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# Methodological approaches to estimate human population exposure to chemical substances

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# Chapter 1.

# Exposure assessment in the framework of risk analysis

## 1.1 General introduction

The industrial activities of the past centuries have resulted in massive increases of the global population exposure to old and new chemical substances, which now are present ubiquitously in the environment and can exhibit various health effects. In particular in recent decades a wide range of modern chemical substances have emerged, especially those used in houses (building materials, floorings, clothing, furnishings etc) and in several consumer products (cosmetics, personal-care products, medicines). Some of these substances have been banned but nevertheless persist in the environment and contaminate food, water and living environment (Briggs, 2003).

There are also a number of sources of naturally occurring inorganic and organic chemicals that can contaminate water, food and soil and can represent a risk for the exposed population. Interestingly when focusing on health problems due to water chemical contamination most of them are caused by chemicals of natural origin rather than those from human-made pollution (Thompson et al., 2007).

When considering in particular the food chain, as additional sources of contamination it must be also considered the treatment of animals with veterinary drugs or the use of agrochemicals in vegetable products, which may leave residues.

Numerous food chemical contaminants are formed during the processing and cooking, other leach from the packaging or storage containers (Borchers et al., 2010). Finally substances like food additives are added voluntarily during the food chain production in order to better preserve or to improve the food qualities.

Chemical safety issues are an increasing concern for scientists working in environment science and food safety. During the past years, chemical hazards have represented one of the consumers' biggest concern, mainly due to their long-term carcinogenic potential effect (Jackson, 2009). More recently, concerns have considered also the potential effects on fertility and the development of foetuses and children by such chemicals as endocrine disrupters (Mantovani and Proietti, 2011). Furthermore, chemical food safety has considerable implication in international trade due to the global nature of the food supply (Satcher, 2000). Therefore, economic and social issues may negatively interfere with the translation of scientific recommendations into updated regulations and controls.

Most of the chemical substances are present in very low concentrations in the media and in the environment, so effects on health are usually far from immediate or obvious: few environmental

exposure of concern today imply large relative risks, thus detecting small effects against a background of variability in exposure and human susceptibility poses severe scientific challenges. Nevertheless, the progressively larger number of people exposed to chemicals means that even small increases in relative risk can add up to major public health concerns (Briggs, 2003; Taubes, 1995).

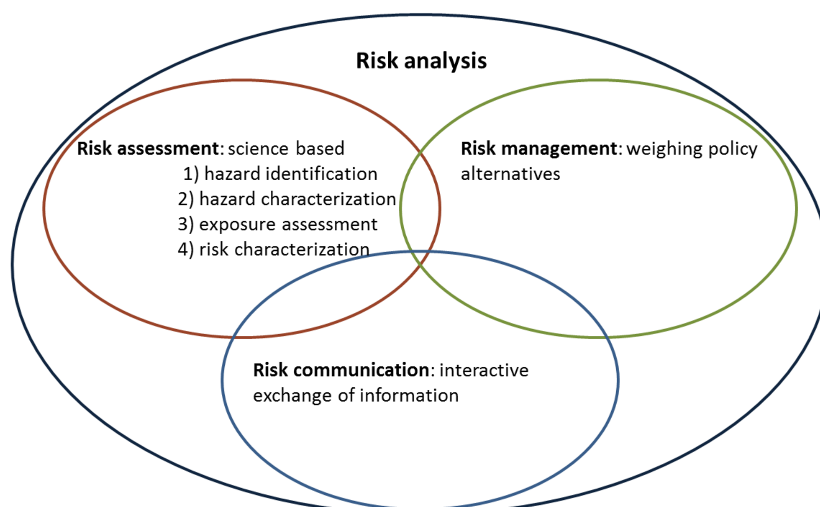
Risk Analysis is a widely recognized approach aimed at protecting consumers' health, by reducing, eliminating or avoiding health risks. Risk Analysis is a multidisciplinary approach that uses all available information to produce reliable estimates of the probability that a specific hazard occurs in certain scenarios, and defines possible measures for risk reduction, ensuring complete and transparent communication to stakeholders. Risk analysis also allows to identify uncertainty related to the estimates.

