resolve the total dose between two routes of uptake (i.e. inhalation and

dermal absorption). In a further elaborated approach, Roy and Georgopoulos (1998) used the combined MLE–PBTK modeling approach with synthetic biomarker data and demonstrated that it is mathematically feasible to reconstruct longer term exposures to VOCs. Some recent studies on population-level exposure reconstruction focused on data sampled from distributions of biomonitoring studies such as NHANES using direct deconvolution of biomarker distributions assuming a linear response (Tan et al. 2006), or a brute-force Monte Carlo sampling approach (Sohn et al. 2004; Tan et al. 2007). However, simplifying assumptions such as linearity are known to produce erroneous exposure characterizations in the forward mode of analysis and therefore they should be considered with great caution in the inverse mode of analysis (Georgopoulos et al. 2009).
From the above, it is clear that a MLE-PBTK modeling approach has the ability to derive the best results for assessing the problem in inverse modeling.

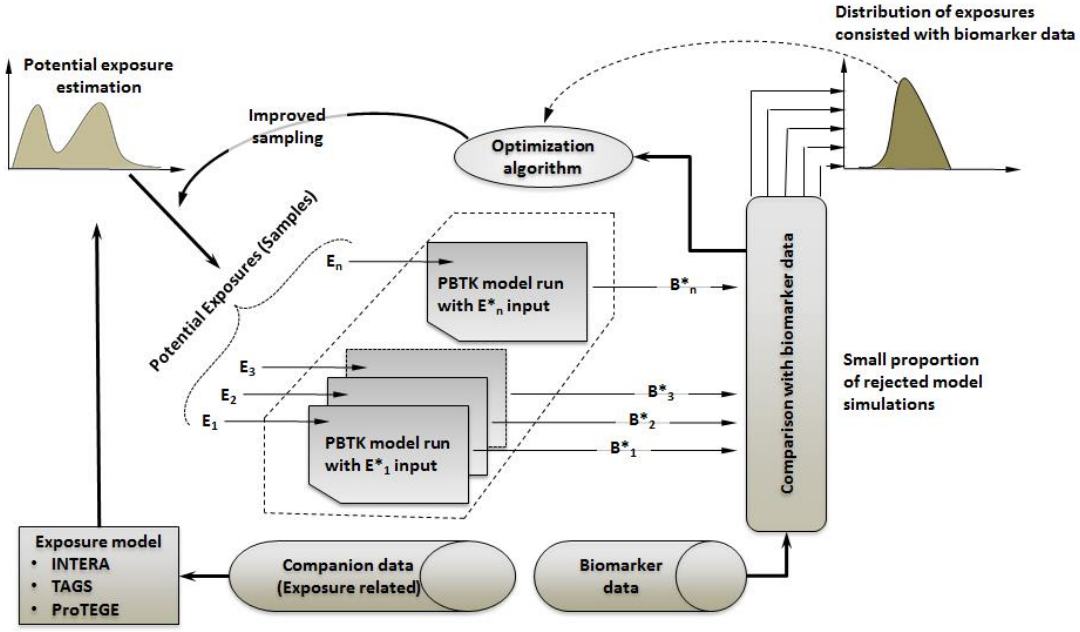


Figure 9: Optimization-aided exposure reconstruction based on HBM data using time-evolving PBTK models (adapted from Georgopoulos et al. (2009))

The need for direct risk characterization based on biomonitoring data, resulted in the establishment of the biomonitoring equivalences (BEs) (Hays and Aylward 2009). BEs values represent quantitative benchmarks of safe or acceptable concentrations of a chemical or its metabolite in biological specimens that are consistent with selected reference values, such as the ADI, TDI, MRL and RfD, using the knowledge about the toxicokinetic properties of the chemical (Boogaard et al. 2011). However, the use of reliable PBTK models is the most convenient way on translating external exposure reference values into BEs. Moreover, in order to utilize the capabilities of *in vitro* testing, PBTK models are used to identify the Biological Pathway Altering Dose (Judson et al. 2011), that is analogous to current risk assessment metrics in that it combines dose-response data with analysis of uncertainty and population variability to arrive at conservative exposure limits. The analogy is closest when perturbation of a pathway is a key event in the mode of action (MOA) leading to a specified adverse outcome.

Interpretation of repeated HBM measurements

Typically, large-scale human biomonitoring surveys gather biomarker measurements from single time points. These data are then used to make inferences about longer periods of toxicant intake, assuming that biomarker values are representative of steady-state conditions. However, steady-state conditions require stable biokinetics, a constant rate of exposure, and a dynamic equilibrium among different body tissues. Figure 10 gives an indication of how a single sampling time may not be representative of steady state biomarker concentrations. At the specific sampling time (t = 10), the biomarker value can be the result of different exposure scenarios. The dashed line implies one high peak exposure episode, the full line a continuous fluctuation around a steady-state situation, and the dotted line a completely steady-state situation. Obviously, information on the biomarker pharmacokinetics is an issue that has a significant effect on both source identification and potential associations with health effects.

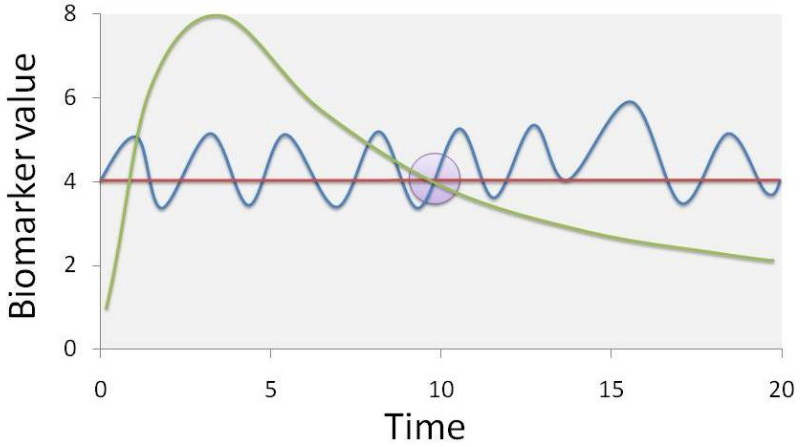


Figure 10: A generic example indicating how a single biomarker value may not be representative for exposure assessment

An example on how this assumption of steady-state may be important for source identification can be found in historical Pb levels in the blood. Fifty years ago, blood Pb levels were very well correlated with the emission of Pb from car emissions, as tetraethyl lead as an antiknocking agent was the main source of Pb emission in the environment. It can be assumed that blood-Pb levels were more or less in a steady state as air concentrations were not changing rapidly. Recently, this has been validated for different European blood Pb datasets (Smolders et al, 2010). Since the outphasing of tetraethyl lead in gasoline, the contribution of this continuous exposure has declined rapidly, and currently childhood lead exposure in the US is thought to occur primarily through paint, dust, and soil ingestion (Bartell et al, 2004). These activities are episodic in nature and seem to occur sporadically and at varying rates (Wong et al, 2000).

Hence, assuming that biomarker values are representative of a steady-state concentration in the measured matrix may not be justified, and additional investigation may be required. By repeated sampling of individuals with particularly high and particularly low biomarker values (e.g. > P_{95} and < P_5), more insight in the pharmacokinetic behavior of the biomarker can be obtained. In combination, having an additional detailed questionnaire aimed at recording specific exposure scenarios during the time period between samplings, more insight into source identification can be obtained. The time lag between sampling obviously depends on the half-life of chemicals, and the matrix that is used for biomarker determination.

Chen et al (2009) outlined a stochastic approach using Markov Chain Monte Carlo (MCMC) simulations and a PBTK model to estimate inhalation exposure to TCE, based on repeated

measurements in venous blood. Their procedure illustrated that estimating environmental exposure from repeated biomarker measurements could be achieved with very high precision given known exposure duration. An additional approach, proposed by Bartell et al (2004) could be the inclusion of measuring the same chemical, but in different matrices, thus reflecting different pharmacokinetic properties of the biomarker. For example, measuring cadmium in both blood and urine could give an indication of the long-term steady state exposure (urine) but also of the more recent, dynamic exposure (blood). Combining these both data may provide increased resolution for source identification. Already earlier, Henderson (1995) proposed the use of multiple biomarkers of varying half-lives to distinguish among different possible exposure scenarios. Recently, the findings of an INTARESE/ENVIRISK workshop on the use of human biomonitoring for environmental health impact assessment reconfirmed the added value of this type of biomarker batteries (Smolders et al 2010). By combining a PBTK model (which describes the expected distribution of a chemical among different matrices in the body) with biomarker battery data (quantifying the parent compound or metabolites in different matrices), greater sampling flexibility may be achieved. Mosquin et al (2009) argued that when a compound is simultaneously measured in for example blood and urine, they can be considered different biomarkers. The availability of multiple biomarkers then leads to a trade-off in the design of studies: is it more efficient to sample one biomarker over multiple time points or more biomarkers at a single time point? From a practical point of view, the latter may be preferred, as it is generally much easier and cost efficient to collect multiple biomarkers at a single sampling event in general population studies. In illustrating this observation by using a well-validated PBTK model for chlorpyrifos, Mosquin et al (2009) concluded that collecting biomarker samples at additional time points tends to be more effective than measuring multiple biomarkers at one time point, based on reducing the mean absolute percentage error (MAPE).

Linking biomonitoring data to health effects

Linking biomonitoring data to health effects will include a) identification of exposure biomarker profiles/biological responses/health outcomes b) examination of links between exposure data and effect data/health outcomes; and c) identification of exposure biomarker profiles that are differentially expressed in the examination survey population, thus linking exposure data with health outcomes at the individual level.

Internal doses will be coupled to health impacts on the local population through advanced statistical methods to derive the dose–response functions which account for differences in exposure patterns, susceptibility differences and inter-individual variation in health response. The approach starts from the biomarker values measured in the respective biological matrices to estimate through the application of the lifetime generic PBTK model the biological effective dose in the target tissue, which is consistent with the biomarker level measured.

We methodology we propose to estimate the health impact borrows the GWAS methodology to create a model Environmental-Wide Association Study (EWAS), to search for environmental factors associated with disease on a broad scale (Patel et al. 2010).

Analogously to GWAS studies, EWAS consist of two methodological steps: first, we consider a panel of unique “environmental assays” measured across cases and controls in the population studies available. This leads to the identification of several environmental factors with significantly high association with the health outcome to be assessed. Second, we validate the associations using data from other cohorts available in CROME and used as a

validation dataset. In this way the results from EWAS can better inform about environmental factors that need to be measured in genetic studies to begin to provide us insight in regards to disease etiology.

We will analyze all environmental factors from the population studies available to the consortium that are a direct measurement of an environmental attribute, such as the amount of pesticide or heavy metal present in urine or blood. Next step will be to categorize all the environmental factors in several groups in order to discern patterns among related groups of factors, analogous to chromosomal units in GWAS. Then, we will use used survey-weighted logistic regression to associate each of the identified environmental attributes with the health end point selected while adjusting for different covariates (age, sex, socio-economic status etc.) considering the interdependence of the covariates. The general formulation of the approach is based on the mathematical linkage of health end points (expressed in terms of odds ratio, *p* with different covariates (age sex, socio-economic status, lifestyle choices such as smoking, etc.) and the internal dose in the target tissue (X_{factor}) such as the expression below:

$$\text{logit(p)}=\ln\left(\frac{p}{1-p}\right)=\alpha+\beta_0\cdot\text{cov}_0+\beta_1\cdot\text{cov}_1+\beta_2\cdot X_{factor}\ldots+\beta_n\cdot\text{cov}_n$$

where *cov* represents the different covariates used in the model and α and β are the regression coefficients which take into account the interdependence between covariates.

Logic regression searches for Boolean (logic) combinations of binary variables that best explain the variability in the outcome variable, and thus, reveals variables and interactions that are associated with the response and/or have predictive capabilities (Kleinbaum et al. 1998). The logic expressions are embedded in a generalized linear regression framework, and thus, logic regression can handle a variety of outcome types, such as binary responses in case-control studies, numeric responses, and time-to-event data.

The outcome in logistic regression analysis is often coded as 0 or 1, where 1 indicates that the outcome of interest is present, and 0 indicates that the outcome of interest is absent. If we define *p* as the probability that the outcome is 1, the multiple logistic regression model can be written as follows:

$$p=\frac{e^{b_0+b_1X_1+b_2X_2+\ldots\ldots\ldots+b_nX_n}}{1+e^{b_0+b_1X_1+b_2X_2+\ldots\ldots\ldots+b_nX_n}}$$

where *p* is the expected probability that the outcome is present; X_1 through X_n are distinct variables or covariates; and b_0 through b_n are the regression coefficients. To obtain the corresponding *logit* function from this, we calculate:

$$logit\left(p\right)=\ln\left(\frac{p}{1-p}\right)=e^{b_0+b_1X_1+b_2X_2+\ldots\ldots\ldots+b_nX_n}$$

The survey-weighted logistic regression model is a variation of the logistic model that is more appropriate for survey data obtained using complex sampling techniques, such as stratified random or cluster sampling. Like the conventional logistic regression models survey-weighted logistic regression specifies a dichotomous dependent variable as function of a set of explanatory variables (Jewell 2004). The survey-weighted logit model reports estimates of model parameters identical to conventional logit estimates, but uses information from the survey design to correct variance estimates introducing *weights* to compensate for over- or under-sampling of specific cases or for disproportionate stratification (e.g. males vs females or minority groups vs general population).

Protocol for cross-Mediterranean study

Introduction

Children from pre-existing Mediterranean cohorts established (1) within the PHIME project, involving Slovenia, Croatia, Italy, Greece and (2) within the INMA Project (Environment and Childhood; Guxens et al., 2012) project in Spain will be followed up at 6-8 years of age (14 years in some cases in Spain) in CROME cross-Mediterranean study. The PHIME cohorts consist of 286 children from Slovenia, 161 from Croatia, 632 from Italy, 350 from Greece and 2493 from Spain. Children of the PHIME Mediterranean cohorts were tested for neurodevelopment (Bayley III test) at 18 months of age. Mother hair, cord blood, cord tissue and meconium have been sampled at birth, breast milk and mother's hair 1 month after birth. Hair samples have been analysed for mercury, cord blood and breast milk for mercury, cadmium, lead and arsenic, as well as for essential elements (selenium, zinc, copper). Analysis for GSTT1/GSTM gene deletion polymorphism has been done on a subset of samples.

The results of the PHIME cohort have already been evaluated. However, the effect of toxic metals is variable among individuals, even if they are exposed to the same level of Hg/MeHg. These differences still remain unclear, what might be explained by genetics. In the follow-up study, we would like to find those genetic polymorphisms which could modulate the detrimental effects of Hg and MeHg at low exposure in Mediterranean cohort. In this regard, we will be focused on gene polymorphisms of GSH related genes (*GSTM1, GSTT1, GSTP1, GSTM3, GPX1*), metal binding protein genes (*MT2A, MT4, SEPW1, SEPP1*), genes involved in scavenging of ROS (*CAT, SOD1, SOD2, GSR*), and genes affecting neuropsychological performance as found in literature (*PON1, BDNF, PGR*) to associate with Hg/MeHg levels in biological tissues (cord tissue, child's hair, or/and child's urine). Examination will include collection of urine and hair samples for analyses of metals and collection of saliva samples for genotyping. Children will be tested for neuropsychological performance using *Wechsler Intelligence Scale for Children (WISC III*).

Background

Epidemiological studies have demonstrated that the developmental neurotoxicity is associated with prenatal methyl mercury (MeHg) exposure (Grandjean and Landrigan 2006); however, susceptibility to MeHg toxicity may be modified by genetic factors (Grandjean and Julvez 2013).

As recently observed by (Grandjean and Julvez 2013)in a longitudinal cohort study ALSPAC, any overall MeHg toxicity was not detectable at the low average exposure levels, even when adjustments for beneficial dietary factors from maternal seafood intake and social class were included in the models. However, adverse associations among genetically susceptible groups were discovered in analyses that were stratified by the SNP allelic variants. While the wild type was associated with benefits from increased MeHg exposure (as a marker of maternal seafood diet) one or two mutations in the genes led to lower IQs at age 8 years. Further, the importance of such genetic predisposition is illustrated by the fact that 21 % of the cohort subjects had at least four minor alleles in the four SNPs identified; this subgroup showed MeHg-associated cognitive deficits. In some analyses, the difference between the groups suggests that children with at least four mutations lose as much as 25 IQ point more

than wild type children at a 10-fold increase in prenatal MeHg exposure. Therefore, environmental neuroepidemiology studies need to include a new focus on genetically susceptible groups in order to assess a more realistic potential risk of neurotoxicant exposures at low levels (Grandjean and Julvez 2013). The variability in susceptibility to neurotoxicity between population groups appears to be an important research priority (highlighted by a recent NIH workshop (Bookman et al. 2011)). Genetic changes of proteins involved in absorption, transport, elimination or distribution of toxic metals in human body could either protect or increase risk of toxic effects in an individual, especially at low exposures.

Gene mutations seem to affect the retention of inorganic Hg and MeHg in the body, e.g., genes that affect glutathione and metallothionein metabolism (Engström et al. 2008; Gundacker et al. 2010; Wang et al. 2012). Other studies have also considered absorption-distribution-metabolism-elimination (ADME) genes that may be of importance. Thus, MeHg is eliminated from the liver as GSH conjugates, and the rate-limiting enzyme for GSH synthesis is glutamyl-cysteine ligase (GCL), which is composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM). Further, the glutathione-S-transferases (GST) catalyze the conjugation of GSH (Gundacker et al. 2010). A recent study in Sweden indicates that a *GCLC* polymorphism affects MeHg retention, and that *glutathione S-transferase pi 1* (*GSTP1*) may play a role in conjugating MeHg with GSH (Engström et al. 2008). The *GCL rs1555903* also showed a highly significant main effect on mercury retention in the umbilical cord in a UK birth cohort (Julvez et al. 2013). Mutations in *glutathione S-transferase mu 1* (*GSTM1*) and *GCLM* also seem to affect the retention of MeHg from fish and seafood (Barcelos et al. 2013).

ABC transporters (ABCC1, ABCC2 and related ABCB isoforms) are key molecules involved in decreasing cellular MeHg concentration and toxicity. There are clear observations of gene expression/protein activity induced by Hg, but no clear results regarding relations with identified SNPs, so far (Gundacker et al. 2010). Llop et al (submitted) demonstrated that the ABC transporters appear to play a major role in transport of MeHg across the placenta and accumulation of MeHg during early development. They showed in three large Mediterranean birth cohorts that the association between maternal fish intake and Hg in cord blood has different magnitudes depending on the children's genotype *ABCB1*, *ABCC1*, and *ABCC2*. The findings strengthen the hypothesis that ABC transporters play a role in mercury transport across the placenta and accumulation of MeHg during early development. As these genes appear to influence MeHg internal dose they might offset MeHg neurotoxicity (Llop et al, submitted).

Selenoprotein P is the major selenium (Se) transport protein in blood. It regulates Se homeostasis, delivery of Se in tissues (particularly in brain and testes), antioxidant activity, decrease of lipid hydroperoxides, and metal binding (including Hg). Se intake and genetic factors have linked effects on stress response, inflammation and apoptotic pathways (Hesketh and Meplan 2011). As shown by Goodrich et al (2011), dental professionals with *SEPP1'3 UTR* had lower urine Hg levels. Furthermore, *SEPP1* hypomethylation was associated with increasing hair Hg levels of dental professionals, significant for males (Goodrich et al, 2013).

Another possibility is that certain genotypes are less resistant to particular toxic effects. Deficits in neuropsychological performance in association with inorganic Hg exposure (dentists and dental assistants) occurred most frequently if the subject had a mutation in the coproporphyrinogen oxidase gene (*CPOX4*) (Echeverria et al. 2010). The same SNP also showed a significant main effect on general cognitive function in a UK birth cohort (Julvez et al. 2013). As observed by Woods, et al, (2012, 2013) (Woods et al. 2012; Woods et al. 2013)

CPOX4 rs1131857 modified toxic effects of chronic mercury exposure (urinary Hg). A mutation in catechol O-methyltransferase (*COMT* rs4680) showed increased frequency of symptoms as well (Heyer et al. 2009); a different *COMT* mutation showed a main effect on MeHg levels (Julvez et al. 2013). Mutation of the gene responsible for formation of metal-binding metallothioneine (*MT*) may also result in a predisposition to adverse effects from elemental Hg exposure in children (Woods et al. 2013). In the UK cohort, *MT2A rs10636* showed a significant main effect on general cognitive functioning (Julvez et al. 2013). In student population in Austria, *MT4 rs 11643815* was associated with increased hair Hg level (Gundacker et al 2009). As demonstrated by Kayaalti et al (2011), *MT2A* GG genotype individuals may be more sensitive to metal toxicity.

The study by Julvez et al. (2013) aimed to assess prenatal MeHg exposure and genetic predisposition to cognitive deficit in children of the ALSPAC longitudinal cohort. The authors took into account four major biological pathways considered important for neurodevelopment or metal neurotoxicity (Gundacker et al. 2010; Harris et al. 2007): brain development and neurotransmitter metabolism, cholesterol metabolism, iron regulation, and peroxidative defence and other miscellaneous pathways. The findings suggest that 4 SNPs (rs2049046, rs662, rs3811647 and rs1042838) functionally related to the *BDNF*, *PON1*, *TF* and *PGR* genes appeared to modify the MeHg-outcome associations with cognitive deficits in children with the minor alleles (mutations). *TF rs3811647* was associated with Hg concentrations; *PON1 rs662*, *BDNF rs2049046* and *PGR rs1042838* were associated with WISC III total IQ (Julvez et al. 2013).