Risk analysis, as defined by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), consists of three components, which are themselves defined as follows:

- Risk assessment: is the scientifically based process consisting of the following steps: 1) hazard identification, 2) hazard characterization, 3) exposure assessment and 4) risk characterization.
- Risk management: is the process of weighing policy alternatives, in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection.
- Risk communication: is the interactive exchange of information and opinions throughout the risk analysis process, including the explanation of risk assessment findings and the basis of risk management decisions (FAO/WHO, 2009) (Figure 1).

This approach can be applied to risks related to chemical substances as well as biological and physical agents, in food, water, air, soil or, more broadly, in the environment people live in.

**Figure 1**: Diagram of the process of Risk Analysis, including the three components: Risk assessment, Risk management and Risk communication.



## 1.2 Human health risk assessment of chemical substances

Risk assessment, the central scientific component of risk analysis, is a process intended to estimate the risk for a given target organism, system or (sub)population, following the exposure to a particular hazard.

It is good to give a definition of hazard and risk in this contest. For hazard we mean a potential source of harm or adverse health effect on a person or people; while risk is the likelihood that a person may be harmed or suffer adverse health effects if exposed to the hazard.

In case of chemicals, risk assessors must take into account the inherent characteristics of the substance of concern as well as the characteristics of the specific target population and identify and quantify the uncertainty of the analysis (IPCS, 2004). The risk assessment process was developed primarily with the intent of supply a specific tool for decision makers to protect health in the face of scientific uncertainty (FAO/WHO, 2009). Human health risk assessment of chemicals refers to methods and techniques that in some cases may differ from approaches used to assess risks associated with biological and physical agents (IPCS, 2004). Risk assessments of chemicals can be performed to evaluate past, current and even future exposures to any chemical found in air, soil, water, food, consumer products or other materials.

As mentioned above, the risk assessment process is composed of four steps:

1) hazard identification: consists in identifying the type and nature of adverse health effects of exposure to the chemical substance. Hazard identification is the first stage in hazard assessment.

2) hazard characterization: is the qualitative and/or quantitative description of the nature of the adverse health effects associated with the chemical substance. When possible, this step should include a dose-response assessment and its related uncertainty. Hazard characterization is the second and last stage in the process of hazard assessment.

3) exposure assessment: it can be described as the qualitative and/or quantitative evaluation of the exposure of an organism, system, or (sub)population to the chemical agent and/or its derivatives.

4) risk characterization: is the qualitative and/or quantitative determination, including attendant uncertainty, of the probability of occurrence and severity of known and potential adverse health effects of the substance in a given organism, system, or (sub)population, based on hazard identification, hazard characterization and exposure assessment (FAO/WHO, 2009).

Ultimately, chemical risk assessments rely on scientific understanding of substance's behaviour, exposure, dose and toxicity. In general terms, risk depends on the following factors:

• the amount of a chemical present in an environmental medium (e.g. soil, water, air), food and/or a product;

• the amount of contact (exposure) a subject or population has with the chemical in the medium, identifying subgroups that can be more exposed;

• the toxicity of the chemical, highlighting more vulnerable subgroups.

The lack of information on one or more of these points is often the main limit of the risk assessment and results in the associated uncertainty, which should be characterized as much as possible (IPCS, 2010). In order to avoid that this uncertainty may lead to an underestimation of the risk, the assumptions underlying the risk assessment often represent the worst-case scenario with estimates that are pessimistic and protective for the population. An interesting question is how much the worst-case scenario is realistic, e.g., the scenario is over-conservative or, conversely, there might be actual situations where exposure is even higher than the worst case.

## 1.3 Exposure assessment to chemical substances

"*All substances are poisons; there is none which is not a poison. Therefore, the right dose differentiates a poison and a remedy*" Paracelsus, 16th century

Exposure assessment is "the process of estimating or measuring the magnitude, frequency, and duration of exposure to an agent, along with the number and characteristics of the population exposed. Ideally, it describes the sources, pathways, routes, and the uncertainties in the assessment" (IPCS, 2004).

The expression "pathway of exposure" refers to the physical course taken by a chemical as it moves from a source to a point of contact with the subject (via food, dust or water); while "route of exposure" refers to the way of absorption (intake through ingestion, inhalation or dermal absorption). The identification of both the pathway and the route of exposure is important in the process of risk assessment (IPCS, 2010). The quantification of exposure, both in individuals and in populations, is a prerequisite for the quantification of risk, indeed the risk is determined by both the hazard and the exposure: if there is no exposure there will be no risk.

Exposure assessment is used to determine whether people are in contact with a potentially hazardous chemical and, if so, to how much, by what route, through what media and for how long. Consequently, the magnitude of exposure include intensity, frequency, route and duration of the exposure, in addition, the exposed population should be always characterized. The goal of the exposure assessment is to obtain an estimate of exposure in terms of concentration per time period and compare it with the appropriate guidance value in order to evaluate if this value is exceeded (European Commission, 2009; IPCS, 2010)

Toxicologists usually divide exposure into four categories: acute (exposure for less than 24 hours), sub-acute (for one month or less), sub-chronic (for 1-3 months) and chronic (more than 3 months). In general acute exposure is sudden and severe, characterized by a rapid absorption of the toxicant and usually involves one single, large exposure. The effects of acute exposure can be immediate and extremely evident, but can also be less obvious with long term effects. In fact, a single acute exposure may have genotoxic effects that persist over time and hence are irreversible (Zeljezic et al., 2008; Yılmaz et al., 2014; Tsao and Wright, 1993). Chronic exposure instead is prolonged or repeated over many days, months or years. The consequences of the chronic exposure are usually not immediately apparent but when the consequences reveal themselves are often irreversible (Chen, 2014; Gurjar and Mohan, 2003). The main difficulty of the estimation of acute exposure is that fluctuations of the chemical concentration in medium are difficult to catch with monitoring plans and equally difficult to model. Instead when considering chronic exposure, the chemical concentration in the medium can be assumed to average out in the long run, hence, concentration variability can be ignored, and only the mean chemical's concentration is needed. The difficulty of chronic exposure estimation is that it is not simple to collect individuals' information on behaviour, consumption patterns and general use of consumer products over long periods of time.

When estimating exposure to chemical substances, apart from the duration in time of the contact between the organism and the substance, that distinguishes acute and chronic exposure, other aspects must necessary be taken into account. For example, a large group of substances, named persistent organic pollutants (POPs) are characterized by an extremely stable structure and are resistant to environmental degradation through chemical, biological, and photolytic processes. When these substances enter into organisms, they accumulate in the fat cells, organs and muscles. Due to their stability and their lipophilic structure, POPs are hardly metabolized via enzymatic route and eliminated by the organism. Thereby, these substances bioaccumulate in the body increasing the body burden (IPCS, 1995; El-Shahwai et al., 2010). Bioaccumulation refers to the continuous increase in the concentration of a chemical in an organism, compared to the chemical's concentration in the environmental media to which the organism is exposed. The longer the biological half-life of the substance, the greater the risk of chronic poisoning, even if environmental levels of the substance are not very high (Tonnelier et al., 2011). The possibility of bioaccumulation is a crucial aspect to consider when estimating total exposure.

Furthermore, chemicals' exposure can occur also during the pre-natal life. In fact, during pregnancy the mother can transfer to the foetus through the placenta different chemicals, which, besides having a potentially toxic effect on foetal development, contribute to the body burden of the future baby yet before birth. The quantitative assessment of prenatal exposure to chemicals is necessary for the assessment of risks (Mattison, 2010; Zhang and Qin, 2014). Finally, it is also important to

consider the exposure of infants via breast milk: the milk for certain substances, especially for lipophilic substances, is the major route of elimination, therefore high concentrations of chemicals are transferred to the baby during breastfeeding. In fact, in order to estimate the exposure of infants is essential to consider also maternal exposure to chemicals (Ettinger et al., 2014)