Apolipoprotein E (*APOE*) variants were recognised as modifying the adverse effects of cord blood Hg on neurodevelopment (Ng et al. 2013).

Table 6: Gene mutations affecting absorption-distribution-metabolism-elimination and neuropsychological performance

Gene mutation affecting ADME	**Role**	**References**
GCL (glutamyl-cystein ligase), heterodimer enzyme encoded by two genes: - catalytic subunit *GCLC* - modifier subunit *GSLM*	The first and rate-limiting enzyme for GSH (glutathione) synthesis	*GCLC* polymorphism affecting retention of MeHg (Engström et al. 2008)[a]; *GCLS* rs1555903 – Hg retention in umbilical cord (Julvez et al. 2013)[b]; *GCLM* – retention of MeHg (Barcelos et al. 2013)[a]
GSTs (glutathione-S-transferases) *GSTP1, GSTM1, GSTT1, GSTO,..*	catalyse conjugation of GSH (via –SH groups) to xenobiotic substrate in order to make substrate more soluble.	*GSTP1* polymorphism (Engström et al. 2008) [a]; *GSTM1* (Barcelos et al. 2013)[a]
GSS (GSH synthetase)	The second step in GSH synthesis	
GPx (GSH peroxidase) *GPx1, GPx3, GPx4, ..*	Selenoproteins combating the oxidative stress in general and the stress created by Hg and other metals.	Se intake and genetic factors have linked effects on stress response, inflammation and apoptotic pathways (Hesketh and Meplan 2011).
MT (metallothionein) *MT2A, MT3, MT4, MT1A,*	Metal-binding protein involved in regulation of	*MT2A* rs10636 - main effect on general cognitive functioning

MT1M… (mutation in gene promotor region!)	metal homeostasis (Zn, Cu), toxic metal binding and protection against oxidative stress. MT2A is the most expressed isoform in humans.	(Julvez et al. 2013)[b]. *MT4 rs 11643815* is associated with increased hair Hg content (student population in Austria) (Gundacker et al 2009) [a]. *MT2A*, -5A/G (AA, AG, GG genotypes) core promotor region – GG genotype individuals may be more sensitive to metal toxicity (Kayaalti et al 2011) [a].
ABC transporters (superfamily of ATP binding cassette transporters): MRP1, MRP2, MDR1 (multidrug resistance associated proteins 1 and 2, multidrug resistance protein 1 also named glycoprotein P) and others (ABCC1, ABCC2, ABCB1) *MRP1=ABCC1,MRP2= ABCC2,MDR1= ABCB1*	Responsible for active transport of various compounds across biological membranes incl. therapeutical drugs and xenobiotics. They modulate kinetics of drugs and toxicants and confer a multidrug resistance phenotype.	MRP1, MRP2 and related MRP isoforms are key moleculs involved in decreasing cellular MeHg concentration and toxicity. There are clear observations of gene expression/protein activity induced by mercury, but no clear results regarding relations with identified SNPs, so far (Gundacker et al. 2010). *ABCB1*, *ABCC1*, *ABCC2*: a role in mercury transport across the placenta and accumulation of MeHg during early development, Mediterranen birth cohorts (LLop et al, submitted) [a]
SelP, Sepp1 (s**elenoproteinP**) *SEPP1 gene or gene regulatory region* *Hypomethylation of SEPP1*	The major Se transport protein in blood. Se homeostasis, delivery in tissues (particularly brain and testes), antioxidant activity, decrease of lipid hydroperoxides, metal binding (including Hg).	Se intake and genetic factors have linked effects on stress response, inflammation and apoptotic pathways (Hesketh and Meplan 2011). A trend of *SEPP1* hypomethylation with increasing hair Hg levels of dental professionals, significant for males (Goodrich et al 2013) [c]. Dental professionals with *SEPP1'3 UTR (rs 7579)*, had lower urine Hg levels (Goodrich et al 2011)[c].
Gene mutation affecting neuropsychological performance	**Role**	**References**
CPOX (coproporphyrinogen oxidase) (*CPOX*)	related to brain development and neurotransmitter metabolism (MeHg could interact to their receptors) (Echeverria et al. 2010)	Deficits in neuropsychological performance occurred most frequently if the subject (dentists) had a mutation in the *CPOX4* (Echeverria et al, 2006)[c] *CPOX4* – modified toxic effects of chronic Hg exposure (Hg in urine) of children and adolescents (Woods et al, 2012)[c]

COMT (catechol O-methyltransferase)		*COMT* rs4680 – modifying child neurodevelopment (Heyer et al. 2009); COMT mutation – main effect on MeHg levels (Julvez et al. 2013)[b]
MT (metallothionein) isoforms 1, 2, 3 and 4	metal-binding (Regulation of metal homeostasis (Zn), protection against oxidative stress) – see also above	*MT2A* rs10636 - main effect on general cognitive functioning (Julvez et al. 2013) [b].
APOE (apolipoprotein) Isoforms ApoE2, ApoE3, ApoE4 *APOE* genes, allel variants *3/3, 3/2, 3/4, 4/4*	A protein transporter expressed by several cell types, but with highest expression in liver and brain (astrocytes, microglia); Epsilon4 allele associated with poor neural repair function (a risk factor for AD)	*APOE* variants modified the adverse effects of cord blood Hg on neurodevelopment (Ng et al. 2013)[a]. *Importance of genotyping for multiple allele variants!* MT gene expression may be impaired by *APOE4* genotype (Graeser et al 2012, mice study)
BDNF (brain-derived Neurotrophic Factor)	related to brain development and neurotransmitter metabolism (MeHg could interact to their receptors) (Echeverria et al. 2010)	The findings suggested that four SNPs (rs2049046,rs662, rs3811647, and rs1042838) functionally related to the Brain-Derived Neurotrophic Factor(*BDNF*), Paraoxonase1(*PON1*), Transferrin(*TF*) and Progesterone Receptor(*PGR*) genes appeared to modify the MeHg-outcome associations with cognitive deficits in children with the minor alleles (mutations) Julvez et al.(2013)
PON1 (Paraoxonase 1)	enzyme that inhibits oxidation of lipoproteins through hydrolysis of lipid peroxides. Such oxidative damage can be induced by MeHg (Ayotte et al. 2011; Hernández et al. 2009).	
TF (transferrin)	Iron uptake (Woods et al. 2013)	
PGR (progesterone receptor)	Related to brain development and neurotransmitter metabolism (MeHg could interact to their receptors) (Echeverria et al. 2010)	

Remark: DNA extracted from: [a] blood, [b] cord, [c] buccal cells

Objective

Within the PHIME Mediterranean study, birth cohorts were recruited in Slovenia, Croatia, Italy and Greece, aiming to assess the impact of low levels of MeHg exposure through fish consumption during pregnancy on the neurodevelopment of children at 18 months. The study considered demographic, nutritional and toxic environmental factors as well. This was the largest study ever conducted in the general European population on the impact of Hg through food consumption. THg in mother´s hair and in cord blood did not predict Bayley scores but a

moderate beneficial effect of fish consumption in pregnancy was observed. Other chemical elements were not associated with the outcomes (PHIME 2006).
In Spain the priority tasks are related to the assessment of the mechanisms of transfer of environmental pollutants into children at the early age stages. For this purpose, it is important to clarify how the physiological changes of the mothers have an influence in this transfer during the fetal period of children and, at the same time, what are the body burdens of pollutants such as organochlorine and organobromine compounds and metals in the pregnant women. The follow-up study examining children at 6-8 years of age, aims to assess potential modification of Hg-neurodevelopment association by selected gene variants. Along with the genes responsible for retention/elimination of Hg in/from the body (glutathione-related genes), gene mutations affecting neuropsychological performance will be studied in association with children's IQ as assessed by the *Wechsler Intelligence Scale for Children (WISC III*).

Methodology

Study subjects

Children from Slovenia (n=286), Croatia (n=161), Italy (n=632) and Greece (n=350) recruited at birth in 2007-2009 within the PHIME project WP I:3 (PHIME 2006); and children from Spain (n=2493) from the INMA project (Guxens et al., 2012). Mothers of the existing birth cohorts will be invited to participate in the follow-up at the age of child 6-8 years. Mother-child pairs will be appointed for a visit.

Table 7: Overview of the samples collected within the PHIME project (status on 13/09/2010)

Sample	Number of samples	Italy	Croatia	Greece	Slovenia	SUM
Mother's hair (at birth)	Stored at JSI	895	234	457	582	2168
Analysed:	THg	891	234	454	574	2153
	>1000 ng/g THg	323	51	257	23	654
	MeHg	323	50	248	21	642
Hair (1 month after birth)	Stored at JSI	762	196	-	353	1311
Cord blood	Stored at JSI	626	207	391	443	1667
Analysed:	THg	610	207	391	443	1651
	MeHg	227	33	207	15	482
Maternal blood	Stored at JSI	872	225	-	-	1097
Analysed:	THg	857	224	-	-	1081
	MeHg	332	27	-	-	359
Milk	Stored at JSI	603	125	52	290	1070
Analysed:	THg	603	124	36	272	1035
	MeHg	224	-	-	-	224
Cord Tissue	Stored at JSI	46	215	-	333	261
Meconium	Stored at JSI	-	205	225	-	430
Baby's urine	Stored at JSI	-	184	66	-	250
	Total Hg	-	141	43	-	184
Maternal Urine	Stored at JSI	676	225	326	(24)	1227

	Total Hg	345	29	230	-	604
Child hair	Stored at JSI	244	-	-	-	244

Data collection and sampling

Available data

The data available from the PHIME project is summarized in Table 8. Mother's hair, mother's blood (Italy and Croatia), cord blood, cord tissue and meconium have been sampled at birth, breast milk and mother's hair 1 month after birth. Hair samples have been analysed for mercury (total Hg and MeHg for the samples having more than 1000 ng/g total Hg in hair), blood and breast milk for mercury (total Hg and MeHg for the samples having more than 1000 ng/g total Hg in hair), cadmium, lead and arsenic, as well as for essential elements (selenium, zinc, copper). *GSTT1/GSTM* gene deletion polymorphism has been done on a subset of samples. Children were tested for neurodevelopment (Bayley III test) at the age of 18 months.

Detailed questionnaire data is available, including pregnancy history (mother's age at delivery, BMI before pregnancy, weight gain increase, cigarettes smoked throughout the pregnancy, weekly alcohol intake, dental visits and new/replaced dental fillings, detailed nutritional data (consumption frequency of different types of food including different fish species)), data on child (sex, birth weight and length, weight and length at 18 months, breastfeeding history (any vs. none), duration and exclusiveness up to 4 months, daycare attendance at 18 months, duration of the intake of fresh and homogenized fish up to 18 months) and socio-economic status data (home area (<50, 50-100, >100 m^2); home ownership; parental education (the higher of the two); number of children in the family; marital status of the mother at delivery).

Follow-up

Child's hair, child's urine and saliva sample of both mother and child (Oragene DNA self-collection kit) will be collected from each child-mother pair at the time of appointment. Mothers will be asked to complete a questionnaire, children will undergo neuropsychological testing by paediatricians.

Table 8: Available data/measurements and follow-up from the PHIME project

		At birth	1 month after birth	18 months after birth	Follow-up (6-8 years)
Exposure assessment	Total Hg, MeHg	Cord blood (whole blood); cord tissue; meconium	Maternal hair, breast milk; detailed questionnaire (no. of amalgam fillings; frequency of fish consumption and other relevant food)		Hair, urine
	Cd, Pb, As	Cord blood (whole blood)	Breast milk; detailed questionnaire (food frequency questionnaire; smoking,…)		Urine
	Se, Mn, Cu, Zn	Cord blood (whole blood, plasma, serum	Breast milk; detailed questionnaire (food frequency questionnaire)		Urine
	No. amalgam fillings		Questionnaire		
	Frequency of fish consumption	Questionnaire	Questionnaire		Questionnaire
	Frequency of consumption for various food items (vegetables, fruit, cereals, offal, diary products, oil, etc.)		Questionnaire		Questionnaire
	Mother's education & occupation		Questionnaire		
	Mother's life-style		Questionnaire		
	Other socio-economic		Questionnaire		Questionnaire?
Biol. asses.	GST polymorphism	Cord			Saliva
	Other relevant polymorphism (see Table 6)				Saliva
Health data	Anthropometry, baby	Questionnaire data		Questionnaire data	
	Chronic disease(s)		Questionnaire		
	Neurodevelopment			Bayley testing	WISC III

Table 9: Available data/measurements and follow-up from the INMA project

Cohort	Measurement	Cord blood serum	Placenta	Hair	Venous blood serum at 4 years of age	Maternal venous serum	Maternal colostrum	Maternal urine
Asturias	PCBs, hexachlorobenzene, hexachlorocyclohexanes, DDT, DDE, PBDE	325	50			308		
Sabadell	PCBs, hexachlorobenzene, hexachlorocyclohexanes, DDT, DDE, PBDE	631					256	
Sabadell	Co, Ni, Cu, Zn, Se, As, Mo, Cd, Sb, Cs, Th, Pb							489
Gipuzkoa	PCBs, hexachlorobenzene, hexachlorocyclohexanes, DDT, DDE, PBDE	628					113	
Valencia	PCBs, hexachlorobenzene, hexachlorocyclohexanes, DDT, DDE, PBDE	499				499		
Menorca	PCBs, hexachlorobenzene, hexachlorocyclohexanes, DDT, DDE, PBDE	410			285			
Menorca	Mercury			302				
TOTAL	PCBs, hexachlorobenzene, hexachlorocyclohexanes, DDT, DDE, PBDE	2493	50		285	807	369	

Chemical analyses

Concentration of total mercury in hair and urine will be determined by the Direct Mercury Analyser DMA-80 (Milestone Srl, Italy). The system integrates thermal decomposition sample preparation, amalgamation, and atomic absorption detection. The typical working range for this method is 0.05 - 600 ng. The mercury vapour is first carried through a long path-length absorbance cell and then a short path-length absorbance cell. The same quantity of mercury is measured twice, using two different sensitivities, resulting in dynamic range that spans at least four orders of magnitude (SW-846 Method 7473). Other elements in urine will be determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7500ce, Agilent, Japan). Results in urine will be corrected for creatinine levels.

The analyses of α-HCH, β-HCH, γ-HCH, δ-HCH, HCB, PeCB, 2,4'-DDT, 4,4'-DDT, 2,4'-DDE, 4,4'-DDE, 2,4'-DDD, 4,4'-DDD, 7 PCB congeners (CB28, CB52, CB101, CB118, CB153, CB138 and CB180) and 14 PBDE congeners (BDE17, BDE28, BDE47, BDE66, BDE71, BDE85, BDE99, BDE100, BDE153, BDE154 BDE138 BDE183 and BDE190 and BDE209) in serum have been performed by chromatographic methods, 1 mL of serum was spiked with the surrogate standards tetrabromobenzene (TBB) and decachlorobiphenyl (CB209) and vortex stirred for 30 s at 2,000 rpm. n-Hexane (3 mL) was added, followed by concentrated sulfuric acid (2 mL). After reaction, the mixture was stirred for 30 s and the supernatant n-hexane phase was separated by centrifugation. The remaining sulfuric acid solution was re-extracted twice with 2 mL of n-hexane (each by 30 s stirring and centrifugation). The combined n-hexane extracts (7 mL) were additionally cleaned with sulfuric acid (2 mL, stirring 30 s). Then, the n-hexane phase was separated by centrifugation and reduced to a small volume under a gentle nitrogen stream. The extract was transferred to gas chromatography (GC) vials using four 25 μl rinses of isooctane. CB142, BDE118 (20 μl) and [^{13}C]-BDE209 (10 μl) were added as internal standards before injection. Organochlorine compounds (OCs) were determined by GC with electron capture detection. BDE congeners were analyzed by GC coupled to mass spectrometry in chemical ionization and negative ion recording.

Total cholesterol and triglycerides were determined by enzymatic methods in maternal and cord serum samples and total serum lipid concentrations were calculated as described elsewhere (Phillips et al. 1989).

Biological analyses

a) Selection of the candidate genes. Selection of genes in our study is based on previously known biochemical data as well as from gene networks using IPA bioinformatics software (Ingenuity Pathway analysis). The selection of genes is focused on enzymes involved in GSH pathway and the interceptors of antioxidant enzymes, as well as on enzymes related to brain development and neurotransmitter metabolism.

b) Selection of SNPs in the candidate genes (GSTT1/GSTM, GCLC, GCLM, GSTP1, GSTM3, GSR, GPX1, SOD1, SOD2, CAT; PON1, BDNF, PGR)

c) DNA isolation from child's and mother's saliva sample

d) SNP genotyping, Hardy Weinberg equilibrium and haplotype reconstruction

In all participants, involved in the study, the selected tag SNPs genotype will be determined by appropriate genotype techniques.

Neuropsychological testing

Wechsler Intelligence Scale for Children (WISC III) will be used to assess the children's IQ. This technique allows to obtain three composite scores: Verbal IQ (based on Information, Similarities, Arithmetic, Vocabulary and Comprehension), Performance (nonverbal) IQ (based on Picture Completion, Coding, Picture Arrangement, Block Design, Object Assembly), and Full Scale IQ (based on all the tests included in the Verbal and performance IQ scales). WISC III will be performed in dedicated rooms located in the University Medical Centre. The test will be administered by psychologists or trained medical doctors and graded by the psychologists within one week after administration.

Covariates

Questionnaire will include potential confounders relevant for the relationship between methyl mercury exposure and IQ of children: sex, age at WISC III assessment, WISC III examiner;
n-3 fatty acid intake due to seafood consumption, 'healthy component' during pregnancy, estimated processed food intake at age 6-7 years, maternal age, parity, house ownership status, parental education, social class recorder during pregnancy.

National targeted environmental problems

Case study Greece – Human biomonitoring for Cr^{6+} in Greece

Introduction

A major local environmental issue in Greece is related to the presence of hexavalent chromium Cr(VI) in drinking water of the Oinofyta municipality, within the wider area of Asopos basin and the related cancer mortality. The Oinofyta municipality (Figure 11) is situated 50 km North of Athens, Greece, and it includes four villages that were initially rural but transformed into industrial areas in the early 1970s. In 1969, a ministerial decision gave permission for depositing processed industrial waste in the Asopos river, which runs through Oinofyta. This decision, furthered by a presidential decree in 1979, permitted free disposal of processed liquid industrial waste into the river. The Oinofyta industrial region is located within Voiotia prefecture at the border of Attica prefecture (that includes the capital city of Athens). Due to the proximity to Attica, the number of industries in the Oinofyta region increased quickly after 1984, when a new law imposed restrictions on the establishment of various industries within Attica. According to the Technical Chamber of Greece (TCOG 2009), there were about 700 industries operating in the Oinofyta area, of which 500 generated liquid industrial waste. Initial concerns were raised after Oinofyta area citizens complained about the discoloration and turbidity of their drinking water. Regular protests ensued from the 1990s onward. In 2007, the Ministry of Environment, Regional Planning and Public Works of Greece imposed fines on 20 industries for disposing industrial waste with high levels of hexavalent chromium into the Asopos river. Official limits on total chromium have been set by both the United States Environmental Protection Agency (US-EPA), equal to 100 μg/l, and the European Union (Council directive 98/83/EC), equal to 50 μg/l. However, as of yet, there are no limits set by any international body for Cr(VI). In 2009, the California Environmental Protection Agency proposed a public health goal level of 0.06 μg/l for Cr(VI) in drinking water (OEHHA 2009).