A variety of different approaches exist for quantifying human exposures. Traditionally, exposure can be estimated by measuring the contaminant's concentration in the medium considered (e.g., air, soil, water, or food) and estimating the quantity that gets in contact with the population. This approach is necessary to identify the main sources of exposure, but it must be completed with information about exposure pathways and routes in order to predict the internal dose, meaning the dose that actually penetrates the organism. Thus to predict the chemical body burden, complex exposure models are constructed which involve information or assumptions about the individual and/or population activities, dietary choices, behaviour, as well as information or assumption on the substance, such as metabolism, kinetics and bioaccumulation. Typically, indirect exposure assessment can be distinguished in dietary, environmental, consumer product, occupational, and cumulative exposure (Fryer et al., 2006).

On the other side, direct exposure assessment methods, as biomonitoring, have been lately implemented. With the biological biomonitoring, the exposure estimate is obtained by measuring the chemical substances or their metabolites in human specimens, such as blood or urine. Indeed human biomonitoring can be defined as the direct measurement of the individual exposure to toxic substances considered. Biological markers of exposure are considered measures of individual internal dose (Noort et al., 2002; IPCS, 2004).

### 1.3.1 Dietary exposure estimate

As said in the introduction, the estimate of the exposure to chemicals via food, defined as dietary exposure estimate, is paramount for a science-based risk assessment.

Dietary exposure estimate is necessary to ensure that safety requirements for food are protective for the public health, consistent among countries, and appropriate for use in international trade. More specifically, the Codex Alimentarius Commission Procedural Manual defines exposure assessment as "the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant" (FAO/WHO, 2011).

Dietary exposure assessments combine food consumption data with data on the concentration of chemicals in foods. The resulting estimate is then compared with the relevant toxicological reference value for the food chemical of concern (FAO/WHO, 2005).

Several methods have been developed for carrying out dietary exposure assessment (e.g. duplicate diet studies, theoretical daily intake etc), and the choice of a method will be driven by the purpose of the assessment, the nature of the chemical and the resources available.

To estimate dietary exposure three main issues must be considered:

1- how to determine quantitatively the presence of the chemical in the food (food chemical concentration data);
2- how to determine the consumption pattern of the foods containing the substance (food consumption data);
3- how to draw together the probability of subjects eating large quantities of a given food and the probability of the chemical substance being present in that food at high levels (dietary exposure models) (Kroes et al., 2002). The development of such issue (the worst-case scenario) may be important to pinpoint subgroups with exposures significantly higher than the general population (EFSA, 2012), as well for risk assessment of chemicals that may induce acute non-lethal effects, such as neurotoxic agents (EFSA, 2011a).

A stepwise or tiered approach is recommended when doing a chemical exposure assessment. The initial step relies on conservative screening methods and if no safety concerns are identified, no additional exposure assessment is required. Where potential safety concerns are identified, the subsequent step is to use methods that incorporate progressively more refined consumption and concentration data (FAO/WHO, 2005; Diouf et al., 2014).

### *Food chemical concentration data*

Levels of chemicals in foods can vary significantly and the representativeness of the data changes according to the measurement method, whether it is based on estimated levels or actual analytical results, the sampling strategy and the market coverage (EFSA, 2011b; Kroes et al., 2002).

Possible sources of chemical concentrations data in food that may be used in dietary exposure assessment are listed below (FAO/WHO, 2005).

- Maximum permitted levels (MPL) or maximum residue limit (MRL): MPL, the highest legal additive concentration that can be added to the different foods in which the use is permitted, is defined for all food additives authorized. The MRL is the upper legal concentration for pesticide residues in food or feed based on good agricultural practices. When no information on actual chemicals' levels in food are known, the MPL and the MRL can be used. Since this data are conservative, the result is usually an overestimation of the dietary exposure picturing the "worst case scenario" (Leclercq et al., 2000).

- Manufactory use levels: referring to additives, industries may provide data on the quantity added during the production process. These data can be used to estimate consumers' exposure assuming

that the amount added to a food during the production will equal the amount remaining in the food as consumed (Wapperom et al., 2011).