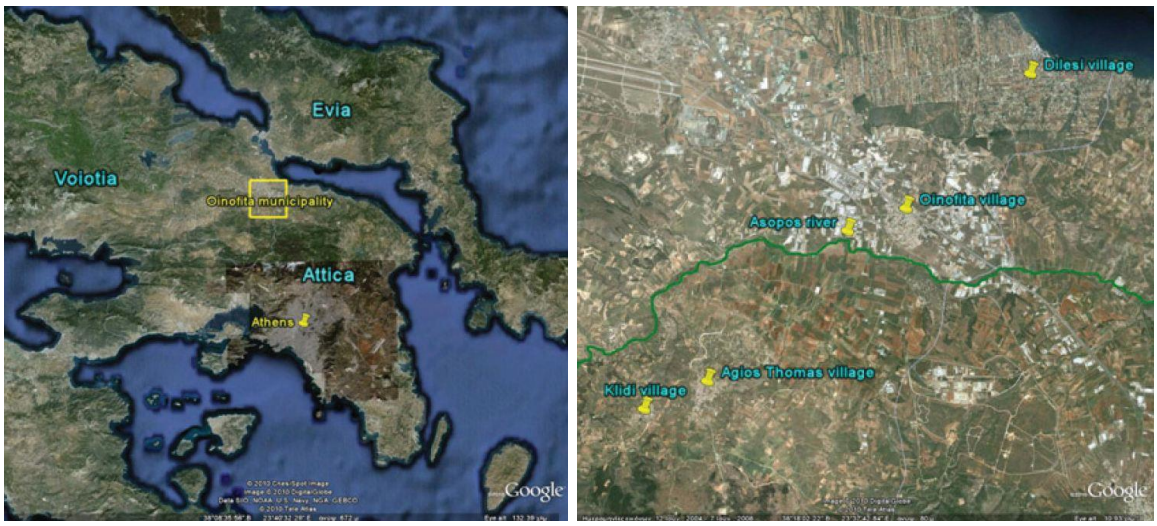


Figure 11: Asopos Basin and Oinofyta municipality

At the cellular level, Cr(VI) is a highly active carcinogen (Costa 1997; Proctor et al. 2002). A key issue is whether Cr(VI) ingested through the oral route, converts to trivalent Chromium Cr(III) (which does not cross the cell membrane that easily) before entering a living cell (Costa 2003). A recent study (Stout et al. 2009) revealed that rats and mice exposed to Cr(VI)-contaminated drinking water developed gastrointestinal abnormalities, including oral and intestinal tumors. An earlier study (Borneff et al. 1968) also found an increased incidence of benign and malignant combined forestomach neoplasms in mice orally exposed to Cr(VI), whereas a more recent publication (O'Flaherty 1996) presented a physiologically based model of chromium kinetics according to which non reduced hexavalent chromium after oral exposure could be metabolized in the red blood cells, liver, kidney and bone. Because areas characterized by high Cr(VI) concentrations in drinking water are relatively uncommon, human epidemiologic studies are scant; the study carried out by Zhang and Li (1987) is one of the most cited and controversial studies analyzing the effects of oral exposure to Cr(VI) on population cancer mortality rates conducted near a chromium smelting plant in the Liaoning Province, China.

Thus, based on ecologic studies and animal studies, one could hypothesize that several organs could be targets of chromium carcinogenicity including the liver, kidney, bladder, gastrointestinal tract, the hematopoietic system and even bone. In order to further examine the potential effects of elevated oral exposure to hexavalent chromium, an ecological mortality study (Linos et al. 2011) was performed in an industrial area of Greece where the water consumed by the population was contaminated with hexavalent chromium (maximum levels ranging between 41 and 156 μg/l in 2007-2009, and presumed exposure for at least 20 years). The goal of the study was to examine the cancer mortality in an area of Greece, historically satisfying its potable needs with a Cr(VI)-contaminated aquifer.

Proposed methodological scheme

Environmental data and external exposure assessment

Since 2007, three independent sets of hexavalent chromium measurements are available for the Oinofyta area. These include: a) a study of the Institute of Geology and Mineral Exploration (IGME 2008) during the period November 2007 to February 2008, which detected 35 samples (out of 87) taken from different wells in the same area, where levels above 10 μg/l with a maximum value 156 μg/l were detected; b) a study conducted by the faculty of the Geology and Geo-environment department of the University of Athens (Vasilatos et al. 2008) during the period September 2008 to December 2008, in which Cr(VI) levels ranged from 41 up to 53 μg/l in three samples taken from the public drinking water supply of Oinofyta; and c) repeated measurements by the Oinofyta municipality in the public drinking water supply during the period July 2007 to July 2010, in which there are 13 measurements with levels above 10 μg/l and with a maximum value of 51 μg/l. Notably all 16 measurements made in 2007 and 2008 by the Oinofyta municipality, record Cr(VI) levels above 8 μg/l. According to official Oinofyta municipality authorities, in early 2009 the main drinking water supply of Oinofyta was diverted to receive water from Mornos lake (reservoir) which is part of the drinking water supply network of the city of Athens. Therefore, more recent measurements made by the Oinofyta municipality (June 2009- July 2010) record relatively lower levels of Cr(VI) (<0.01-1.53 μg/l). To the best of our knowledge, there are no systematic measurements of Cr(VI) before 2007. However, a measurement made by the

Oinofyta municipality in 1996, showed Cr(VI) levels of 54 μg/l in the public drinking water supply.
Moreover, in the study carried out by Economou-Eliopoulos et al. (2011), groundwater samples from the Asopos aquifer showed a wide spatial variability, ranging from <2 to 180 ppb Cr total content [almost same to the Cr(VI)-values] despite their spatial association. The presence of Cr(VI)-contaminated ground water at depths >200m is attributed to a direct injection of Cr(VI)-rich industrial wastes at depth rather than that Cr(VI) is derived from the Asopos river or by the interaction between water and Cr-bearing rocks.
The data mentioned above, will be used as a starting point for deriving exposure distributions for the population of the area. In addition, other potential exposure pathways will be investigated, related to the mobility of chromium within other environmental media, so as to synthesize potential aggregate exposure profiles. For this purpose, the TAGS computational platform (Sarigiannis et al. 2012) will be employed.

Linking biomonitoring data to exposure

Biomonitoring data acquisition

Humans are exposed to two major oxidation states of Cr, Cr(III) and Cr(VI). Cr(VI) is the most toxic and carcinogenic form of Cr. The high toxicity of Cr(VI) results from its active accumulation into cells, whereas Cr(III) is much less toxic because it is not able to enter cells well. Inside the cell, all of the Cr(VI) is reduced to Cr(III) and this reduction process and the formation of Cr(III) is responsible for the induction of DNA damage and other cytotoxic effects of Cr(VI). Monitoring of occupational exposure to Cr(VI) compounds is usually based on measurements of Cr in the urine and serum. However, Cr(III) is an element that is found in the diet and background levels in biological fluids can be high masking low levels of Cr(VI) exposure that come from environmental/occupational exposure. Additionally, Cr is readily excreted and redistributed from serum and hence, Cr measurements in human serum and urine reflect relatively recent exposures and are used primarily for heavy industrial exposures. Also, measurement of Cr in erythrocytes is more informative being indicative of exposure to hexavalent Cr since Cr(III) cannot enter the red blood cell while Cr(VI) readily enters the cells. In fact, Cr-hemoglobin complexes are persistent and therefore a single determination can potentially estimate cumulative Cr(VI) exposure going back in time. Thus, the measurement of Cr in red blood cells is a relatively good indicator of Cr(VI) exposure. A second indicator of Cr(VI) exposure involves its adduction with DNA. In the cell, the hexavalent form of Cr is reduced to the trivalent form which actually binds to the phosphate backbone of DNA and can cause the formation of ternary complexes involving glutathione, amino acids such as cysteine and histidine, and DNA. Cr(III) can also adduct protein to DNA forming ternary protein-Cr(III)-DNA complexes. There has been a substantial amount of work using the DNA-protein crosslinks induced by chromate in white blood cells of humans as a biomarker of chromate exposure and early toxic effects. Recently, a UvrABC system and ligation-mediated PCR has been used to study and detect Cr-DNA adducts in exons 5 and 7 of the p53 gene in human A549 cells. Preliminary results suggests that this technique can detect and map the sites of Cr-DNA adducts at a single nucleotide level in exons 5 and 7 of the p53 gene. Interesting, some of these sites appear to be hotspots for p53 mutations in human cancer. Some studies are also investigating whether Cr-DNA adducts that were tightly bound to DNA form these coordinate covalent bonds preferentially on guanines that are neighbored by methylated cytosines since previous studies have suggested that guanines near methylated

cytosines are sites for preferential adduct formation. This phenomenon addresses the issue of epigenetic susceptibility with regard to chromate carcinogenesis since the degree of methylation of a promoter region of the gene can vary depending upon the individual and the tissue. Therefore, the sites of Cr adduct formation may also vary depending upon the degree of methylation of the gene's coding region and this may give rise to differences in adduct formation and differences in genetic susceptibility to adduct formation. The biomarkers available to assess chromate exposure and effect represents useful types of biomarkers. In fact, since a portion of the Cr bound to DNA can be eliminated by exposing the DNA to Ethylenediaminetetraacetic acid (EDTA), one can address whether the adducts detected were Cr-DNA adducts. There are few adducts that display this unique chemical property of EDTA reversibility, and therefore it is a useful way to identify DNA adducts that contain Cr.

In order to understand the significance and the limitations of Cr measurements in biological samples, it is important to bear in mind that all measurable Cr is in the trivalent form. Even under conditions of high exposure to Cr(VI), there is a relatively rapid reduction to Cr(III) upon absorption of Cr(VI) into biological fluids or tissues. Measurements of total Cr in urine or plasma are most frequently used to assess occupational exposure to Cr compounds. Ease of the sample collection and the suitability for the direct analysis by graphite furnace atomic absorption spectrometry (GF-AAS) were the major reasons for a broad adoption of Cr measurements in these fluids for biomonitoring purposes. Measurements of Cr in urine are usually adjusted for creatinine concentrations to take into account dilution effects. High levels of Cr in urine and blood were found in chrome platers and stainless steel welders, which are professional groups with a considerable exposure to Cr(VI)-containing fumes.

One of the important findings in these biomonitoring studies was the detection of significant amounts of absorbed Cr in some occupational groups that had low ambient levels of Cr in the workplace. For example, analysis of Cr in blood and urine samples obtained from various categories of welders found that mild steel welders had significantly elevated Cr levels despite relatively low levels of atmospheric Cr. The toxicological significance of urinary measurements is diminished under conditions of low-level exposure. Urinary and serum measurements can be significantly influenced by Cr intake from dietary sources which is expected to lead to a substantial day-to-day variability. The inability of urinary and serum analyses to differentiate between exposure to toxic Cr(VI) and innocuous organic complexes of Cr(III) makes it difficult to perform risk analysis at environmental exposure levels. The contribution of dietary Cr is not expected to be significant in persons with high industrial exposure, and in these situations serum and urinary Cr measurements can be useful markers of internal dose.

The major problem encountered in the biomonitoring of exposure to toxic Cr(VI) using measurements of chromium concentrations in biological fluids is the inability to determine whether measurable Cr is derived from the dietary sources or originated from toxic Cr(VI) compounds (Figure 12).

Since all Cr in the body is trivalent Cr, the assessment of Cr(VI) exposure needs to be based on the mechanistic differences in the interaction of harmless Cr(III) and toxic Cr(VI) forms with cells. Although experiments with animals provided significant support for the idea that Cr concentrations in erythrocytes could preferentially reflect Cr(VI) exposure, uncharged Cr(III) complexes with organic ligands are also likely to enter cells. Measurements of Cr(III)-DNA adducts in lymphocytes or other biological samples should greatly improve risk assessment by providing evidence about the formation of DNA damage which could only result from exposure to toxic Cr(VI). At present, only assays for DNA-protein crosslinks have

sufficiently high sensitivity to detect current levels of human exposure to Cr(VI), but the Cr specificity of these determinations still needs to be improved. Inductively coupled plasma mass spectroscopy (ICP-MS) techniques have high analytical sensitivity for the detection of Cr; however, this method suffers from background problems during analysis of biological samples. For example, the current detection limit for ICP-MS is one Cr per 5,000 DNA bases, which means that 10^2 –10^3-fold increase in the sensitivity must be achieved before this methodology can be applied to analysis of human samples.

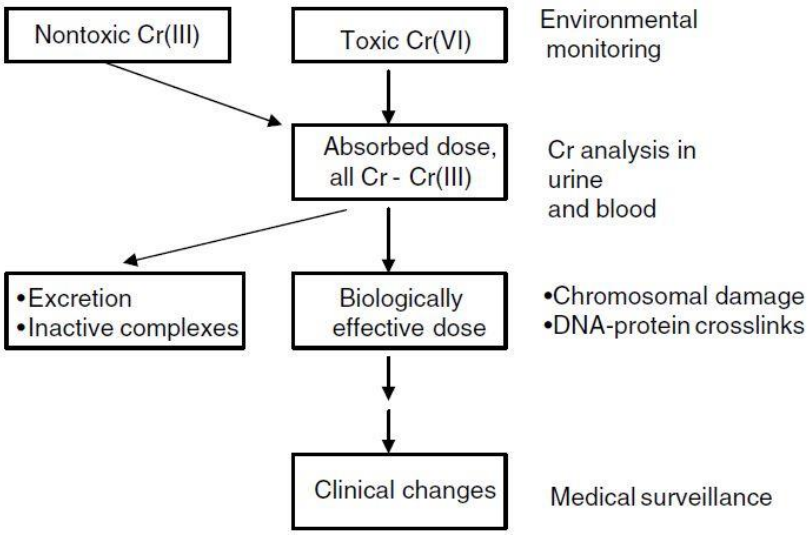


Figure 12: Major approaches for assessment of exposure to chromium compounds

One approach for overcoming background problems could be a combination of capillary electrophoresis or HPLC with on-line detection of Cr by ICP-MS.

Since no single biomarker can determine unambiguously exposure to Cr(VI), the most appropriate strategy would be to utilize two biomarkers:

- one measuring Cr in the biological sample and another quantifying DNA protein crosslinks. Depending on whether chronic or short-term exposure is suspected, Cr determinations in erythrocytes or urine can be used as a biomarker of absorbed dose. Given that the K-SDS crosslink assay and analytical measurements of Cr in blood and urine are relatively inexpensive and have high sample capacity, we envision these two types of analyses could be performed for the screening the magnitude of exposure.
- More complex Cr-specific analyses (second tier biomarkers), which are being developed for specific Cr(III)-DNA adducts, could be performed only for individuals characterized by elevated levels of Cr in the biological fluids mentioned above.

<u>Assimilating biomonitoring data</u>

From the above, it is clear that understanding the dynamics of Cr(VI)-Cr(III) dynamics is a key for identifying actual exposure to Cr(VI) based on the evaluation of Cr in commonly assessed biological fluids. The link of biomonitoring data to exposure will be carried out by the parameterization of the generic PBTK model developed in the frame of CROME. The parameterization will be based upon recently developed PBTK modeling studies for Cr(VI) regarding humans (Kirman et al. 2013) and rodents (Kirman et al. 2012). One of the major related parameter is the kinetics of Cr(VI) reduction in stomach. Kinetics of Cr(VI) reduction in rodent stomach contents was previously reported that occurred by a mixed second-order

model in which the rate of Cr(VI) reduction was dependent upon the concentrations of Cr(VI) and reducing agents present in the stomach contents (Proctor et al. 2012). The experiments in rodent stomach contents were conducted at a range of Cr(VI) spiking concentrations and dilutions so as to assess reduction under conditions consistent with that of recent rodent 90-day and 2-year bioassays (Thompson et al. 2011). Second-order rate constants (k) in mice and rats at pH = 4.5 were 0.2 and 0.3 L/mg/h, respectively, and the reducing capacity of gastric contents was approximately 16 mg Cr(VI) reducing equivalents per L of stomach contents in both species (Proctor et al. 2012). Similar to rodent gastric contents, the kinetics of Cr(VI) reduction in fasted human stomach fluid exhibited mixed second order reaction kinetics. Based on the data collected, a simple, empirical relationship between the reduction rate constant and pH can be described as follows:

$$K_{red} = 44.5 \cdot \exp(-pH)$$

The constant of 44.5 L/mg/h estimated for humans (at pH = 0) is slightly higher than, but within precision limits of the pH-dependent values derived previously for rats (27 L/mg-h) and mice (18 L/mg-h) (Kirman et al. 2012). A range of estimates obtained for reducing equivalents in fasted human stomach fluids (4 to 10 mg/L gastric fluid; mean = 7 mg/L) is consistent with the range of reducing equivalents (or reductive capacity) reported by De Flora et al. (1987) in stomach fluid during the fasted state (<10 mg/L). In De Flora et al. (1987), peak reductive capacity was observed during the 1–4 h periods after each meal and the range of reducing equivalents was reported for the fed state to be 10–60 mg/L with a median of approximately 30 mg/L. Fed stomach fluid samples could not be obtained for Kirman et al. (2013) *ex vivo* experiment; thus, data from De Flora et al. (1987) for the fed state were used to parameterize the human PBTK model. Therefore, estimates of reductive capacity (or reducing equivalents – mg/L) in the fed and fasted state were approximately 30 and 7 mg/L, respectively. The second order rate constant was assumed to be the same in the fed and fasted condition. Datasets that provide time-course data (i.e., more than a single data point) for chromium in plasma following Cr(III) exposure (Kerger et al. 1996; Mohamedshah et al. 1998) will be used to estimate model parameters for systemic clearance of Cr from plasma. Similarly, data sets that provide time-course data for chromium in plasma following Cr(VI) exposure (Kerger et al. 1996; Paustenbach et al. 1996; Stift et al. 2000), will also provide useful information on the systemic clearance of Cr(III) from plasma, since systemic levels largely reflect Cr(VI) that has been reduced to Cr(III) in the GI tract and portal system.

Association to health effects

Association to health effects will be based upon meta-analysis incorporating the CROME-LIFE methodology on existing epidemiological data already published by Linos et al. (2011). According to the study, a total of 474 deaths were observed, of which 118 were cancer related. These figures (i.e. one in four deaths being cancer related) are in accordance to the general Greek, EU15 and EU27 averages (Eurostat, 2011). The all cause standardized mortality ratio (SMR) for the Oinofyta municipality was similar to that of the prefecture of Voiotia (SMR = 98, 95% CI 89-107). The SMR for all cancer deaths over all the years was slightly increased but not statistically significantly (SMR = 114, 95% CI 94-136). There were eight observed deaths of the hepatobiliary system, and more specifically: six primary liver cancers, one bile duct, and one gallbladder. For primary liver cancer, the observed deaths were eleven fold higher than the expected number of deaths (SMR 1104, 95% CI 405-2403, $p < 0.001$); statistically significant SMRs for primary liver cancer were observed among both

males and females. Observed deaths associated with kidney and other genitourinary organ cancers (five deaths with ICD-9 code 189, and one death with ICD-9 code 184) were more than threefold higher than expected in women (SMR 368, 95% CI 119-858, p = 0.025). The SMR for lung cancer was also statistically significantly elevated (SMR 145, 95% CI 101-203, p = 0.047). Furthermore, elevated SMRs were noted for several other cancer sites, including cancers of lip, oral cavity and pharynx, stomach, female colon, female breast, prostate, and leukaemia, but did not reach statistical significance. Tests for linear trend performed after grouping the period specific SMRs into 3 time intervals, i.e. 1999-2002, 2003-2006, 2007-2009, did not reveal any significant evidence of a linear trend. However, there was a statistically significant SMR of 193 (95% CI 114-304, p = 0.015) for all cancer deaths that was found for the year 2009.

Within the frame of CROME-LIFE project, these data will be meta-analyzed in the following way:

- Development of Cr(VI) external exposure probability distributions, taking into account the data of environmental contamination (starting from data on drinking water concentrations)
- Translating them into internal exposure in the target tissues with the use of internal dosimetry PBTK model.

Critical data for the completion of the case study

Filling the gap

Human biomonitoring data are the critical data missing for the application of the CROME methodology in Asopos basin. Given that environmental concentrations and actual exposure have been decreased after 2007, we need to identify recent, as well as past exposure. For this purpose, a combination of biomonitoring data will be applied, including urine samples (for assessing current exposure levels), as well as hair samples for assessing exposure burden from the past. For the purposes of the analysis, a sample of 20 residents will be collected. Details on the analytical techniques that will be follow are given below.

Methods

Electrothermal Atomic Absorption Spectroscopy (ETAAS) for assessing Cr(VI) in hair

The proposed analytical method, is the one described by Afridi et al (2006). In that study, biological samples were collected from a total of 56 long-term exposed steel production workers (PW), 35 quality control workers (QCW) and 75 unexposed normal controls (all male, age range 25 55 years). The working solution of Cr was prepared daily from certified standard solutions of all analytes under study in 2M nitric acid. All solutions were stored at 4^{o}C. Hair samples were collected from the occipital region of the head. The samples of hair were obtained using stainless steel scissors from the nape of the neck. The hair samples were cut into approximately 0.5-cm pieces in length and mixed to make a representative hair sample. In the case of each person, hair strands were washed with diethyl ether-acetone (3+1) mixture, non-ionic detergent solution (distilled water) and ultrapure water, respectively. After washing, the hair samples were dried at 80^{o}C for 6 h. Hair samples for each participant were placed in separate plastic envelopes, which indicated the identification (ID) number of the participant. Duplicate 0.5 mL of each certified urine and blood samples, while 0.2 g of human hair samples BCR 397, were placed into 50-mL Pyrex flasks. A 5-mL volume of a freshly

prepared mixture of concentrated HNO_3 -H_2O_2 (2:1, v/v) was added to each flask, and the solutions were heated on an electric hot plate at 80^{o}C for 2-3 h, until the clear transparent digests were obtained. Final solutions were made up to 10 mL with 2M HNO_3. The final solutions were collected in polyethylene flask for determinations of Cr by ETAAS. Blank digestions were also carried out. Duplicate samples of QCW, PW and normal controls were treated as described above. Detailed instructions on the operation of the Perkin-Elmer model 4110 ZL are described in the operator's manual. The sample (calibration blank, standards, reagent blank, and control sample) and matrix modifiers were introduced to the furnace by an auto sampler. The calibration was periodically verified by analyzing the standard at the frequency of 10 readings. A microwave-assisted digestion procedure was carried out in order to achieve a shorter digestion time. For digestion of biological samples, duplicate samples of dried scalp hair (200 mg) three replicate samples of CRM 397, were accurately weighed into Teflon PFA digestion vessels directly, to which 2 mL of HNO_3 and 1 mL of 30% H_2O_2 were added and left to stand for 10 min, then the vessels were sealed and placed in a PTFE reactor. This was then heated following a one-stage digestion programme (250 W, 15 min for hair samples). After cooling the digestion vessels in an ice bath for 20 min before opening, the resulting solution was evaporated almost to dryness to remove excess acid, and then diluted to 10.0 mL in volumetric flasks with 2M HNO_3. Blank extractions (without sample) were carried through the complete procedure of both methods. The concentrations were obtained directly from calibration graphs after correction of the absorbance for the signal from an appropriate reagent blank.

Urine Samples - Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The proposed methodology for identifying Cr(VI) in urine samples is the one proposed by Scheepers et al. (2008). Spot urine samples were stored at 4 ^{0}C in the dark and transferred to a laboratory for further storage at −18 ^{0}C. The analysis of Cr was performed according to Lewalter et al. (1985). Urine was diluted with a solution of magnesium nitrate with Triton-X and sulphuric acid (matrix modifier). Cr levels were determined at 357.9nm using electro thermal atomic absorption spectrometry, AAS (Solaar M, Thermo Analytical) with Zeeman background correction. The LOQ was 0.10μg/L (corresponding to 0.10μg/g creatinine if a creatinine value of 1 g/L is assumed) of urine and the coefficient of variation was 7.1% at 0.31μg/L. Creatinine concentrations in urine samples were determined using the Jaffe reaction.

Case study Greece – Health effects of urban biomass combustion

Introduction

Over the last couple of years, the use of biomass as heating source was allowed in Greece as a CO_2-neutral means of space heating in the large metropolitan areas of Athens and Thessaloniki affecting more than half of the country's population. At the same time the use of light heating diesel was heavily taxed. In the same period Greece faces a financial crisis with significant repercussions on the average household income. That combination of parameters resulted to increased use of biomass for residential heating in year 2012, followed by a significant increase of ambient air, indoor air and exposure to PM10 and PM2.5. In this study, we aim to quantify the health and socioeconomic effects related to that shift from light heating diesel to biomass burning, as well as to evaluate alternative scenarios of residential heating energy share. For this purpose, a complex methodology incorporating measurements and

modelling will be implemented; a conceptual representation of the methodology is illustrated in Figure 13:

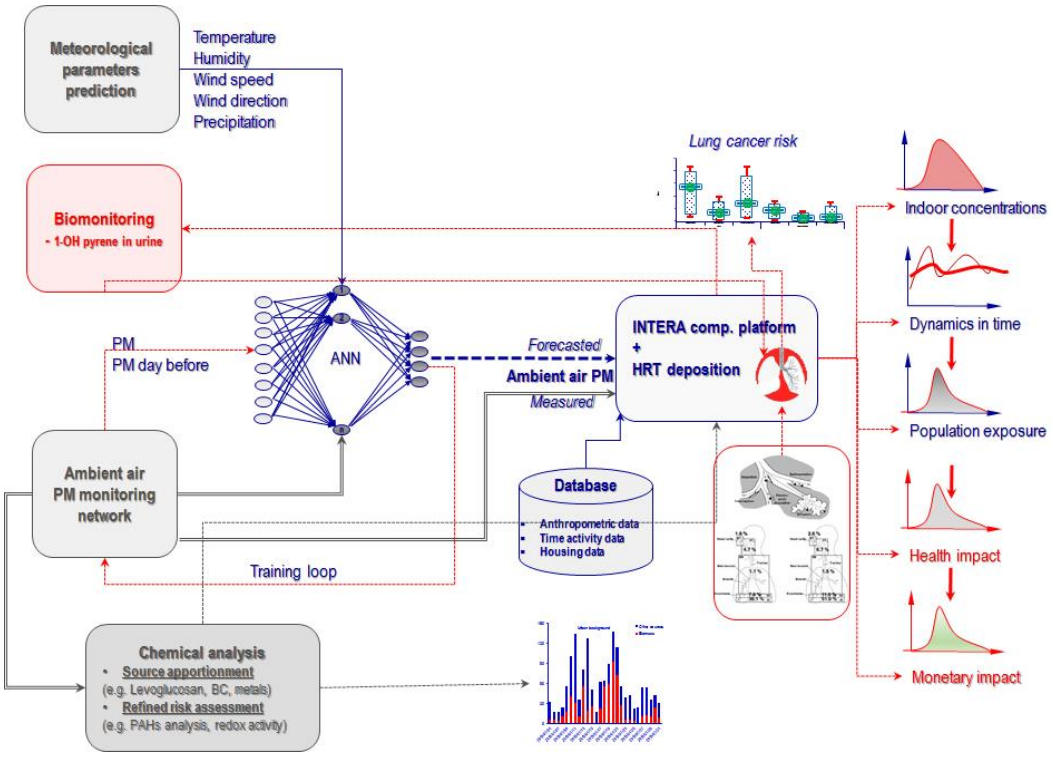


Figure 13: Conceptual representation of the methodology

Proposed methodological scheme

Environmental data and external exposure assessment

Preliminary data for the verification of the case have been delivered by Sarigiannis et. al (2013). Interesting conclusions are given by the comparative study of daily values for the same dates between the years 2011 and 2012. During the warm period of early October-early November the year 2011 at the traffic station, PM10 concentrations were 59.8 and PM2.5 47.0 μg/m^3, while during the cold period, amounted to 82.9 and 68.3 μg/m^3 respectively. During the warm period of year 2012, the respective concentrations remain lower, being 53.1 and 29.5 mg/m^3 for PM10 and RM2.5 during the warm period, while a more significant elevation occurs during the cold period (76.5 and 59.7 μg/m^3 for PM10 and PM2.5 respectively). Since average wind speed was similar to both seasons, reduced concentrations of year 2012 at the traffic station are attributed to reduced traffic emissions, which in turn are due to the reduced traffic load by 30% in 2012, as evidenced by *in situ* traffic measurements carried out. Moreover, while in 2011 the ratio of RM2.5/RM10 remains almost constant (~ 0.8) between the two periods (warm-cold), in 2012 increases significantly during the cold period in 2012 (from ~ 0.55 up to 0.78); the latter indicates that additional contribution beyond traffic sources is becoming important. Instead, at the background station, whereas concentrations of PM10 and RM2.5 were higher the warm period of 2011 (41.4 and 31.1 μg/m^3), versus 30.6 and 19.4 μg/m^3 for 2012 respectively, during the coldest period of 2012, PM concentrations are significantly higher (73.1 and 62.7 μg/m^3 for PM10 and PM2.5 respectively), versus 53.1 and 43.5 for the year 2011. The increase of concentrations in year

2012 is accompanied by a sharp increase in the ratio PM2.5/PM10 (from 0.63 to 0.86), in contrast to 2011, where the corresponding change was smaller (from 0.75 to 0.82). Between the two years, there is a significant variation among the emission patterns, where the traffic component appears to be reduced in 2012, while during the colder period the component associated with the biomass heating is dramatically increasing. Given that temperatures during the cold season of 2012 are close to the ones of 2011 (daily average 11.1 ^{0}C for both years), these differences cannot be attributed to increased need for domestic heating. During the last winter (2013-2014), the pattern of traffic and biomass burning emissions seems to be similar to the previous year, thus, the problem of biomass burning still remains.

The contribution of biomass burning to PM air pollution was verified by levoglucosan analysis of PM, which is considered the most specific tracer of biomass burning (Belis et al. 2013; Perrone et al. 2012; Zhang et al. 2008) and the empirical function proposed by (Caseiro et al. 2009), according to which:

$$\text{wood smoke PM (in } \mu g/m^3) = \text{Levoglucosan (in } \mu g/m^3) \cdot 10.7$$

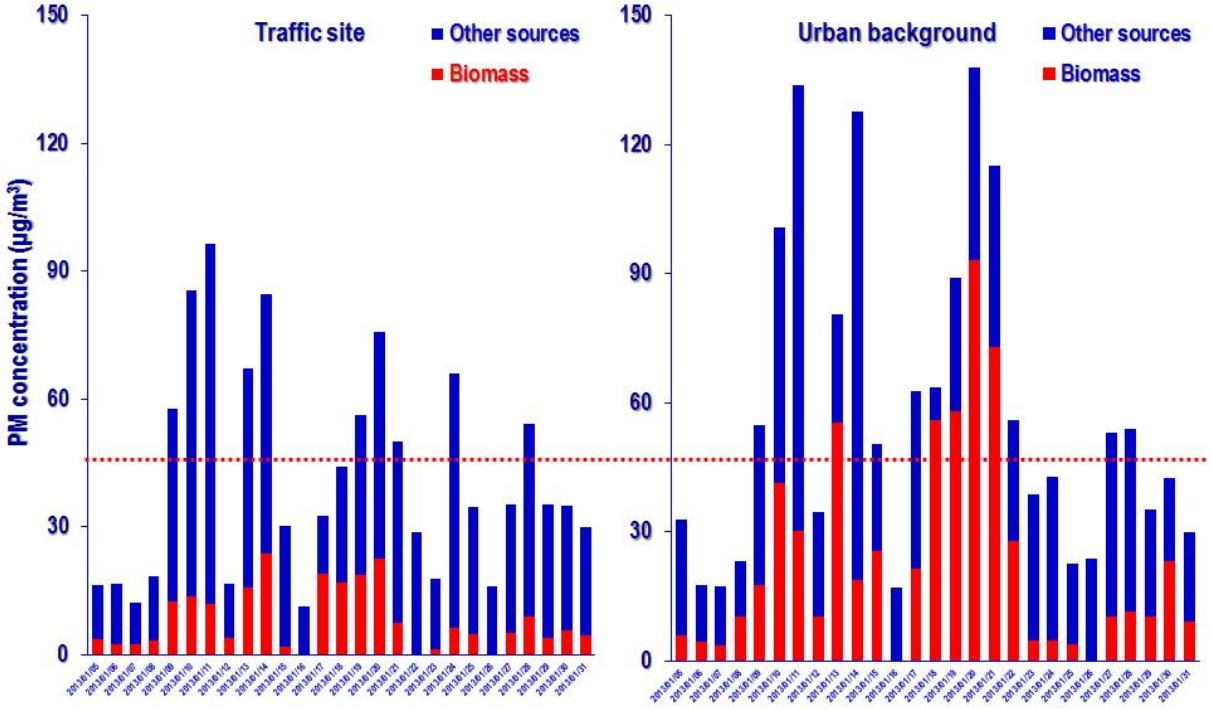


Figure 14: Biomass burning contribution in two distinct sites (traffic and urban background) in the city of Thessaloniki

Increased biomass emissions, are linked to elevated exposure to PM, as well to toxic compounds adsorbed on them, PAHs (IARC 2010), being the ones of highest concern among them. It is also interesting that higher concentrations of PM due to biomass burning, where accompanied by equally high concentrations of PAHs; the latter shows that toxic potency of biomass emitted PM is at least equally toxic to traffic emitted PM (Figure 15).

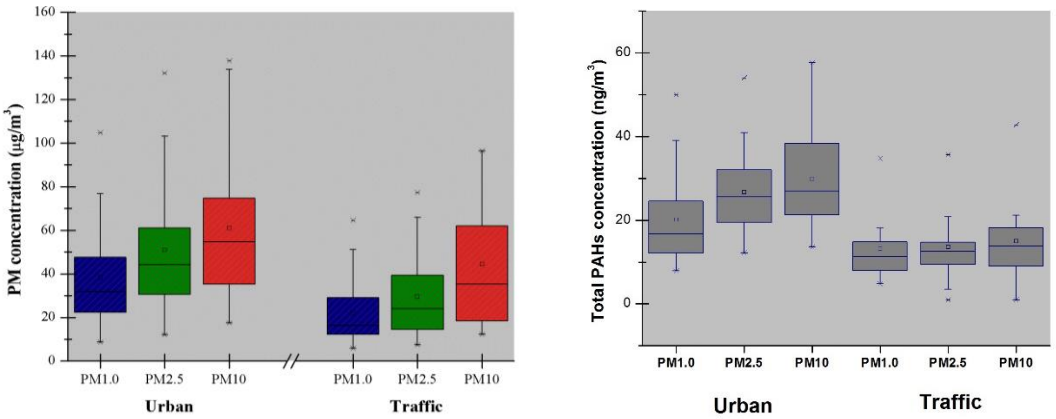


Figure 15: PAHs concentration between urban and traffic site for the different PM fractions

Linking biomonitoring data to exposure

Biomonitoring data acquirement

The direct, adverse effects, especially the carcinogenic effects, of PAHs on human health are well documented. The PAHs themselves are not carcinogenic, but some PAHs derivatives are carcinogenic when converted by the body to compounds that can be excreted (Ciarrocca et al. 2014). Therefore, the toxic effects of PAHs are secondary to their biotransformation. This process is principally driven by the action of mixed-function monooxygenase cytochrome P450 enzymes (CYP1A1, CYP1A2, CYP1B1 and CYP3A4). These phase I enzymes convert the PAHs into reactive epoxides. The detoxification of these highly reactive, mutagenic and carcinogenic compounds is reliant upon their combination with glutathione in a reaction catalysed by glutathione S-transferase class M (GSTM). For the enzymes CYP1A1 and GSTM1, genetic polymorphisms affecting their catalytic activity are known. As a metric of internal exposure to PM, 1-OH pyrene in urine, is considered as a reliable biomarker (Bouchard and Viau 1999; Miao et al. 2014).

Assimilating biomonitoring data

To assimilate the internal dose data for PAHs, we need to model the deposition of PM across human respiratory tract (HRT), as well the fate of PAHs that is adsorbed on the different PM size fractions deposited across the different regions of the HRT. Thus, we will need to use two types of modelling approaches:

HRT modelling

Internal exposure of PM will be carried out using the Multiple-Path Particle Dosimetry (MPPD) model. The algorithms in MPPD calculate the deposition and clearance of monodisperse and polydisperse aerosols in the respiratory tract of rats and human adults and children (deposition only) for particles ranging from ultrafine (0.01 microns) to coarse (20 microns) sizes. The models are based upon single-path and multiple-path methods for tracking air flow and calculating aerosol deposition in the lung. The single-path method calculates deposition in a typical path per airway generation, while the multiple-path method calculates particle deposition in all airways of the lung and provides lobar-specific and airway-specific information. Within each airway, deposition is calculated using theoretically derived efficiencies for deposition by diffusion, sedimentation and impaction within the airway or airway bifurcation. Filtration of aerosols by the head is determined using empirical efficiency functions. Results using the model show good agreement with experimental data for regional deposition in the rat and human lung (Heyder et al. 1986).

PBTK modelling

For simulating the inhalation pharmacokinetics of PAHs, we will adapt the CROME-LIFE generic PBTK model using the parameterization proposed by Chiang and Liao (2006). A key issue for the proper evaluation of biomonitoring data is entero-hepatic recirculation, that needs to be taken into account when assessing the fate of 1-OH-pyrene within human tissues (Jongeneelen and Ten Berge 2012), as well as the strong partition coefficient of PAHs to lung epithelium (Boström et al. 2002; Gerde et al. 1997).

Association to health effects/Socioeconomic cost

Indoor biomass burning for residential heating, is a major source of indoor and outdoor air degradation. One of the major components of biomass burning is emitted PM. The association of biomass burning PM with potential health effects, as well as their toxicity potency in comparison to other combustion sources PM have been investigated through controlled exposure study of human exposure to woodsmoke, epidemiological studies (observational or interventional), as well as with toxicological tests - a very comprehensive review on both perspectives was carried out by Naeher et al. (2007).

To date, only a single controlled exposure study of human exposure to woodsmoke itself seems to have been published (Barregard et al. 2006). Thirteen subjects were exposed to realistic concentrations of woodsmoke (200–300 μg/m^3 PM2.5) generated under controlled conditions for two 4-h sessions, spaced 1 wk apart. In this study, exposure to woodsmoke resulted in small exposure-related changes in levels of inflammatory mediators and coagulation factors. In addition, evidence of increased free radical-mediated lipid peroxidation was observed in 9 of the 13 subjects. Although this is the only controlled study of woodsmoke exposure published to date and it observed a small number of subjects, it is suggestive of woodsmoke-associated systemic inflammatory effects.

The majority of information regarding direct human health effects associated with woodsmoke exposure is derived from a relatively large number of epidemiologic studies have documented respiratory effects of residential woodburning, especially in children (Naeher et al. 2007). One of the earliest studies was conducted in Michigan by Honicky et al. (Honicky et al. 1985) who compared respiratory symptoms in 31 children who lived in homes with wood stoves with 31 children who lived in homes without wood stoves. Symptoms were categorized as mild, moderate, and severe. The two groups did not differ with respect to mild symptoms, but differed significantly for severe symptoms ($p < .001$). Related health effects research in Seattle shows associations between PM2.5 and lung function decrements in children (Koenig et al. 1993), visits to emergency departments for asthma (Norris et al. 1999), hospitalizations for asthma (Sheppard et al. 1999), and increases in asthma symptoms in children (Yu et al. 2000), as well as increases in exhaled nitric oxide (Koenig et al. 2005). Since woodburning is the primary source of fine particles in the Seattle airshed, the health effects studies suggest a causal relationship. Another study examined the relationship of woodstoves to otitis media and asthma in a case-control study of home environmental air pollutants in Springville, NY (Daigler et al. 1991). That study found use of woodstoves was more likely to be present in homes of children with otitis media (OR 1.7, CI=1.03, 2.89). In a panel study of adults (ages 18–70) in Denver, CO (Ostro et al. 1991), the use of a fireplace or woodstove was associated with an increase in daily moderate or severe shortness of breath (OR 1.3, CI 1.1, 1.4). Use of woodstoves or fireplaces was second only to the presence of smokers in the home, and more strongly associated with shortness of breath than use of gas stoves or occupational exposures.

In the study of Happo et al, (2013) health related toxicological properties of PM1 emissions from five modern and two old technology appliances were examined. The collected samples were weighed and extracted with methanol for chemical and toxicological analyses. Healthy C57BL/6J mice were intratracheally exposed to a single dose of 1, 3, 10 or 15 mg/kg of the particulate samples for 4, 18 or 24 h. Thereafter, the lungs were lavaged and bronchoalveolar lavage fluid (BALF) was assayed for indicators of inflammation, cytotoxicity and genotoxicity. Lungs of 24 h exposed mice were collected for inspection of pulmonary tissue damage. There were substantial differences in the combustion qualities of old and modern technology appliances. Modern technology appliances had the lowest PM1 (mg/MJ) emissions, but they induced the highest inflammatory, cytotoxic and genotoxic activities. In contrast, old technology appliances had clearly the highest PM1 (mg/MJ) emissions, but their effect in the mouse lungs were the lowest. Increased inflammatory activity was associated with ash related components of the emissions, whereas high PAH concentrations were correlating with the smallest detected responses, possibly due to their immunosuppressive effect.

A recent meta-analysis of 15 epidemiologic studies covering a wide range of countries found that people exposed to biomass smoke had combined odds ratio (OR) of 2.44 for developing chronic obstructive pulmonary disease as assessed by lung function measurements or symptom-diagnosed chronic bronchitis, and that this was true for both women and men. Moreover, cigarette smoking appeared to have a synergistic effect with biomass smoke, increasing the OR for chronic obstructive pulmonary disease development to 4.39 (Diette et al. 2012). The authors concluded that inhalation of woodsmoke, at a relatively low level, had the potential to alter host pulmonary immune defense mechanisms in such a way as to lead to an increased susceptibility to infectious lung disease. Inhalation of woodsmoke can have a significant impact on pulmonary homeostasis and/or exacerbations of ongoing lung disease processes.

Till now, limited studies have evaluated the results of shifting between biomass and conventional for domestic heating (intervention studies). The most comprehensive study up to now is the one based on a real cohort by the study carried out by Johnston et al. (Johnston et al. 2013) who evaluated the effect of reductions in air pollution from biomass smoke on daily mortality. Age stratified time series analysis of daily mortality with Poisson regression models adjusted for the effects of temperature, humidity, day of week, respiratory epidemics, and secular mortality trends, were applied to an intervention and control community, (Central Launceston, Australia, a town in which coordinated strategies were implemented to reduce pollution from wood smoke and central Hobart, a comparable city in which there were no specific air quality interventions. The participants number was 67 000 residents of central Launceston and 148 000 residents of central Hobart. Interventions include community education campaigns, enforcement of environmental regulations, and a wood heater replacement program to reduce ambient pollution from residential wood stoves started in the winter of 2001.Mean daily wintertime concentration of PM10 fell from 44 μg/m^3 during 1994-2000 to 27 μg/m^3 during 2001-07 in Launceston. The period of improved air quality was associated with small non-significant reductions in annual mortality. In males the observed reductions in annual mortality were larger and significant for all cause (−11.4%, 95% confidence interval −19.2% to −2.9%; P=0.01), cardiovascular (−17.9%, −30.6% to −2.8%; P=0.02), and respiratory (−22.8%, −40.6% to 0.3%; P=0.05) mortality. In wintertime reductions in cardiovascular (−19.6%, −36.3% to 1.5%; P=0.06) and respiratory (−27.9%, −49.5% to 3.1%; P=0.07) mortality were of borderline significance (males and females

combined), while there were no significant changes in mortality in the control city of Hobart. In the study carried out by Haluza et al. (2012) in Austria, it was estimated that replacement of light oil by biomass as well as fossil gas (scenario 2) will lead to about 101 additional deaths per year (7.2 deaths per 100,000 population) because of the increase of PM10. Replacement of light oil by biomass only (scenario 3) would result in an increase of 174 deaths per year (12.4 per 100,000) from the increase in PM10. Scenario 2 would result in more than 203 additional annual hospital admissions (14.5 per 100,000) and scenario 3 in additional 353 hospital admissions (25.2 per 100,000 inhabitants). Annual mean values in $\mu g/m^3$ of PM for rural areas and urban areas for current energy mix were 25.4 and 31.9 $\mu g/m^3$ respectively. For replacement of light fuel oil by other energy sources were for rural 27.2 $\mu g/m^3$ and for urban 34.7 $\mu g/m^3$. For replacement by biomass were for rural 28.5 $\mu g/m^3$ and for urban 36.8 $\mu g/m^3$. Although interesting from the perspective of scenario analysis, the use of PM10 as an input for the concentration response functions, does not seem to fully capture the variability on PM concentrations related to biomass burning, thus differences within the scenarios might be actually underestimated.