- Monitoring and surveillance data: data obtained both from a stratified sampling plan and from a targeted sampling can be used. The stratified sampling plan is a representative picture of chemical levels present in food, whereas the targeted sampling plans includes those products expected to contain higher levels of the investigated chemical substance (Kroes et al., 2002). Data collected with monitoring and surveillance plans must be used knowing that they may not be representative of all food available in the market and that not all commodities entering the food chain are monitored. Furthermore, the sampling design, the analysis and the reporting procedures must be clearly specified in order to obtain comparable chemical concentration data. In any case these data generally provide a better figure of the concentration of chemicals in foods as purchased by consumers (Huybrechts et al., 2011).

-Total Diet Studies (TDSs): a TDS can determine the population dietary exposure to both beneficial and harmful chemical substances across the entire diet. With this approach concentrations of chemicals are measured in foods after they have been prepared as for normal consumption. In this way, any variation in the chemical concentration that may occur during the food production process is automatically included, the impact of cooking on less stable chemicals and on the formation of new ones are taken into account as well. TDSs are designed to cover the whole diet and, consequently, to estimate the population total intake of each chemical substance of interest (Hulin et al., 2014; EFSA/FAO/WHO, 2011).

### *Food consumption data*

Food consumption data reflect what either individuals or groups consume in terms of solid foods, beverages, including drinking-water, and supplements (FAO/WHO, 2005). When choosing the sources of food consumption data, it is always necessary to evaluate the fitness-for-purpose of the data available. Indeed the aspects that should be considered are the time frame represented by the food consumption survey, the population groups to which the data refer, the food group for which the data are available and the overall amount and quality of the data (Lambe, 2002).

Food consumption data should include information on all factors that may influence food consumption patterns or the dietary exposure such as demographic characteristics (age group, sex), body weight, geographic region, day of the week on which the data are collected, and season. It is also important to consider food consumption patterns for sensitive subpopulations and for individuals at the extreme end of the distributions (95th-99th percentiles) (FAO/WHO, 2005).

Methods for food consumption data collection used in exposure assessment are listed below.

- Population-based methods: represented by the national food supply surveys, mainly based on food balance sheets (FBSs) or food disappearance data. They provide gross annual estimates of the national availability of food commodities. The major limitation of national food supply data is that they reflect food availability rather than food consumption. Indeed population-based methods give only an estimate of the average consumption that does not account for losses prior to consumption, due to processing, spoilage, trimming and waste. These data may be used for a preliminary or rough estimation of the population exposure to chemicals assuming that all foods bought are actually eaten (Sheehy and Sharma, 2013; Kroes et al., 2002)

- Household-based methods: information regarding food availability or consumption at the household level may be collected by "budget surveys" and by "consumption surveys". These methods include data on foodstuffs purchased by a household, follow-up of consumed foods, or changes in food stocks, but it does not provide information on how food is handled within the household, or on actual consumption by its members (Iwaoka et al., 2001; Kroes et al., 2002).

- Individual-based methods: data collected by individual-based methods provide detailed information on food intake and its distribution over various well-defined groups of individual. Indeed individual-based methods provide data that more closely reflect actual consumption; however, they may be prone to bias and difficult to collect.

*Food record survey*, also called "food diary", requires that the subject reports all foods and beverages consumed during a specified period, usually 1 to 7 days. The respondent is also asked to keep note of the quantity of the food and beverages actually consumed (Rothausen et al., 2012).

*24-h dietary recall* (24-HDR): consists in listing foods and beverages consumed on the previous day or during the 24 h prior to the interview. The quantities are usually assessed by using household measures, pictures (photos) or food models (Kahn et al., 1995).

*Food frequency questionnaire* (FFQ): consists of a structured list of individual foods or food groups. For each item on the food list, the respondent is asked to estimate the number of times the food is usually consumed per day, week, month, or year. FFQs may be qualitative, semi-quantitative or quantitative (Shu et al., 2004).

*Diet history survey*: also called "meal-based diet history", is designed to assess usual individual food consumption. It consists of a detailed listing of the types of foods and beverages commonly consumed at each eating occasion over a defined time period, which is often a "typical week". The aim is to define an individual's total usual intake and meal pattern (Tapsell et al., 2000).

*Food habit questionnaire*: it is designed to collect either general or specific types of information, such as food perceptions and beliefs, food likes and dislikes, methods of preparing foods, use of dietary supplements, and social settings surrounding eating occasions. These types of

information are frequently included along with the other four methods, but should also be used as the only information source (Bashour, 2004).

## *Dietary exposure models*

Dietary modelling refers to the mathematical technique used to generate exposure estimates. Dietary modelling combines food consumption data with food chemical concentration data to estimate dietary exposure to the food chemical. This is usually summed for all foods containing the chemical in order to obtain the overall dietary exposure estimate. The estimate is usually expressed as a concentration per body weight. In the broadest sense, the model to represent dietary exposure can be considered as:

*consumption * concentration= dietary exposure*

There are however a number of different models for combining or integrating consumption and concentration data (Lambe, 2002):

- point-estimate assessment = this approach, also called deterministic approach, multiplies a single fixed value for food chemical concentration by a single food consumption amount for each food that contains the chemical and then sums the intake from all implicated foods. The result is a single dietary exposure value. The single data points used in the deterministic assessments are generally means for the population group being assessed, but sometimes medians or high percentile values, depending on the purpose of the dietary exposure assessment (Oldering et al., 2014). The deterministic assessment is straightforward to conduct and the output is relatively easy to interpret (FSANZ, 2009). A point estimate is not inherently "conservative" or "realistic": the conservatism incorporated into the analysis is determined by the data and the assumptions that are used in calculating the estimate. In any case a point estimate doesn't incorporate variability and does not quantify uncertainty (FAO/WHO, 2005).

- simple distribution = even called semi-probabilistic approach, employ distributions of food consumption data with a single point chemical concentration per food or food group, to generate a range of individual dietary exposures (FSANZ, 2009). The results are more informative than point estimates because they take into account the variability that exists in food consumption patterns, but, they retain conservative assumptions related to the presence and concentration of the chemical in foods (Fierens et al., 2014).

- probabilistic models = involves using distributions of both food consumption and food chemical concentration data to produce a distribution of estimated dietary exposures. Consequently, the probabilistic approach takes into account each value the variables can take and weights each possible scenario by the probability of its occurrence. The two primary advantages of this approach

are that: 1) it ensures that any variability and/or uncertainty are reflected in the model output, 2) it permits the exposure assessor to consider a distribution of exposure according to the probability, from minimum to maximum, with all modes and percentiles (Holmes et al., 2005).

It must be beard in mind that, by their very nature, dietary exposure assessments, independently from the approach used, can only approximate the real situation with regard to the dietary exposure to food chemicals. The reliability, accuracy and value of the estimates depend on the quality of the input data.

### 1.3.2 Other indirect exposure estimates

A part from the dietary exposure assessments, other existing indirect exposure models can be broadly categorised according to the following types of exposure source: environmental, consumer product and occupational, and cumulative exposure.

#### *Environmental, consumer product and occupational exposure models*

Environmental exposure models have been developed in an effort to quantify human exposures to chemicals via contact with the surrounding natural environment. Keeping in mind that the EU is the largest chemical producing area in the world, accounting for 32 % of an estimated global turnover for chemical production of euro 1 632 billion in 2001, it is essential to keep monitored environmental exposure in order to protect human health and the integrity of the ecosystems. Of particular concern are the substances that do not degrade (persistent) since they accumulate in the environment and are now ubiquitous worldwide. Air pollution and generically presence of natural and antropogenic chemical substance in the environment are also of concern (CEFIC, 2002; Sonne et al., 2012; Rhind, 2009; Goldberg and Luce, 2012).