Within the frame of the CROME-LIFE study, evaluation of multiple health endpoints will be carried out, including mortality, infant mortality, lung cancer, chronic bronchitis, respiratory and cardiovascular hospital admissions. Well established concentration-response functions will be used for translating actual exposure into health associated risks.

Table 10. CRFs for the selected health endpoints

Health endpoint	CRF	Reference	Background rate	Age group
Mortality (all cause)	6% (95% CI: 2%, 11%) change per 10 $\mu g/m^3$ $PM_{2.5}$	(Hurley et al. 2005; IOM 2011)	8417 annual deaths/881288 population (EUROSTAT 2011; WHO 2008)	Adults aged 30 years and older
Infant Mortality	4% (95% CI: 2%, 7%) change per 10 $\mu g/m^3$ PM_{10}	(Hurley et al. 2005; IOM 2011)	145 post-neonatal deaths per 100,000 live births (9141 annual births) (EUROSTAT 2011; WHO 2008)	1 month to 1 year
Chronic bronchitis	22% (95% CI: 2%, 38%) change per 10 $\mu g/m^3$ PM_{10}	(IOM 2011; Schindler et al. 2009)	390 new cases annually per 100,000 adults at risk (adjusted for remission - remission rate of 56.2%) (Schindler et al. 2009)	Adults aged 18 years and older
Cardiovascular hospital admissions	0.6% (95% CI: 0.3%, 0.9%) change per 10 $\mu g/m^3$ PM_{10}	(Hurley et al. 2005; IOM 2011)	(Hurley et al. 2005): 723 emergency cardiac admissions per 100,000 population, all ages, per year	All Ages
Respiratory hospital admissions	0.9% (95% CI: 0.7%, 1.0%) change per 10 $\mu g/m^3$ PM_{10}	(Hurley et al. 2005; IOM 2011)	(Hurley et al. 2005): 617 emergency respiratory hospital admissions per 100,000 population, all ages, per year	All Ages

For estimation of lung cancer risk, a more comprehensive methodology will be follow, incorporating internal dosimetry of PAHs including HRT deposition of PM and actual internal exposure of PAHs, based on the amount of PAHs adsorbed on the deposited PMs across the

different regions of HRT. Calculation of the overall toxicity of the mixture of the main 19 PAHs will be assessed using Toxic Equivalent Factors (TEFs), on the assumption that the TEF for B[a]P is equal to 1:

$$TEQ = \sum_{i=1}^{19} \left(C_i \times TEF_i \right)$$

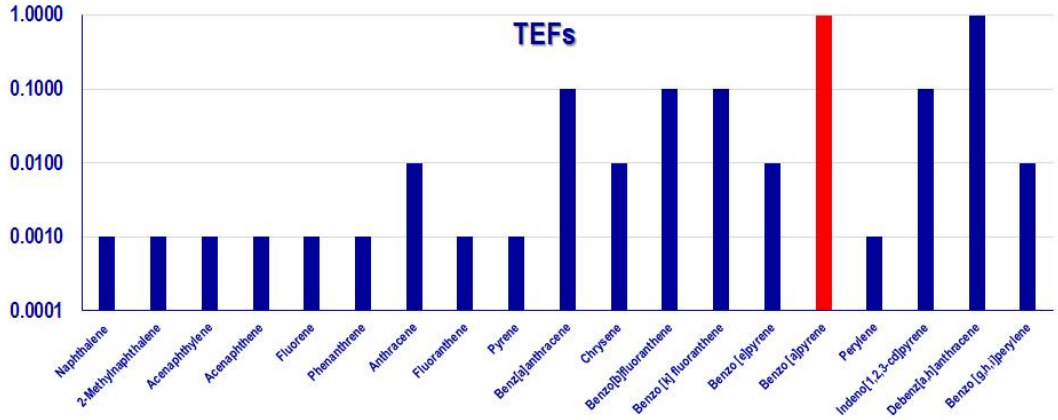


Figure 16: Relative carcinogenic potency of PAHs

A slope factor will be derived, based on the Inhalation Unit Risk of B[a]P, based on the bodyweight normalized dose of an average adult (bodyweight 70 kilos, inhalation rate 20 m^3/day)

$$SF = 3.5 \times 10^{-3} \times IUR_{B[\alpha]P}$$

Based on all the above, the actual carcinogenic risk will be given by the following formula:

$$ICR_i = DF_i \times [PM] \times \left(\frac{TEQ}{[PM]} \right) \times \frac{IR_i}{BW_i} \times SF$$

In order to evaluate the financial cost of increased morbidity and mortality due to biomass PM exposure, as well as the benefit from potential intervention policies, monetary valuation of health associated costs will need to be estimated. The starting point for the valuation of health end-points is the identification of the components that comprise changes in welfare. These components should be summed to give the total welfare change, assuming no overlap between categories. The three components include:

- Resource costs i.e. medical costs paid by the health service in a given country or covered by insurance, and any other personal out-of-pocket expenses made by the individual (or family).
- Opportunity costs i.e. the cost in terms of lost productivity (work time loss (or performing at less than full capacity)) and the opportunity cost of leisure (leisure time loss) including non-paid work.
- Dis-utility i.e. other social and economic costs including any restrictions on or reduced enjoyment of desired leisure activities, discomfort or inconvenience (pain or

suffering), anxiety about the future, and concern and inconvenience to family members and others.

The welfare changes represented by components (i) and (ii) can be proxied using market prices that exist for these items. This measure - in best practice - needs to be added to a measure of the affected individual's loss of utility, reflected in a valuation of the willingness-to-pay/accept (WTP/WTA), to avoid/compensate for the loss of welfare associated with the illness. For the endpoints of consideration mentioned above, Table 11 presents a summary of the unit values derived in the course of the HEIMTSA project (UBath 2011). These values are the result of both an evaluation of the evidence available in the existing literature.

Table 11: Monetary evaluation (€)

Mortality/morbidity indices	Lower estimate	Medium estimate	Highest estimate	Reference
Mortality (all cause)	1,120,000	1,650,000	5,600,000	(Alberini et al. 2006)
Infant Mortality	1,120,000	2,475,000	11,200,000	(Holland et al. 2004)
Chronic bronchitis	43,000	60,000	100,000	(Krupnick and Cropper 1992)
Cardiovascular hospital admissions	2,990	2,990	8,074	(Holland et al. 2004; Navrud 2001)
Respiratory hospital admissions	2,990	2,990	8.074	(Holland et al. 2004; Navrud 2001)

For each health end-point, the unit values are identified on the basis of an informal meta-analysis of the evidence, accounting for the distribution of available values and an assessment of the quality and geographical focus of each study, incorporating considerable uncertainty in health valuation. The uncertainty derives from a combination of the paucity of the evidence base, the difficulty that people have with identifying their preferences for (avoidance of) health conditions, and the lack of maturity in the study methods themselves.

Critical data for the completion of the case study

Filling the gap

There are plenty of detailed environmental data related to PM concentrations and chemical speciation. This data incorporated to advanced modelling framework described above will allow the accurate assessment of external and internal exposure to PM. Exposure to PAHs and consequently to PMs, will be verified by PAHs metabolites measurements in urine. Health data for the endpoints mentioned in Table 10 will be retrieved by the Hellenic Statistical Authority and the hospitals of the wider Metropolitan area of Thessaloniki.

Methods

Within the CROME-LIFE project, 1-OH pyrene in urine will be used as a metric of internal exposure to PM, which is a well validated (Bouchard and Viau 1999; Miao et al. 2014) major metabolite traced after exposure to PAHs. The straightforward HPLC method proposed by is a good basis for the analysis of 1-OHP in human urine. It is generally sensitive, specifc and can be made very reproducible with some minor modifications (Bouchard and Viau 1999).

Alternative assays should be validated by comparison with this assay since quality of data and standardization of the analytical method are fundamental for interlaboratory comparison and interpretation of results. In addition, since urinary levels of 1-OHP are dependent on urine output, 1-OHP urinary excretion values should be adjusted for creatinine levels. Urine should be collected in standard polyethylene or polypropylene tubes and we suggest the addition of a small amount of thymol to prevent bacterial growth. Tests conducted in our laboratory have further shown that storing of urine samples (collected over thymol) at room temperature, 4 °C or –20 °C causes no loss of the analyte for a period of several weeks.

Case study Slovenia - Human Biomonitoring in Slovenia

Introduction

Legislative basis

The legislative basis for the implementation of the Human Biomonitoring programme in Slovenia is defined in the *Article 49 Act of Chemicals* of the *Chapter IX. Protection of Human Health and the Environment* (O.J. RS No. 16/2008) under the topic related to protection of people or the environment, prohibitions and restrictions. It is stated that the Minister (i) shall prohibit or limit the trade or the use of chemicals, already regulated by EU-provisions or provisions of other international agreements, and (ii) may determine the conditions under which chemicals classified as dangerous or products containing them may be placed on the market or used if they have adverse effects on human health or the environment.

In the articles Articles 50 and 51 of the same Act, dealing with provisional measures and the monitoring, it is stated that the Government (i) may temporary prohibit or restrict the production, marketing or use of certain chemical and introduce other measures, when there is a justified reason to suspect that the chemical may have irreparable consequences for human health and the environment due to the geographic, environmental and health specifics of Slovenia, limiting or preventing the consequences to an acceptable level, before concrete and reliable scientific evidence on the effects and action of such chemical are available; (ii) shall study the introduced provisional measures whenever new findings on the chemical become available and no later than within one year of their introduction, (iii) shall analyse the effect of the measures introduced and whether they were justified and adopt a decision on further measures.

While in the Article 51a. which deals with the biomonitoring of chemicals it is stated that for the purpose of preparing and monitoring of measures to limit the risk of chemicals to people and the environment, monitoring of presence of chemicals and their breakdown products in people and organisms (hereinafter: biomonitoring) shall be conducted in professionally justified intervals of time.

In Slovenia the biological monitoring is coordinated by the competent authority for chemicals and carried out by health and other public institutes authorised by the Minister, for people and organisms together or separately (hereinafter: biomonitoring providers).

Biomonitoring providers shall cooperate with the competent authority for chemicals and among themselves on: preparing a short- and long-term biomonitoring programme, its intersectional coordination, monitoring of its implementation, performing expert evaluation and proposals for measures. Conditions regarding the professional and technical competence of public institutes for conducting the biomonitoring from the preceding paragraph shall be set out by the Minister. Provisions for biomonitoring from this article do not infringe upon

provisions for biological monitoring at the workplace which are governed by regulations on occupational safety and health.
The EU and National law on prohibitions and limitations of marketing and use of certain dangerous chemicals among others include the POPs and PIC conventions, and former experiences with setting up the monitoring of pesticides in foodstuffs and in drinking water (from 1998).
In addition, it is also important to mention the necessary compliance with the National Programme on Public Health-2012 in correlation with National Programme On Chemical Safety 2006-2010, and the Action Plan on Chemical Safety of Children (2011), the European Environment and Health Strategy and Action plan 2004-2010, and WHO Guidelines.

Objectives

Short-term objectives of the HBM programme is to provide data on exposure of the inhabitants to chemicals and related health impact throughout Slovenia, reference (background) values, and spatial differences in exposure including rural, urban environments and contaminated sites. Provision of institutional framework for the implementation of the programme on a long-term basis is also one of the key objectives to be settled.
Long-term objectives include the exposure and risk assessment for health, implementation and monitoring of implemented measures, science based risk evaluation (awareness, case-by-case consulting, risk communication), time trends of exposure, and providing input for policy making, based on surveillance activities.
The objectives of HBM in Slovenia comply well with the main problem targeted by CROME-LIFE which is the assessment of the impact on human health due to exposure to chemical agents originating either from environmental contamination (air, soil, water), or from consumer products (food contact materials, construction materials, cosmetics, clothes, etc.) through multiple routes.

Proposed methodological scheme

Environmental data and external exposure assessment

In Slovenia, availability of environmental data and related spatial data infrastructure was recently analyzed into detail within the EU project EGIDA (Coordinating Earth and Environmental cross-disciplinary projects to promote GEOSS (FP7-ENV-2010), GA 265124, 2010-2012). Among others, data availability and needs for exposure and human health risk assessment was addressed. The following was concluded. People are exposed to large numbers of chemicals in the environment, but the exposure is relatively poorly defined. In Slovenia, biological monitoring is an essential means of systematic collection of information on exposure to various pollutants in environment. Human biomonitoring program includes investigations of various biomarkers. The results of biological monitoring are therefore indicative cumulative indicator of the living environment in terms of burden of POPs and other pollutants. In addition, spatial (geographic) differences in exposure of population due to the specifics of local environment must be taken into account (e.g. locations of past and current industrial activities, spatial distribution of pollutants in different environmental compartments...).
In terms of data needs to investigate the cause-effect relationships between the living environment and the health outcomes, the following can be concluded. Various data exist in different forms and formats (layer-shaped, ASCII, tabulated or raster data) and are usually

organized according to the DPSIR network, depending on the data source and provider. As most important sources, the Environmental Atlas (web mat viewer), Inspire GEOportal http://www.geoportal.gov.si/ (hosted by the Surveying and Mapping Administration), GEOportal http://gis.arso.gov.si/geoportal/catalog/main/home.page (hosted by the national Environment Agency), EIONET-SI http://nfp-si.eionet.europa.eu/ (interactive maps, tables, texts or downloadable shape files) and GERK http://rkg.gov.si/GERK/viewer.jsp (land parcel identification system hosted by the Ministry of Agriculture and Environment) were identified. Based on the outcome of these analyses, a catalogue with more than 100 indicators identified by DPSIR framework is available along with the data source and provider within different Societal Benefit Areas (natural disasters, health, energy, climate, weather, ecosystems, agriculture and biodiversity). There is a schematic presentation of different GIS data sets usable in the interpretation of HBM results on Figure 17.

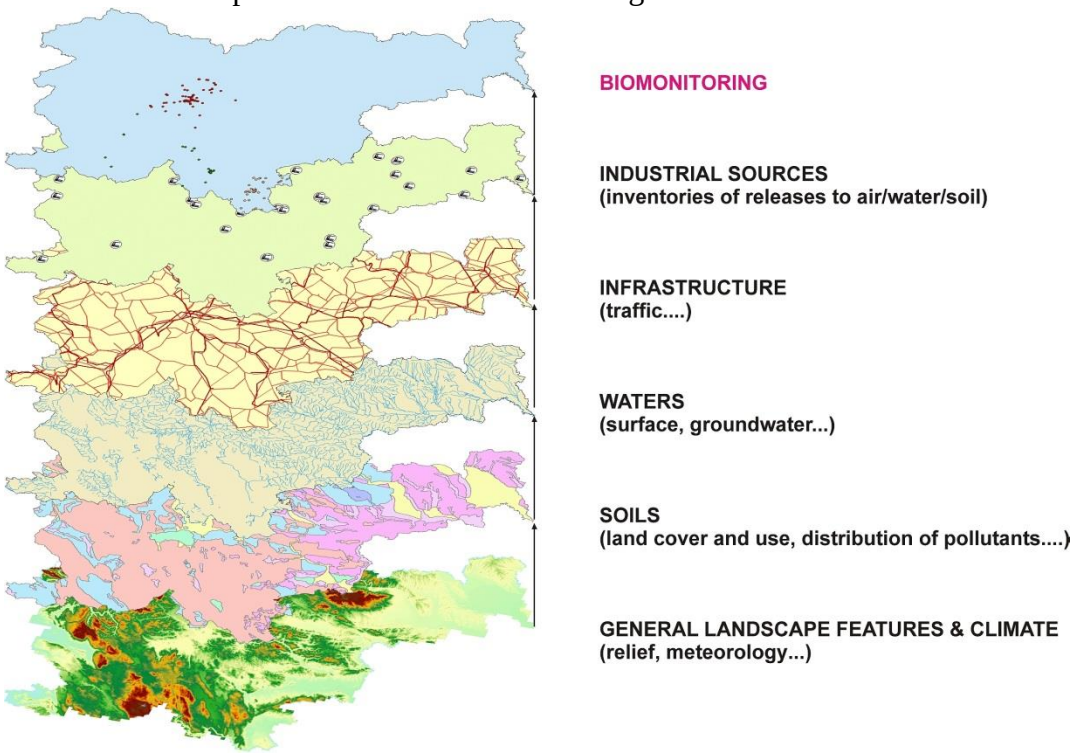


Figure 17: Scheme of GIS data sets usable in the interpretation of HBM results

Linking biomonitoring data to exposure

Assimilating biomonitoring data

Biomonitoring provide a link between exposure and processes in the body. Collection of as much as possible of exposure information is important to identify pathways of exposure and to eliminate sources. Complexity of the problem led to development of modeling tools. Modelling framework is described in the initial chapters of this report.

Human biomonitoring data acquirement

The pilot phase of the HBM was implemented in the time period between 2007 and 2010 and the second phase of HBM was between 2011 and 2014.

Study design

The *study population* includes lactating women and men from the same area in the age from 20-40 years, 50 women and 50 men from each area (1200 subject all together). Twelve areas were selected:

a) urban (3 areas)
b) rural (3 areas)
c) contaminated sites (6 areas) selected on the basis of past activities (former Hg mine, former Pb mine, former factory of transformers and capacitors – PCBs pollution) and also present industry (smeltres, cement factory, power plant, glass factory, etc.)

Inclusion criteria strictly followed are: (i) residency in the area at least 5 years, (ii) first child, (iii) breastfeeding only one child not twins; (iv) normal healthy pregnancy; (v) availability for sampling 6-8 weeks after delivery, (vi) age 20-40 years.

Exclusion criteria include the following: chronic diseases, occupational exposure, smoking, alcohol or drug abuse, reside near known emissions of pollution except on contaminated areas.
The study population included in the study is presented in Figure 18.

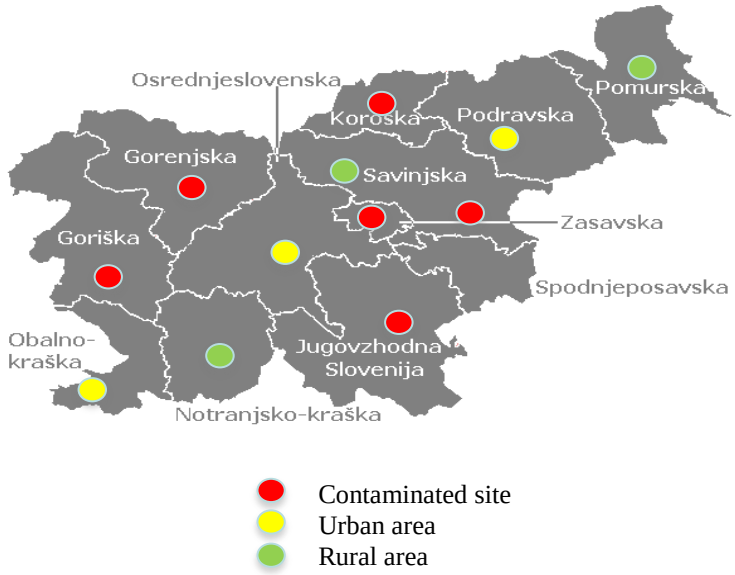


Figure 18: Selected areas

A schematic presentation of study protocol is presented in Figure 19

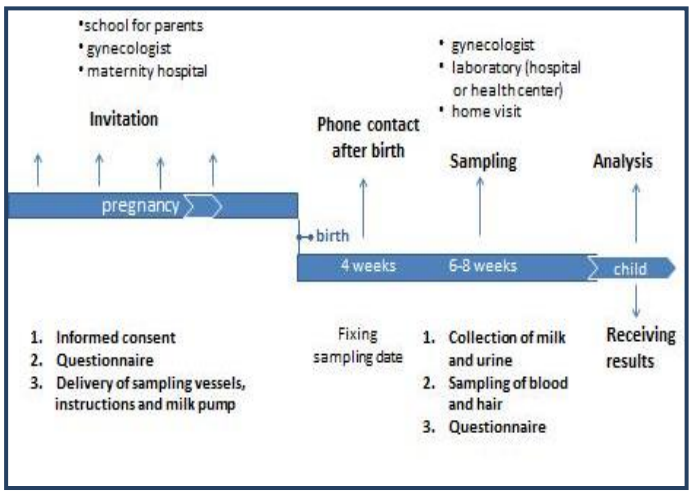


Figure 19: The timelines of the study protocol

A questionnaire was designed to verify the absence of exclusion criteria and to provide information about the residence, occupation, hobbies, health status, pregnancy, smoking status, and general dietary habits - food frequency assessment of consumption of vegetables/fruits, milk/milk products, eggs, meat, fish: fresh/frozen/canned, and alcoholic beverages, coffee/tea, supplements).
The measurements and the matrix table are presented in Table 12.

Table 12: List of biological samples/matrixes and measurements included in the HBM in Slovenia

Sample	***Individual samples***	***Pooled samples***
Breast milk	*Pb, Cd, Hg, As, Cu, Zn, Se* *organochlorinated pesticides* *marker PCBs (28, 52, 101, 138, 153, 180)* *triglycerides, cholesterol*	*PCDD,* *PCDF,* *dioxin like PCB,* *PBDE*
Blood - women	*Hemogram* *Pb, Cd, Hg, As, Cu, Zn, Se* *creatinine* *TSH*	
Blood - men	*Hemogram* *Pb, Cd, Hg, As, Cu, Zn, Se* *organochlorinated pesticides* *marker PCBs (28, 52, 101, 138, 153, 180)* *triglycerides, cholesterol, creatinine* *TSH*	*PCDD,* *PCDF,* *dioxin like PCB,* *PBDE*
Urine	*Pb, Cd, Hg, As, Cu, Zn, Se* *Markers of kidney function* *(albumin, alpha-1-mikroglobulin, IgG, NAG)* *Creatinine*	
Hair	*Hg*	

Funding for HBM was provided by the Ministry of Heath, Chemical Office of the Republic of Slovenia, the implementation was performed by the Jožef Stefan Institute, Department of Environmental Sciences and the subcontracting institutions: University Medical Centre

Ljubljana, Institutes of Public Health Maribor, Celje, Ljubljana, Kranj, Nova Gorica, Ravne, Murska Sobota, Koper, Novo Mesto and regional hospitals and health centers.

Association to health effects

Biochemical markers of kidney function

Kidney is one of the major target organs for the toxic metals (Pb , Cd , Hg), and various organic compounds (PCBs, furans, dioxins, etc.) , which can accumulate there and thereby cause damage.

Proteinuria is a general term for the presence of increased amounts of protein in the urine. Proteinuria may reflect abnormal loss of plasma proteins due to a) increased glomerular permeability to large molecular weight proteins (albuminuria, IgG or glomerular proteinuria), b) incomplete tubular reabsorption of normally filtered low-molecular-weight proteins (tubular proteinuria), or c) increased plasma concentration of low-molecular-weight proteins (overproduction proteinuria, such as immunoglobulin light chains).

Proteinuria may also reflect abnormal loss of proteins derived from the kidney (renal tubular cell constituents due to tubular damage) and lower urinary tract. Albuminuria, tubular proteinuria and renal tubular cell constituents are pathognomonic of kidney damage. In addition, findings from experimental and clinical studies have suggested an important role for proteinuria in the pathogenesis of disease progression of chronic kidney disease.

Although many biochemical markers are known to detect kidney dysfunction, but due to simplicity and compliance with the guidelines (K/DOQI), we use serum creatinine to calculate the assesment of glomerular filtration rate (OGF) by MDRD formula (traceable to IDMS). Low molecular weight urinary protein and enzyme are used to detect tubular kidney damages: alpha-1-microglobulin and the enzyme N-acetylglucosaminidase (NAG). Urinary Albumin and IgG are used to assess glomerular kidney disfunction. All proteins are divided by urinary creatinine.

Thyrotropin , thyroid -stimulating hormone TSH

Thyroid is one of the most important endocrine glands in the human body that regulates the metabolic rate, heart rate and breathing, affects the growth and development of the nervous system, and many other processes in the body. The main function of the thyroid gland is the secretion of the two most important thyroid hormones T3 and T4. The amount of hormones synthesized in the thyroid gland depends not only on the thyroid gland, but also on higher control centers in the body. Primary control center is located in large brains and is responsible for regulation of the internal environment of the body, therefore also for the control of the amounts of hormones. This part of the brain is hypothalamus, which releases TRH (thyrotropin releasing hormone). TRH stimulates activity in the lower regulatory center - the pituitary gland to secrete TSH (thyroid stimulating hormone). TSH acts directly on the pituitary gland subordinate - the thyroid gland and encourages it to produce more hormones in situations where it is necessary. Some data from the literature shows that at the time of poisoning or exposure to Pb, Cd, Hg and organic pollutants there is a dysfunction of the pituitary gland, the hypothalamus or the thyroid itself and thus the proper secretion of TSH . The main consequence may be hypo-or hiper tyroidism

Critical data for the completion of the case study

Filling the gap

In Slovenia human biomonitoring data, health outcome data and some exposure data are available. Exposure data will be improved during CROME-LIFE project. All this data will be used in advanced modelling framework described in initial chapters of this report. Results of this process will allow the assessment of external and internal exposure to selected metals.

Methods

External exposure assessment will be improved by the use of sensors for outdoor and indoor monitoring. The indoor environment quality is, however, a very broad term comprising essentially all kinds of factors that people are exposed to indoors. Air quality data parameters that will be monitored on-line by sensors are temperature, relative humidity, CO_2, NO_2, dust, O_3, noise, VOC and radon. In the EU CITI-SENSE project use of sensors for online monitoring of the indoor air environment will be implemented in a pilot study in just one city. In CROME_LIFE project this will be expanded to more locations.

Case study Italy - Human Biomonitoring in Italy

Introduction

In Italy the first national biomonitoring programme of the general population has been launched in 2008. The pre-existing situation was related to the internal dose assessment of metals in population groups, available only for a few geographical areas of the country. In 1990, data for 20 metals in inhabitants of the region Lombardy were published (Minoia et al.,1990), and, after a gap of many years, in 2004, a survey on 26 metals in the region of Latium was conducted (Alimonti et al., 2005a). Moreover, for some metals data were still missing for the Italian general population, and information on internal exposure have not been systematically examined in relation to other characteristics of the population groups in study (i.e. gender, age, smoking or dietary habits, etc.). HBM data production is a time-consuming and expensive process that requires a deep estimation of several analytical and biological factors that could affect the final results, including route of absorption, the presence of sources of environmental pollution in certain residential areas, the physiological variability and lifestyles. The data obtained from these studies are necessary to obtain Reference Values (RV), statistically derived numbers that indicate the upper margin of background exposure to a given substance in a defined population at a given time (Schulz et al., 2007).

PROBE (PROgramme for Biomonitoring general population Exposure on metals)

To fill this gap a HBM survey on the environmental exposure of healthy adults to metals in Italy (PROBE project, Alimonti et al., 2011) was conducted by the Istituto Superiore di Sanità (ISS) in the 2008-2010 period, funded by the Ministry of Health.

Objectives

Primary objective of PROBE was to supply representative data on the metals' internal dose in adults in order to highlight the environmental impact on the health of Italian population. The activities carried out were devoted to:

- develop, standardize and validate protocols and methods for samples collection and metal analysis as a basis for their reliability, transferability and comparability;
- establish Reference Values (RVs) for the exposure of healthy adults to environmental metals, including a large metal profile (20 metals);
- examine the possible influences of certain variables (demographics and habits) on the metal level of individuals.

Study design

To achieve these objectives, the project was divided into the following phases:

- identification of the adequate skills in the area to be potentially enrolled;
- training to enable the harmonization of procedures for collecting, storing, transporting and processing the human specimens;
- development and validation of laboratory methods for the quantification of metals;
- determination of levels of metals in study population blood and serum;
- stratification of results according to age, sex, alcohol and smoking habits, lifestyle, diet, etc.

Impact

Further to providing information about the serum metal level of urban population living in different geographical areas of Italy, the results obtained by PROBE can be eventually used as support to REACH regulation application in Italy:

- to encourage further investigations (Commission Regulation No. 1907/2006, art. 45, paragraph 5);
- to identify substances of very high concern, persistent, bioaccumulating and toxic or chemicals of equivalent concern (Commission Regulation No. 1907/2006, Annex XIV);
- to assess the efficiency of risk reduction measures or of substitutional choice in authorized substances underlying the minimization requirements (Commission Regulation No. 1907/2006, art. 60, paragraph 10).

Methodological procedures

Population: 1423 subjects (18–35, 36–50, 51–65 yrs) living in urban sites of 5 different Regions (Piedmont, Emilia Romagna, Umbria, Latium, Calabria), 953 were males and 470 females (Figure 20). The 41% were in the mid–age category (36–50 years).

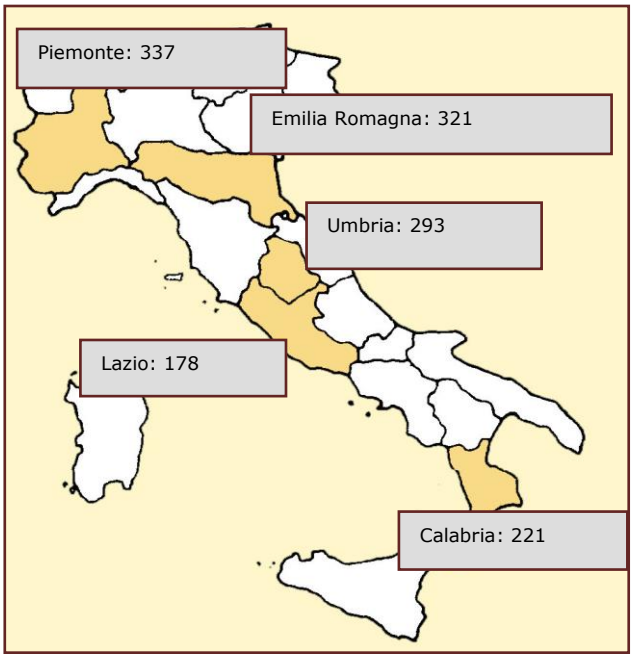


Figure 20: study areas

Each donor was also asked to answer a short interview, in the form of questionnaire in order to collect information on:

- general data such as gender, age, height and weight (body mass index), place of residence and job; anamnesis in terms of acute or chronic diseases (age at diagnosis), recent (last 60 days) drug intake, dental fillings or metal implants (type, number, how long);

- life-styles in terms of alcohol consumption (type, quantity, frequency), smoke (type, quantity, frequency), exercise (type, frequency), traffic at home and at work (type, intensity), home/work distance from potential industrial areas and type of industrial area;
- diet in terms of type (normal, vegetarian, etc.), consumption of fish (weekly amount), consumption of milk and dairy products;
- regular use of hormonal supplementation, contraceptive drugs, mineral supplementation.

Analytes: twenty metals – Sb, As, Be, Cd, Cr, Co, Ir, Pb, Mn, Hg, Mo, Ni, Pd, Pt, Rh, Tl, Sn, W, U and V – were measured in blood and serum of the PROBE participants. The selection of metals was based on a compromise among different requirements:
– gravity of known or suspected health effects subsequent to the environmental exposure to the metal;

- –need to assess the effectiveness of public health actions to reduce exposure to a metal;
- –availability of adequate sample amounts;
- –availability of a multi-elemental analytical technique with adequate accuracy, precision, sensitivity, specificity, and throughput.

Analysis: method based on Sector Field Inductively Coupled Plasma Mass Spectrometry (SF-ICP-MS). The method used for serum analysis was validated by ACCREDIA (the Italian National Accreditation Body) and the following validation performances were assessed: linearity; Limit of Detection (LoD) and Limit of Quantification (LoQ); specificity; accuracy (precision and trueness); and robustness (AOAC, 1998; Commission Decision 2002/657/EC; LGC, 2003; NATA, 2009; Thompson et al., 2002).

Results

PROBE provided the Reference Values for metals of the urban Italian population; the higher percentiles (75^{th}, 90^{th}, 95^{th}) provided for each metal, i.e., the upper limits of derived RVs, are helpful for determining whether levels observed in separate public health investigations or other studies are unusual. The GM is preferred because takes into account all values measured and represents the "ideal" measure of central tendency in the event of logarithmic normal distribution, as usual for metals concentration in blood. Table 13 reports, as an example, the RVs for whole blood for the entire population.

Table 13: Reference values (µg/L) for metals in Blood

	N	P5	P50	P75	P90	P95	AM	GM	CI GM
Antimony	1423	<LoD	0.32	0.44	0.61	0.72	0.37	0.31	0.30–0.32
Arsenic	1423	0.28	1.16	2.01	3.70	5.32	1.70	1.14	1.09–1.20
Beryllium	1423	<LoD	0.090	0.120	0.156	0.184	0.097	0.085	0.083–0.087
Cadmium	1423	0.23	0.52	0.76	1.11	1.42	0.63	0.53	0.51–0.55
Chromium	1423	0.06	0.23	0.41	0.75	1.09	0.38	0.24	0.23–0.25
Cobalt	1423	0.055	0.138	0.225	0.338	0.443	0.192	0.147	0.142–0.152
Iridium	1423	<LoD	9.09	12.5	17.0	20.4	10.2	9.02	8.78–9.26

Lead	1423	7.38	20.2	30.9	43.4	51.7	24.0	19.9	19.2–20.5
Manganese	1423	4.41	8.30	10.4	12.8	14.5	8.74	8.19	8.04–8.35
Mercury	1423	0.35	1.15	1.95	3.40	5.16	1.68	1.19	1.15–1.25
Molybdenum	1423	0.69	1.22	1.52	1.79	2.05	1.28	1.21	1.19–1.23
Nickel	1422	<LoD	0.90	1.38	1.95	2.62	1.23	0.89	0.86–0.92
Palladium	1423	<LoD	25.2	32.2	40.7	47.6	26.9	24.0	23.4–24.6
Platinum	1423	5.28	15.1	20.6	27.3	31.6	16.4	14.1	13.6–14.5
Rhodium	1423	<LoD	18.9	24.0	28.8	32.2	19.6	18.0	17.6–18.4
Tallium	1423	0.018	0.034	0.048	0.074	0.098	0.045	0.037	0.035–0.038
Tin	1423	0.124	0.501	0.975	1.754	2.250	1.261	0.539	0.512–0.568
Tungsten	1423	0.011	0.027	0.039	0.059	0.075	0.038	0.028	0.027–0.029
Uranium	1423	1.84	5.92	8.25	11.5	14.0	6.76	5.65	5.47–5.84
Vanadium	1423	0.027	0.067	0.092	0.120	0.146	0.076	0.064	0.062–0.066

Figure 21 shows the metal level in blood as a function of age (three age categories).

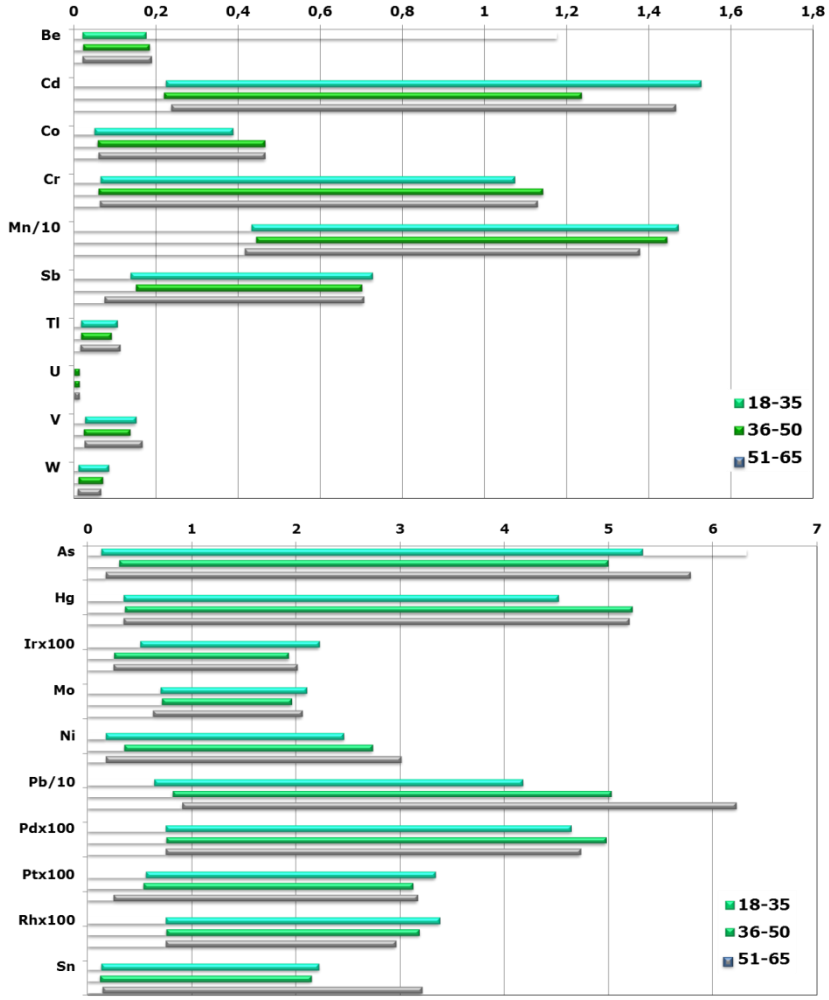


Figure 21: metal level in blood as a function of age

Linking biomonitoring data to exposure

As for other similar studies the main drawback of HBM results is in the association of the internal dose with the external one, i.e., with the environmental level of contamination and, thus, with the identified source. This aspect is correctly tackled in a modelling framework, as described in the initial chapters of the report.

Critical data for the completion of the case study

Filling the gap

PROBE was a classical HBM campaign devoted to obtain RVs for general population. The combination of these RVs with exposure data or particular health outcomes were not among the primary aims of the project. However, during the CROME-LIFE activities, individual georeferencing as well as environmental exposure at the time of blood collection will be implemented in order to obtain a broader meaning of the HBM data in terms of association between external and internal doses. Reanalysis of data sets taking into account life styles and dietary habits could be also performed in the CROME framework.

Tentatively, a follow-up on the general health status of the subjects included in PROBE could be carried out with the final aim to use old and new data to supply the models described in initial chapters of this report. Results of this process will allow the assessment of external and internal exposure as well as the potential health effects to selected metals.

Case study Spain - Human Biomonitoring in Spain

Introduction

The Spanish case study will address the transfer of persistent organic pollutants and metals between mothers and newborns during pregnancy. Four cohorts are considered for study in the Spanish context: Menorca, Sabadell, Valencia, Gipuzkoa and Asturias (Figure 22). In these cohorts data on organochlorine and organobromine compounds such as polychlorobiphenyls (PCBs), hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs), DDT, DDE and polybromodiphenyl ethers (PBDE) in cord blood serum is available (Table 9). In addition, Measurements of these compounds in maternal venous blood serum is also available (Asturias and Valencia, Table 9). These compounds were also measured in venous blood serum of children at 4 year age (Menorca), in placenta (Asturias) and in colostrum (Gipuzkoa and Sabadell) (Table 9). Furthermore, Co, Ni, Cu, Zn, Se, As, Mo, Cd, Sb, Cs, Th, Pb data on maternal urine collected in the first and third trimesters of pregnancy is available from the Sabadell cohort (Table 9).

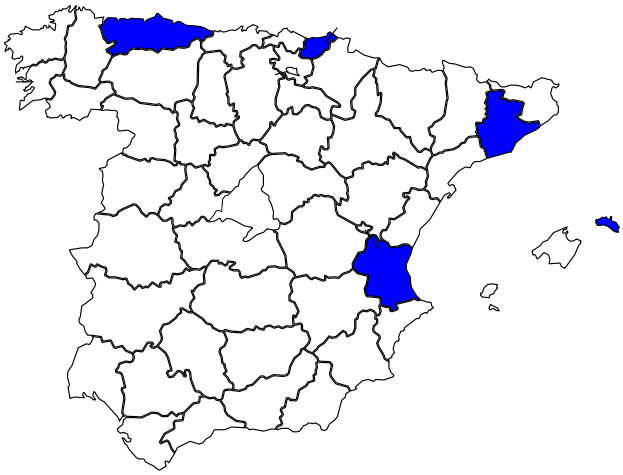


Figure 22: Map showing the geographical location of the cohorts under study in Spain

Proposed methodological scheme

The main target addressed for this data collection is the examination of the pollution load received by children from their mothers during the fetal period. The calculation of this transfer requires an estimation of the pollutant load stored into the mothers and then the identification of the transfer processes.

Critical data for the completion of the case study

Filling the gaps

Metal studies

Concerning metals, one first approach requires the study of metals that are essential for life, e.g. zinc (Zn), copper (Cu) and iron (Fe) and those which are toxic, even at low concentrations, e.g. mercury (Hg), lead (Pb), arsenic (As), thallium (Th), chromium (Cr) or cadmium (Cd). Given the increasing use of these toxic metals in new technologies and the

increasing inputs of them from road traffic and other sources, there is growing concern over the public health implications of continued exposure to them (Järup 2003; Lauwerys and Lison 1994; Rodriguez and Diaz 1995; Schulz et al. 2007; Wells et al. 2011; Zubero et al. 2010).

The study of metal concentrations in humans is of high interest because of the essential metabolic functionality of some of them and the toxic properties of others. Moreover, exposure to metals at the onset of life, both in the fetal period and during the first years, can be associated with negative health effects in later stages (Vahter 2008). Accordingly, assessing the exposure to a large number of metals, particularly in the earliest stages of life, may provide the knowledge necessary for identifying public health problems and implementing prevention policies early on.