People exposed to chemicals in the professional environment compose a specific subgroup of the population that deserves special attention in terms of exposure assessment. Exposure to substances or mixtures in the workplace can occur through inhalation, absorption through the skin or ingestion. The extent to which the worker is exposed depends on the concentration of the substance in the air, the amount of time the worker is exposed and the effectiveness of controls (SWA, 2012). People who are exposed to high levels of chemicals in the working environment, represent a population subgroup at high risk. For this reason occupational exposure assessment need to be conducted separately from exposure assessment of the general population (Van Tongeren et al., 2002; Chen et al., 2012; Farmer and Johnson, 1990).

Furthermore, special attention must be given to exposure through consumer products. The consumer, meaning a member of the general public who may be of any age, either sex, and in any

state of health, may be exposed to a substance by using consumer products or good commodities (ECHA, 2012). The goal is to increase the knowledge on which dangerous substances are used in consumer products, estimate the exposure levels and consequently asses the health risk ascribable to the use of products containing these substances, with special attention to vulnerable subgroups (e.g. infants, pregnant women) (Stapleton et al., 2011; Imm et al., 2009).

#### *Cumulative exposure models*

Concern about the exposure of the population to "chemical cocktails", that is exposure to multiple chemicals at the same time, has increased over the last thirty years (HCNL, 1985). Assessing potential health risk from combined exposures either as intrinsically complex mixtures or as exposures to multiple individually identifiable substances is one of the major challenges for chemical risk assessors (Ferrone et al., 2004; Sarigiannis and Hansen, 2012). The main problem is that substances may interact within the organism and the single effect may sum or, in the worst case, the mixture effects can be larger (synergistic) than the sum of individual effects (EFSA, 2014a).

Until recent times chemical exposure assessors focused on the exposure via one pathway (via food, via air etc) at a time, and via one route (ingestion, inhalation, dermal contact), however, it is increasingly recognized that many of the numerous chemicals the consumers are exposed to everyday are ubiquitous, resulting in exposure from food, water, air, dust, and soil and that aggregated exposure assessment is needed (EPA, 2001; http://www.efsa.europa.eu/en/consultations/call/130725.pdf).

Finally, the cumulative exposure is defined as: “combined exposure to multiple chemicals including all routes, pathways, and sources of exposure to multiple chemicals” (EFSA 2013; Van Klaveren et al., 2009).

### 1.3.3 Biomonitoring

Biomonitoring consists in monitoring the environmental contaminants in tissues and other biological media, such as urine, blood, and breast milk, by measuring biological markers (biomarkers). It follows that human biomonitoring can represent the internal exposure of the individual to the selected chemical substance, in fact knowing the levels of environmental chemicals in the population helps to determine how much people have been exposed to them (Koch and Calafat, 2009).

Biomonitoring data may be interpreted mainly with two different approaches:

- descriptive approach: it produces a review of the data in order to describe which chemicals get into the general population and at what concentration, so that the concentration reference ranges for the chemicals can be established; to evaluate if the exposure levels are higher in some sub-groups than

in others; to highlight possible temporal trends in exposure levels or to assess the effectiveness of actions conducted to reduce the population's exposure.

- risk-based approach: biomonitoring data are compared to measures of toxicity obtained by toxicologic, epidemiologic or pharmacokinetic studies, in order to evaluate the health risk associated with the amount of chemical in the body. This approach allows to determine the prevalence of subjects with chemical levels above known toxicity threshold values and also to set priorities for research on human health effects (Shatkin and Ranalli, 2007).

Biomarkers can include chemicals and their metabolites, as well as DNA mutations or specific proteins associated with exposure to specific chemicals. In other words, a biomarker is a substance, structure, or process that indicates an exposure or susceptibility or predicts the incidence or outcome of a disease (Shatkin and Ranalli, 2007). In general biomarkers can be distinguished in three groups (Nordberg, 2010):

- *Biomarker of exposure*: concentration of the chemical substance or it's metabolites or products of the interaction with an endogenous component;
- *Biomarker of effect*: indicators of a change in biological function in response to a chemical exposure;
- *Biomarker of susceptibility*: factors that may increase the sensitivity to chemicals in certain individuals: they can be genetic (e.g., polymorphisms of metabolising enzymes) or biological factors (e.g., low iodine status for thyrostatic contaminants (EFSA, 2014b)).