Mothers constitute a source of heavy metals for their infants during pregnancy and lactation. However, only a few studies on prenatal exposure to trace metals have been published, most of which focused on a small number of these elements (Messiha et al., 1988; Vahter et al., 2006; Wright and Baccarelli 2007; Al-Saleh et al. 2011; Kippler et al. 2009; Shirai et al. 2010). In some cases, animal models have been used to assess the prenatal effects of these pollutants (Liu et al. 2009; Tokar et al. 2010). However, specific measurements at the individual level can enable a better understanding of the possible influence of exposure to metals on health. Such measurements also facilitate the identification of sources and routes of metal contamination at both individual and the general population levels. These aspects are even more important when dealing with prenatal exposure.

Unfortunately, there are no generally accepted methods for physiologically assessing exposure to metals. Urine is the preferred source of information for heavy metals biomonitoring, can be collected without invasive methods and has been widely used in large environmental studies such as the German Environmental Survey for Children (GerES) and the National Health and Nutrition Examination (NHANES) (Esteban and Castaño 2009). However, one basic requirement for biomonitoring of metals with urine analysis concerns the reproducibility of different urine measurements from each individual in samples collected at different time periods. This aspect is particularly significant for women during pregnancy in which children are in-utero exposed to metals and other compounds by maternal transmission. Thus, analysis of metals in urine collected at different pregnancy periods may provide useful information for assessment of the efficacy of the use of urine samples as exposure markers.

Therefore, we plan to develop and apply an analytical method using acid digestion prior to analysis by inductively coupled plasma quadruple mass spectrometry (Q-ICP-MS) for the simultaneous analysis of the above mentioned metals in maternal urine and to determine their concentrations in urine samples of pregnant women living in a highgly industrialized urban town (Sabadell), which were collected during their first and third trimester of pregnancy. This approach affords an assessment of the steadiness of the concentrations of these metals in urine during the pregnancy period and their usefulness for epidemiological studies, maternal and prenatal exposure estimates.

Specific metal studies

Specific attention will be devoted to cobalt. This is a transition metal of widespread environmental occurrence. Usually it is found in association with copper or nickel (Barceloux, 1999) and is a minor component in a huge amount of minerals (Kim et al., 2006). It has been used for different applications such as pigments, catalysts in oil and gas production, battery electrodes, orthopedic prostheses and others (NHANES, 2009). Human exposure to this metal

depends on diet. Its main sources are fish, green vegetables and fresh cereals (Unice et al., 2012). Cobalt is an essential trace metal used in the formation of vitamin B12 (also named cobalamin) although only a small fraction of human cobalt intake is used for this purpose (Kim et al., 2006).

Occupational and accidental exposures to cobalt have been reported to originate asthma and respiratory problems (Nemery et al., 1992; Swennen et al., 1993), alterations of thyroid hormones (Prescott et al., 1992) and other effects.

The maternal concentrations of metals, including cobalt may change along pregnancy which may also be related to variations in fetal exposure (Figure 23). Measurements of trace metal changes along pregnancy have been considered in some cases but these studies did not include cobalt. Iron depletion is one of the most relevant changes during pregnancy (Goonewardene et al., 2012) which has been reported to lead to possible intestinal absorption increases of heavy metals such as cobalt, manganese, zinc, cadmium and lead (Flanagan et al., 1980).

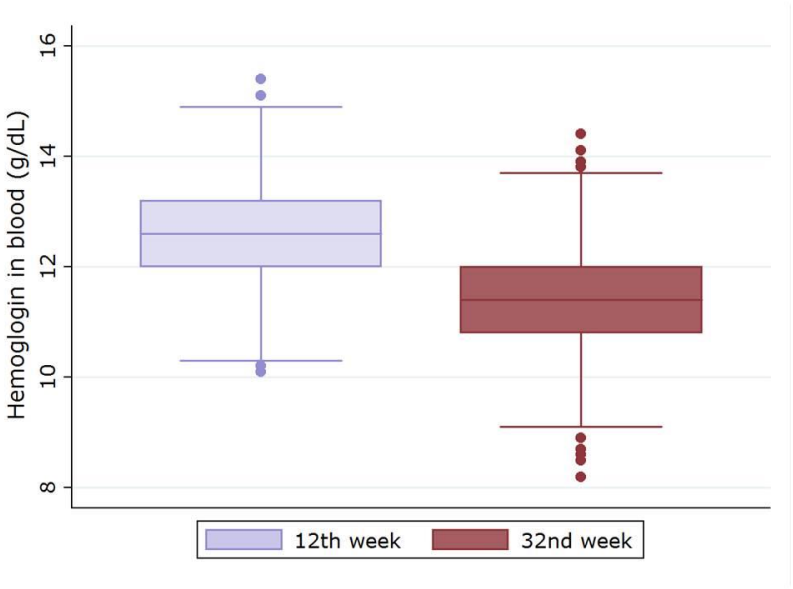


Figure 23: Preliminary data showing the Co concentration differences in urine of pregnant women collected in the first and third trimesters

We will compare the levels of cobalt in urine of pregnant women in the first and third trimester of pregnancy to assess of the possible relationships of iron depletion with the observed changes.

Arsenic is also an element in which specific attention will be devoted. It is a metalloid widely present in the earth crush, occurring in trace quantities in rocks, soils, air and water. It can be found in inorganic forms such as arsenite (As(III)) and arsenate (As(V)) and their methylated species (monomethylarsenic and dimethylarsenic acids – MMA and DMA-, respectively) and is also a constituent of organic compounds such as arsenobetaine, arsenocholine, dimethylarsinylethanol, trimethylarsoniumlactate, arsenic-containing sugars or phospholipids, which are widely present in marine animals (Edmonds & Francesconi 1993). In addition to the natural sources, some anthropogenic activities can increase arsenic levels in the environment, such as mining, smelting of non-ferrous metals or burning of fossil fuels. Arsenic was also present in some pesticides used in the past (Abernathy et al. 2001).

Humans are exposed to arsenic by consumption of food and water (Abernathy et al. 2001). In some areas drinking water is an important source of exposure to inorganic arsenic (Vahter et al., 2006). Nevertheless, food is generally the main contributor to the total arsenic daily intake. Rice may be one of the most important dietary sources. This cereal has higher efficiency for the assimilation of this metalloid from soils than other crops (Williams et al., 2007). Accordingly, areas with high arsenic in the water incorporate this metalloid by consumption of this cereal, e.g. Bangladesh (Vahter et al. 2006). On the other hand, seafood is one of the most important sources of organic arsenic. In some populations the dietary load of this metalloid depends on the amount and kind of seafood consumption (Meltzer et al. 1994).
Exposure to arsenic has been found to be associated with diverse health effects such as skin lesions (Argos et al., 2011) and immunotoxicological (Ahmed et al., 2011; Andrew et al., 2008), cardiovascular (Chen et al., 2011) and endocrine disorders (Chen et al., 2007). Arsenic is carcinogenic (non-threshold, class I human carcinogen; ATSDR 2007). It has been associated to skin, lung, bladder and liver cancer (International Agency for Cancer Research, 2004) and can cross the placental barrier (Concha et al., 1998). Prenatal exposure to arsenic from drinking water has been associated with reduction of birth weight in highly exposed populations (Rahman et al. 2009), as well as neurodevelopmental defects (Hamadani et al., 2011; Parajuli et al., 2013). Increased blood pressure and anemia during pregnancy has also been related to arsenic exposure (Hopenhayn et al. 2006, Kwok et al. 2007).

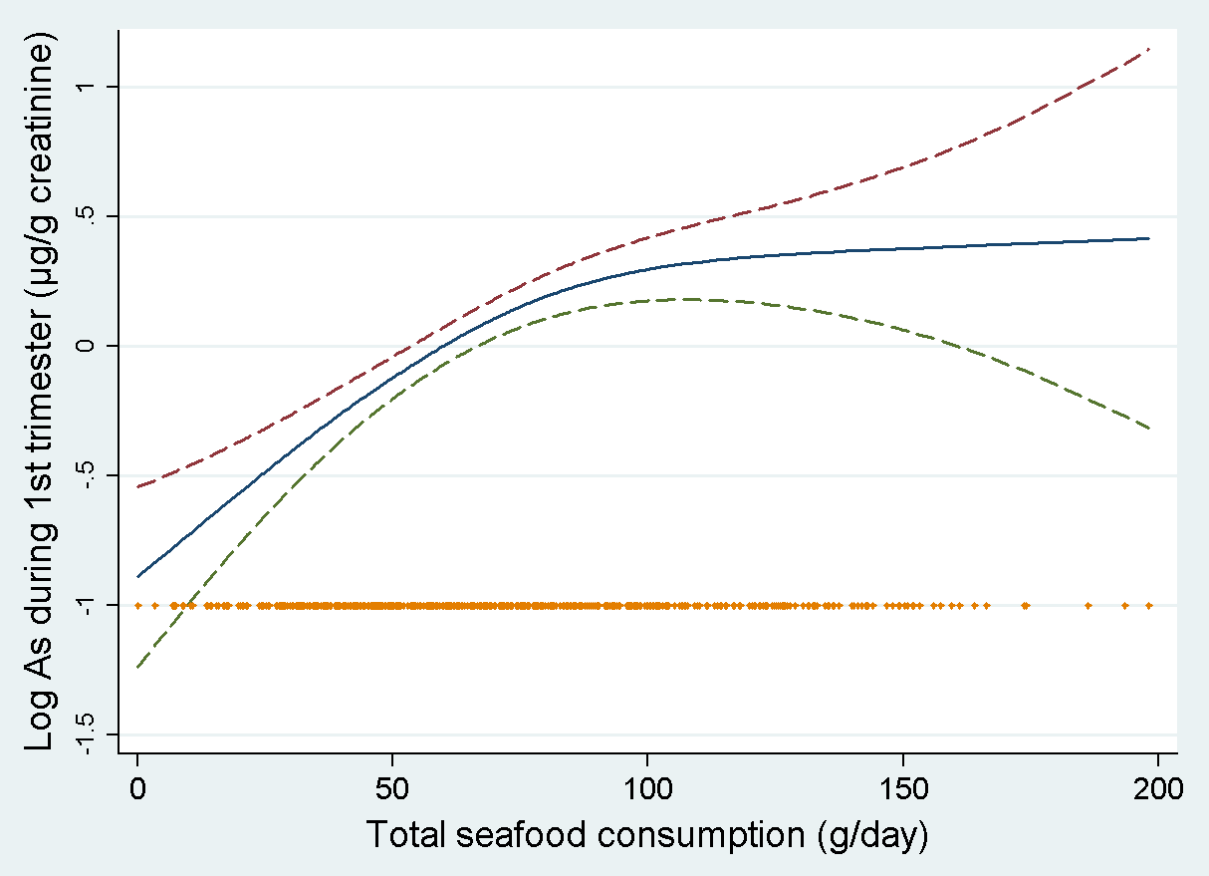


Figure 24: Preliminary results of the dependence between arsenic excretion and fish consumption in pregnant women

Biomethylation is the most important way for detoxification of inorganic arsenic, MMA and DMA are less harmful and rapidly eliminated by urine. During pregnancy and lactation there is increased methylation, which may be a way to protect the fetus from high exposure to toxic arsenic. Nevertheless, during the first stages of pregnancy, when these processes are not fully enhanced and fetal susceptibility to possible harm is higher, prenatal exposure may be more important and may induce changes that could become apparent much later in life (Vahter 2009).

Arsenic in seafood is mostly present in the form of non-toxic organic compounds but seafood, especially shellfish, also contains small amounts of inorganic arsenic (Borak &Hosgood 2007). The arsenobetaine levels increase in humans after seafood intake but this compound is rapidly excreted. Populations with high seafood consumption, such as those in the Mediterranean area, have high arsenic urinary levels. Accordingly, it is important to confirm this origin in studies of urinary arsenic in human population, especially when prenatal exposure is considered. Preliminary results for urinary arsenic excretion and fish consumption in the Sabadell population of pregnant women are shown in Figure 24.

Total urinary arsenic will be determined in the two different stages of pregnancy of Sabadell pregnant women. The influence of diet, with special emphasis in seafood consumption, on the observed concentrations of arsenic in urine will be evaluated. Differences in exposure or excretion that eventually could have effects on prenatal exposure or future children development will be assessed by urinary arsenic during both trimesters.

Antimony is a toxic metal which was used in the past against parasite diseases. Nowadays, it has essentially industrial uses, e.g. it is used in semiconductors, infrared detectors and diodes among other applications (Cooper and Harrison 2009; Sundar and Chakravarty 2010) because of its flame retardant properties. It is also used in brake linings, as Sb_2S_3, after elimination of asbestos, in the late 1990 (Garg et al., 2000; Wahlin et al., 2006). This metalloid is scarcely found in diet so that human intake is essentially related to environmental processes. Long occupational exposure to Sb has been associated with neumococosis forms (Cooper and Harrison 2009). Studies concerning human exposure to environmental Sb and effects are scarce. Very limited information is also available on prenatal and children exposure, although its presence in amniotic fluid has been observed (Caserta et al. 2011).

Copper is an essential metal. It is necessary for the function of some enzymes such as ceruloplasmine or cytocrom c oxydase. Diet is the main source in humans of this metal, being present in a wide variety of foods (Mason 1979). Besides diet and the gastrointestinal system, Cu may also be incorporated through respiration because it is found in atmospheric particulate matter. Industrial activity is the main source of this metal to the environment but vehicular traffic is also important because at present it is used in brake linings (Amato et al. 2009). The population living in sites with high traffic density may be significantly exposed to atmospheric Cu. Deficiencies of this metal are not common but if they occur during pregnancy they can be harmful for the fetal development. At the same time, children are more susceptible to the toxicity of this metal which is a known toxic at high concentrations (Mason 1979).

The concentrations of Cu and Sb in the pregnant women from the Sabadell cohort is shown in Figure 25.

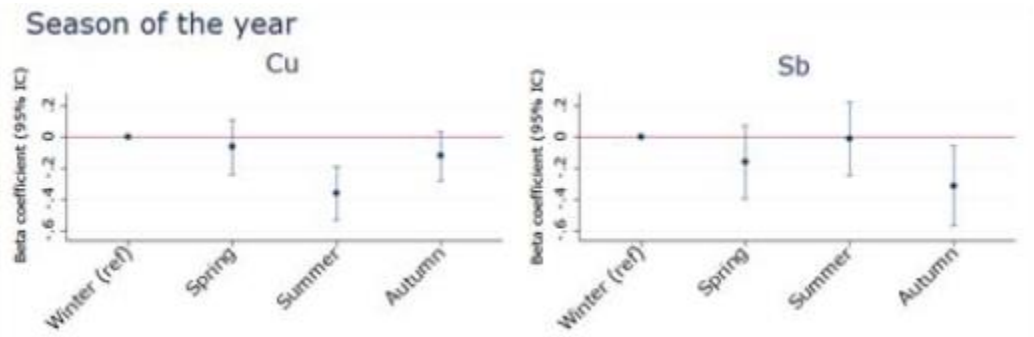


Figure 25: Seasonal distribution of Cu and Sb in the urine of pregnant women from Sabadell.

Atmospheric particles emitted by vehicular activity constitute one of the main pollutant sources in urban areas (Moreno et al., 2006) and health outcomes, e.g. oxidative stress, inflammation, thrombotic complications of atherosclerosis which can eventually be related with cardiovascular or cerebrovascular stroke, epoc and others (Pope and Dockery, 2006; Perez et al., 2009). Studies on the influence of traffic pollution in the composition of atmospheric particles of urban areas are available, e.g. Querol et al (2007) but they rarely consider human exposure to these atmospheric pollutants. Exposure to Sb and Cu is now an important issue due to the changes in brake technology that occurred about 15 years ago. Urine is an adequate matrix to study body burden of these metals since it is easily accessible and non-invasive. The concentrations of these metals in urine are representative of circulating levels in human organism. The study of urine from pregnant women may provide a tool for assessment of the exposure of newborns to these metals during fetal growth.

Organohalogen studies.

Maternal factors influencing on the body burden of organohalogen compounds in newborns: The gestational weight gain.

Gestational Weight Gain (GWG) is itself a potential risk factor influencing the growth and health of the fetus and later outcomes during childhood and adulthood (Viswanathan et al. 2009). Inadequate GWG has been associated with low birth weight and preterm birth (Han et al. 2011) whereas excessive GWG to children obesity (Oken et al. 2007). GWG might be related to pre-pregnancy BMI (Dietz et al. 2006). In view of the increasing prevalence of overweight and obesity among childbearing women, the Institute of Medicine (IOM) provided specific recommendations for GWG. Moreover, it stated the need for research on possible relationships between environmental exposures and GWG (IOM, 2009). Associations between prenatal POP exposure and birth weight (Govarts et al. 2012; Lopez-Espinosa et al. 2011), early obesity (Valvi et al. 2012) and preterm (Bergonzi et al. 2011) have been reported. Given these findings, further knowledge is needed to assess the importance of GWG on newborn POP concentrations.

POPs are able to cross the placenta during pregnancy (Sala et al. 2001). Thus, children already come to life with an initial body burden of these compounds that depends partly on anthropometric and sociodemographic maternal characteristics such as age, pre-pregnancy BMI, GWG, educational level and social class, obstetric and lactational history and diet (Glynn et al. 2007; Sarcinelli et al. 2003; Wolff et al. 2005; Vizcaino et al., 2010).

Modifications of serum POP levels have been related to weight changes in general population (Lim et al. 2011; Wolff et al. 2005a) or in obese individuals (Chevrier et al. 2000). Weight losses increase circulating concentrations of these compounds because they are mainly stored

in adipose tissue and released during lipid mobilization. In contrast, weight gain tends to dilute POP levels in serum. Very little is known on the GWG influence on the fetal POP concentrations despite of the substantial weight changes during pregnancy. In this respect, one study in newborns from Baltimore (n = 297; Herbstman et al., 2007) did not show concluding associations between GWG and cord serum PBDEs or PCBs levels. PCBs were also considered in a similar study of mothers from Lake Ontario (n = 193; Stewart et al., 2000) and the results were not concluding. None of these previous studies considered the IOM GWG recommendations or investigated potential modifier effects of pre-pregnancy BMI on GWG in a wide range of POPs.

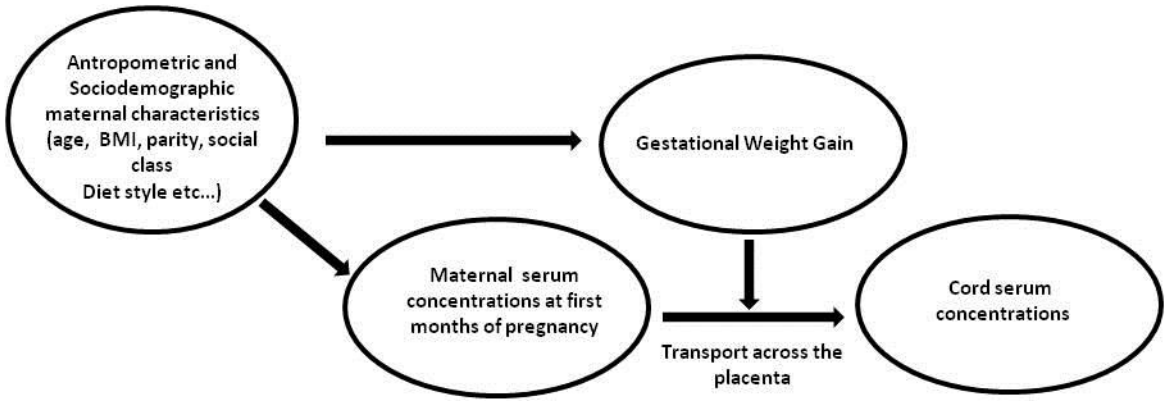
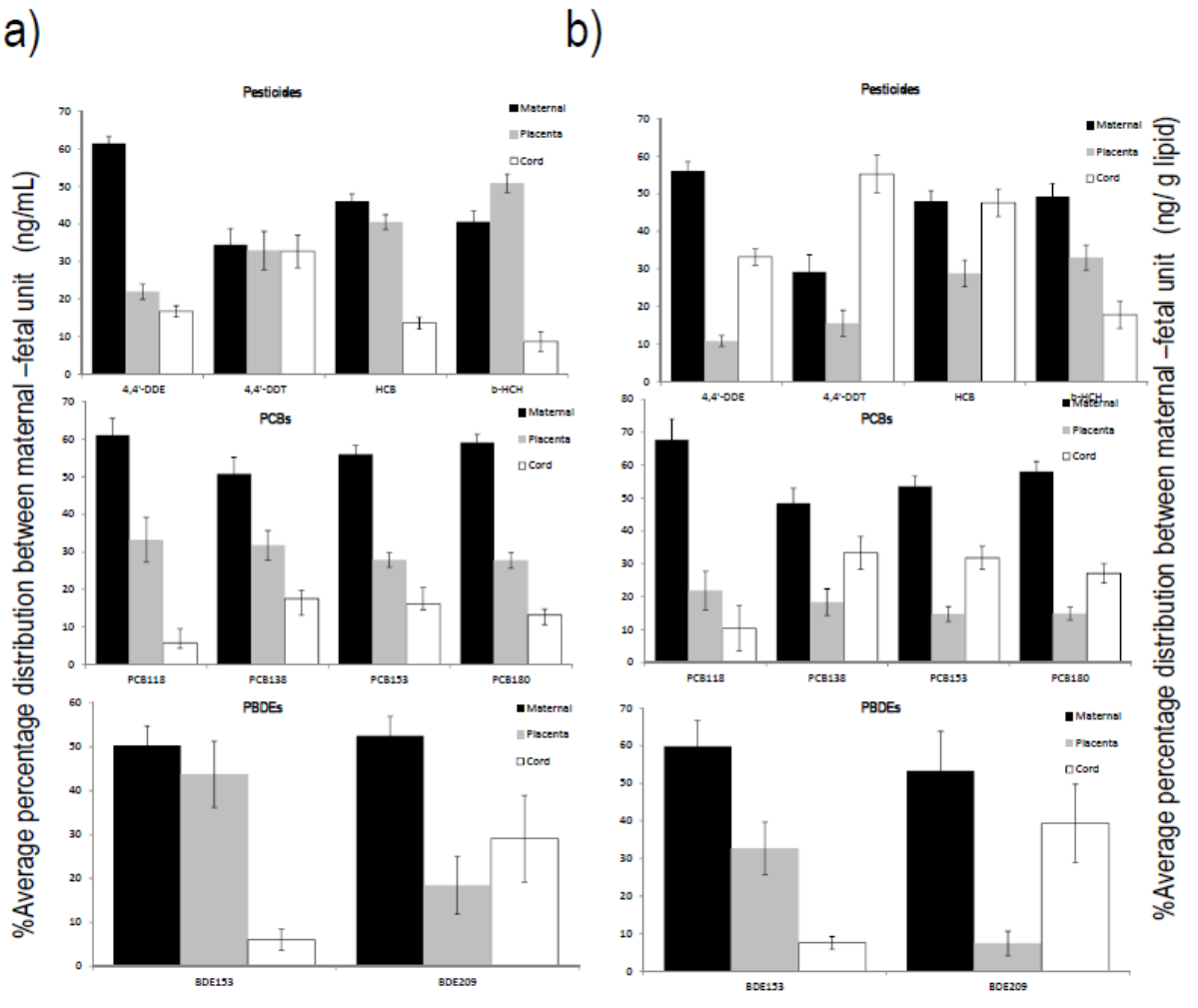


Figure 26: Conceptual hypothesis

In our study the significance of GWG adjusting by other potential determinants of POP concentrations in newborns will be investigated in the context of the IOM guidelines (Figure 26).

Transfer of POPs through placenta

Human exposure to Persistent Organic Pollutants (POPs) begins in the uterine life period by transplacental transfer (Rogan et al. 1986). Placenta may prevent transfer of some pollutants but there is evidence that POPs, even those of high molecular weight can reach the fetuses (Vizcaino et al. 2011). Transfer of contaminants during pregnancy may have implications for fetus health. Fetuses are more vulnerable than adults to chemical exposure as their immune system and detoxification mechanisms are not fully developed. In-utero exposure may lead to severe repercussions for newborns and may predispose to late adult deleterious effects (Boekelheide et al. 2012). Thus, in utero exposure to POPs, including PBDEs, PCBs and organochlorine pesticides, has shown to increase the risk of adverse development outcomes in children (Gascon et al. 2012; Herbstman et al. 2010; Lopez-Espinosa et al. 2011; Park et al. 2008a; Ribas-Fito et al. 2007; Valvi et al. 2012). These results have increased notably the interest of the scientific community on exposure to these compounds during gestation.



$$\%maternal = \frac{[Cm]}{[Cm + Cuc + Cp]}$$

$$\%cord = \frac{[Cuc]}{[Cm + Cuc + Cp]}$$

$$\%placenta = \frac{[Cp]}{[Cm + Cuc + Cp]}$$

Figure 27: Average percentatge distribution of POPs between maternal serum, placenta and fetal serum expressed as ng/ml (a) and lipid adjusted concentrations (b). Interval bars correspond to 95% confidence interval.

Consequently, the number of studies reporting prenatal concentrations of POPs has increased in the recent years. Examination of this previous work evidences difficulties for comparison since there is a lack of standardization regarding subject selection, timing of sampling and reported levels (Jakobsson et al. 2012). Placenta, maternal and cord serum are the most common matrices to assess prenatal exposure to POPs, notwithstanding the processes of transfer of these pollutants from mother to fetus during pregnancy are still not clear (Barr et al. 2005). Previous studies stated that the distribution in body compartments of chemicals with log Kow >4 is driven solely by lipid fraction in tissues and blood (Haddad et al. 2000). Accordingly, partition ratios between matrices of POPs should be close to 1 when adjusted for lipid content. However, there is small experimental evidence from human studies to evaluate this statement. Some exposure assessment studies have shown good correlations between

mother, placenta and cord serum (Bergonzi et al. 2009) but studies describing the distributions and partition ratios of POPs between placenta, cord and maternal serum in humans are very scarce and limited to a reduced number of subjects (Needham et al. 2011) .
Insight into the transfer of POPs through placenta in a population exposed to baseline levels by examination of maternal and fetal distribution of POPs in mother-child pairs and quantification of the partition ratios between placenta, maternal and cord serum samples will be increased. HCB, β-HCH, 44'-DDT and their principal metabolite 4,4'-DDE, PCBs (PCB 118, 138, 153 and 180) and PBDEs (BDE 28, 47, 99, 153, 154 and 209) will be studied (Figure 27).

Transfer of POPs through breast milk.

Newborn are exposed to POPs during breastfeeding. The high lipophilicity of these compounds makes breast milk an important vehicle for pollutant transmission. Carrizo et al., (2007) reported that children who breastfed showed higher concentrations of organochlorine compounds at age 4 than children who did not. Children in its first stages of life are more susceptible to possible harm produced by these compounds, possibly at lower concentrations than adults, due to the fact that their organism is still forming and their detoxification systems are not still totally developed. Recent studies have found an association between high exposure to DDT and slight neurodevelopmental delay (Ribas-Fito et al., 2003; Eskenazi et al., 2006), higher incidence of asthma in children with higher concentrations of 4,4'-DDE (Sunyer et al., 2006), or higher IgE (therefore higher sensibility to allergy) in newborns who had been exposed to high concentrations of HCB in placenta (Reichrtrak et al., 1999).
According to most studies comparing breast milk and maternal serum, breast milk levels of organochlorine pollutants reflect concentrations in serum and adipose tissue (Aylward et al., 2003). Few studies have focused on the relationship between concentrations in both matrices, and they usually deal with a short number of subjects. In our study it will be investigated whether organochlorine compounds are equally transferred to colostrum or they depend on their physical chemical properties to be more or less present in colostrum than in serum. Determination of stable milk:serum partition coefficients may allow to predicting newborn intake (Lakind et al., 2006).

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Annex 1 – Generic PBTK modelling formulation and parameterization

For non-eliminating organs:

Red blood cells

$$V_{rbc\_org}\frac{dC_{rbc\_org}}{dt} = Q_{org}\cdot HCT\cdot\left(C_{rbc\_art} - C_{rbc\_org}\right) + PS_{rbc\_org}\cdot f_u\cdot\left(C_{int\_org} - \frac{C_{rbc\_org}}{K_{rbc}}\right)$$

Where $V_{rbc\_org} = f_{vas\_org}\cdot V_{org}\cdot HCT$

Plasma +interstitial

$$V_{int\_org}\frac{dC_{int\_org}}{dt} = Q_{org}\cdot(1-HCT)\cdot\left(C_{pls\_art} - C_{int\_org}\right) - PS_{rbc\_org}\cdot f_u\cdot\left(C_{int\_org} - \frac{C_{rbc\_org}}{K_{rbc}}\right) - PS_{cell\_org}\cdot f_u\cdot\left(C_{int\_org} - \frac{C_{cell\_org}}{K_{org}}\right)$$

Where $V_{int\_org} = V_{org}\cdot\left[f_{int\_org} + f_{vas\_org}\cdot(1-HCT)\right]$

Cellular

$$V_{cell\_org}\frac{dC_{cell\_org}}{dt} = PS_{cell\_org}\cdot f_u\cdot\left(C_{int\_org} - \frac{C_{cell\_org}}{K_{org}}\right)$$

Where $V_{cell\_org} = f_{cell\_org}\cdot V_{org}$

Kidney

Plasma + Interstitial

$$V_{int\_kid}\frac{dC_{int\_kid}}{dt} = Q_{kid}\cdot(1-HCT)\cdot\left(C_{pls\_art} - C_{int\_kid}\right) - PS_{rbc\_kid}\cdot f_u\cdot\left(C_{int\_kid} - \frac{C_{rbc\_liv}}{K_{rbc}}\right) - PS_{cell\_kid}\cdot f_u\cdot\left(C_{int\_kid} - \frac{C_{cell\_kid}}{K_{kid}}\right) - \frac{CL_{pls\_kid}\cdot Q_{kid}\cdot(1-HCT)\cdot C_{int\_kid}}{\left[Q_{kid}\cdot(1-HCT) - CL_{pls\_kid}\right]}$$

Portal vein

Red blood cells

$$HCT \cdot V_{pv} \frac{dC_{rbc\_pv}}{dt} = Q_{GI\_tract} \cdot HCT \cdot \left(C_{rbc\_GI\_tract} - C_{rbc\_pv}\right) + PS_{rbc\_pv} \cdot f_u \cdot \left(C_{pls\_pv} - \frac{C_{rbc\_org}}{K_{rbc}}\right)$$

Plasma + interstitial

$$\left(1-HCT\right) \cdot V_{pv} \cdot \frac{dC_{pls\_pv}}{dt} = Q_{GI\_tract} \cdot \left(1-HCT\right) \cdot \left(C_{\text{int}\_GI\_tract} - C_{pv}\right) - PS_{rbc\_pv} \cdot f_u \cdot \left(C_{pls\_pv} - \frac{C_{rbc\_pv}}{K_{rbc}}\right)$$

For venous blood

Red blood cells

$$HCT \cdot V_{ven} \cdot \frac{dC_{rbc\_ven}}{dt} = \sum_{org} Q_{org} \cdot HCT \cdot C_{rbc\_org} + PS_{rbc\_ven} \cdot f_u \cdot \left(C_{pls\_ven} - \frac{C_{rbc\_ven}}{K_{rbc}}\right) - Q_{lung} \cdot HCT \cdot C_{rbc\_ven}$$

Plasma +interstitial

$$\left(1-HCT\right) \cdot V_{ven} \frac{dC_{pls\_ven}}{dt} = \sum_{org} Q_{org} \cdot \left(1-HCT\right) \cdot C_{\text{int}\_org} - PS_{rbc\_ven} \cdot f_u \cdot \left(C_{pls\_ven} - \frac{C_{rbc\_ven}}{K_{rbc}}\right) - Q_{lung} \cdot \left(1-HCT\right) \cdot C_{pls\_ven}$$

For arterial blood

Red blood cells

$$HCT \cdot V_{art} \cdot \frac{dC_{rbc\_art}}{dt} = -\sum_{org} Q_{org} \cdot HCT \cdot C_{rbc\_art} + PS_{rbc\_art} \cdot f_u \cdot \left(C_{pls\_art} - \frac{C_{rbc\_art}}{K_{rbc}}\right) + Q_{lung} \cdot HCT \cdot C_{rbc\_art}$$

Plasma +interstitial

$$(1-HCT)\cdot V_{art}\frac{dC_{pls\_art}}{dt}=-\sum_{org}Q_{org}\cdot(1-HCT)\cdot C_{\text{int\_art}}$$
$$-PS_{rbc\_art}\cdot f_u\cdot\left(C_{pls\_art}-\frac{C_{rbc\_art}}{K_{rbc}}\right)$$
$$+Q_{lung}\cdot(1-HCT)\cdot C_{pls\_art}$$

For liver:

Red blood cells

$$V_{rbc\_liv}\frac{dC_{rbc\_liv}}{dt}=HCT\cdot\left(Q_{liv}\cdot C_{rbc\_art}+Q_{pv}\cdot C_{rbc\_pv}\right)$$
$$-\left(Q_{liv}\cdot C_{rbc\_art}+Q_{pv}\cdot C_{rbc\_pv}\right)$$
$$+PS_{rbc\_org}\cdot f_u\cdot\left(C_{\text{int\_org}}-\frac{C_{rbc\_org}}{K_{rbc}}\right)$$

Plasma + Interstitial

$$V_{\text{int\_liv}}\frac{dC_{\text{int\_liv}}}{dt}=(1-HCT)\cdot\left(Q_{liv}\cdot C_{pls\_art}+Q_{pv}\cdot C_{pls\_pv}\right)$$
$$-\left(Q_{liv}+Q_{pv}\right)\cdot(1-HCT)\cdot C_{\text{int\_liv}}$$
$$-PS_{rbc\_liv}\cdot f_u\cdot\left(C_{\text{int\_liv}}-\frac{C_{rbc\_liv}}{K_{rbc}}\right)$$
$$-PS_{cell\_liv}\cdot f_u\cdot\left(C_{\text{int\_liv}}-\frac{C_{cell\_liv}}{K_{liv}}\right)$$

Cellular

$$V_{cell\_liv}\frac{dC_{cell\_liv}}{dt}=PS_{cell\_liv}\cdot f_u\cdot\left(C_{\text{int\_liv}}-\frac{C_{cell\_liv}}{K_{liv}}\right)$$
$$-\frac{CL_{\text{int\_liv}}\cdot C_{cell\_liv}\cdot f_u}{K_{liv}}$$

where $CL_{\text{int\_liv}}$ is the intrinsic clearance of the chemical considered

For lungs:

$$V_{\text{int\_lung}} \cdot \frac{dC_{\text{int\_lung}}}{dt} = Q_{lung} \cdot (1 - HCT) \cdot \left(C_{pls\_ven} - C_{\text{int\_lung}}\right)$$
$$- PS_{rbc\_lung} \cdot f_u \cdot \left(C_{\text{int\_lung}} - \frac{C_{rbc\_lung}}{K_{rbc}}\right)$$
$$- PS_{cell\_lung} \cdot f_u \cdot \left(C_{\text{int\_lung}} - \frac{C_{cell\_lung}}{K_{rbc}}\right)$$
$$+ Q_{vent} \cdot C_{amb\_air} \cdot P_{air}$$
$$- Q_{vent} \cdot \left(\frac{C_{\text{int\_lung}}}{P_{air}} \cdot (1 - V_{ds}) + C_{amb\_air} \cdot V_{ds}\right)$$

For uterus:

Plasma +interstitial

$$V_{\text{int\_uterus}} \frac{dC_{\text{int\_uterus}}}{dt} = Q_{uterus} \cdot (1 - HCT) \cdot \left(C_{pls\_art} - C_{\text{int\_uterus}}\right)$$
$$- K_{d\_uterus\_pla} \cdot \left(C_{placenta} - C_{uterus\_M}\right)$$
$$- PS_{rbc\_uterus} \cdot f_u \cdot \left(C_{\text{int\_uterus}} - \frac{C_{rbc\_uterus}}{K_{rbc}}\right)$$
$$- PS_{cell\_uterus} \cdot f_u \cdot \left(C_{\text{int\_uterus}} - \frac{C_{cell\_uterus}}{K_{rbc}}\right)$$

beside the assumption of equal diffusion flow from uterus to placenta and vice-versa during pregnancy, uterus behaves like other organs.

For placenta:

$$V_{\text{int\_placenta}} \frac{dC_{\text{int\_placenta}}}{dt} = K_{d\_uterus\_pla} \cdot \left(C_{\text{int\_placenta}} - C_{\text{int\_uterus}}\right)$$
$$- K_{d\_pla\_amniot} \cdot \left(C_{\text{int\_placenta}} - C_{amniot} \frac{P_{placenta}}{P_{amniot}}\right)$$
$$+ Q_{placenta\_fetus} \cdot \left(C_{\text{int\_art\_fetus}} - \frac{C_{\text{int\_placenta}}}{P_{placenta}}\right)$$
$$+ K_{glu\_deconj} \cdot Q_{placenta\_fetus} \cdot C_{\text{int\_placenta\_glu}}$$
$$- PS_{rbc\_placenta} \cdot f_u \cdot \left(C_{\text{int\_placenta}} - \frac{C_{rbc\_placenta}}{K_{rbc}}\right)$$
$$- PS_{cell\_placenta} \cdot f_u \cdot \left(C_{\text{int\_placenta}} - \frac{C_{cell\_placenta}}{K_{rbc}}\right)$$

For breast:

$$V\frac{dC_{\_breast}}{dt} = PS_{\_cell\_breast}\cdot f_u\cdot\left(C_{\_int\_breast} - \frac{C_{\_breast}}{K_{\_breast}}\right) - L_{excr}$$

and the related excretion via lactation

$$L_{excr} = Q_{\_milk}\cdot\frac{C_{\_breast}}{K_{\_breast}}\cdot P_{\_milk/blood}$$

$$P_{\_milk/blood} = \frac{K_{ow}\cdot Fl_{\_tissue} + Fw_{\_tissue}}{K_{ow}\cdot Fl_{\_blood} + Fw_{\_blood}}$$

Lifetime scaling

The parameters related to organ volumes (***V***) and blood flows (***Q***) were taken from the ICRP (ICPR 2002) report and fitted to time (***t in hours***) in order to derive continuous time depended non lineal polynomial formulas in the form of:

$V = a\cdot t^b + c\cdot t^d + e\cdot t + f$ for organ volumes

$Q = a\cdot t^b + c\cdot t + d$ for organ flows

Table 14. Regression coefficients for lifetime scaling (from conception to adulthood)

	organ volumes (mL)						**organ flows (mL/min)**			
	a	***b***	***c***	***d***	***e***	***f***	***a***	***b***	***c***	***d***
Portal vein	1.00E-01	1.00E+00	9.80E-02	9.96E-01	0.00E+00	5.70E+01	6.09E-02	-2.61E-02	1.06E+00	1.14E+02
Adipose	2.54E-02	1.00E+00	1.88E+01	5.20E-01	0.00E+00	9.06E+02	1.17E-01	1.00E-01	1.01E+00	3.00E+01
Bones	5.97E-02	1.00E+00	1.26E+00	6.10E-01	0.00E+00	4.52E+02	2.00E-02	1.04E-02	1.05E+00	3.00E+01
Brain	-5.03E-02	1.00E+00	9.07E-01	7.69E-01	0.00E+00	3.95E+02	-3.99E-01	7.10E-01	9.52E-01	1.80E+02
Gonads	8.25E-02	1.00E+00	8.31E-02	9.99E-01	0.00E+00	1.10E+00	3.57E-02	-3.56E-02	1.00E+00	3.00E-01
Heart	4.68E-02	1.00E+00	-3.81E-02	1.01E+00	0.00E+00	2.80E+01	4.08E-03	1.81E-05	1.41E+00	2.40E+01
Kidneys	3.17E-02	1.00E+00	1.44E-02	1.06E+00	0.00E+00	3.80E+01	3.86E-02	-7.90E-03	1.11E+00	1.10E+02
Liver	2.79E-03	1.00E+00	1.10E+00	6.03E-01	0.00E+00	1.60E+02	8.54E-03	-3.51E-04	1.24E+00	3.90E+01
GI tract	8.20E-02	1.00E+00	4.41E-02	1.04E+00	0.00E+00	9.00E+01	6.09E-02	-2.61E-02	1.06E+00	1.14E+02
Muscle	1.26E-01	1.00E+00	7.76E-06	1.76E+00	0.00E+00	9.50E+02	1.00E-01	-1.03E-01	9.92E-01	3.10E+01
Skin	2.88E-01	1.00E+00	2.71E-01	9.98E-01	0.00E+00	2.00E+02	1.06E-02	-2.72E-03	1.10E+00	3.00E+01
Lungs	9.74E-02	1.00E+00	6.33E-02	1.03E+00	0.00E+00	8.40E+01	-4.98E-01	9.94E-01	9.48E-01	5.58E+02
Arterial/venous blood	1.26E-01	1.00E+00	-1.25E-01	9.98E-01	0.00E+00	3.80E+01				

Total blood	1.15E-01	1.00E+00	-1.10E-01	9.92E-01	0.00E+00	1.33E+02				
WEIGHT	3.90E-01	1.00E+00	8.40E+01	3.66E-01	0.00E+00	3.21E+03				
HCT	-1.00E-01	9.66E-12	-1.00E-01	1.55E-06	5.50E-07	5.80E-01				

Table 15. Gestation parameters (from conception to birth)

	organ volumes (mL)						**organ flows (mL/min)**			
	a	***b***	***c***	***d***	***e***	***f***	***a***	***b***	***c***	***d***
Uterus	3.90E-01	1.00E+00	8.40E+01	3.66E-01	0.00E+00	3.21E+03	3.57E-02	-3.56E-02	1.00E+00	3.00E-01
Placenta	3.90E-01	1.00E+00	8.40E+01	3.66E-01	0.00E+00	3.21E+03	3.57E-02	-3.56E-02	1.00E+00	3.00E-01
Amniotic fluid	3.90E-01	1.00E+00	8.40E+01	3.66E-01	0.00E+00	3.21E+03				
Breast										