Therefore, the toxicokinetic and toxicodynamic characteristics of specific chemicals drive the selection of the proper biomarker for a biomonitoring program (Mantovani et al., 2008). In fact, once in the organism, the chemical goes through the metabolic pathways, is transformed and either accumulated or eliminated. There are various compartments that a chemical may cross undergoing various transformations when metabolized. Thus, in order to provide correct interpretation of the biomarker, it is important to know where in this process the measured biomarker comes from, the appropriate biological specimens to be sampled and whether it is a biomarker of exposure, susceptibility or effect.

Running a biomonitoring study, we can quantify the amount of a chemical actually absorbed into the human body. Because of sophisticated techniques used in analytical chemistry, it is now possible to detect extremely low concentrations of chemical substances in human specimens. Biomonitoring requires no assumptions regarding exposure parameters such as ingestion or inhalation rate, frequency of exposure or behaviour habits (Paustenbach and Galbraith, 2006). In fact, biomonitoring data represent an actual measure of integrated exposures via all routes and pathways and it is not susceptible to assumptions or models. Human biomonitoring data integrates all sources of possible

exposure to a chemical, taking into account all relevant routes of exposure (diet, air, water and soil), all routes of absorption (respiratory, oral and skin) and all factors of individual variability (susceptibility, metabolism, lifestyle, etc.). At the same time, since biomonitoring data are integrated measures of exposure from all routes and pathways, in general they do not add any information on where the chemical comes from or how it entered the organism, nor on which route contributes the most to the total exposure. However, in some cases the integrated assessment of different biomarkers of exposure for the same contaminant may provide valuable additional information, when supported by background information. For instance, the measurement of arsenic in nails or hair provides information on chronic exposure, whereas the measurement in urine informs on short-term exposure; moreover, the speciation of urinary arsenic does inform on possible sources (e.g.: arsenobetaina is a seafood specific metabolite) and on the individual capacity to metabolize the inorganic, highly toxic, form (measurement of methylated metabolites) (Cubadda et al., 2012).

Collection of biomonitoring data over an extended period of time can provide information regarding trends in exposure that is particularly important when evaluating the effectiveness of environmental remediation programs or evaluating the reduction in general use of a chemical (Albertini et al., 2006). Furthermore, using biomonitoring data is possible to identify population subgroups that have higher exposure compared to the general population (Paustenbach and Galbraith, 2006). Finally the use of biomonitoring data may help to test and validate exposure models when the results of modelling predictions are compared to biomarker's levels actually measured in exposed population.

Biomonitoring information can be used as well to investigate health effects, but to interpret biomonitoring data in terms of health, studies are needed on the relationships between exposure and levels in the body and between levels in the body and health effect. Indeed the detection of a substance in the body indicates only that an exposure has taken place, it does not indicate whether such exposure has resulted in any adverse health effect (Bates et al, 2005). With biomonitoring studies it is not possible to define the toxic dose: unless toxicology and epidemiology studies have defined the toxicity and the dose-response curve, the simple presence of a chemical in an organism may be difficult to interpret in terms of health consequences. An appropriate approach may be to match exposure and effect biomarkers: this may support testing hypotheses, such as the association of biomarker patterns with given health conditions (La Rocca et al., 2012).

Concluding, we cannot use biomonitoring as an automatic tool, which can be considered by itself, but it has to be integrated with environmental monitoring, toxicological and eco-toxicological data and finally with epidemiological studies (Smolders et al., 2008).

It should be noted that there is no single ideal method for assessing chemical exposures. The choice depends on the objectives of the study: the most important criterion is the appropriateness of the method for answering the research question.

## 1.4 Chemical substances from an exposure assessment point of view

Chemicals can be classified following several different criteria, for example according to their structure, physical properties or use, but considering the topic of this thesis, we have considered three groups according of the health effect:

1. *Chemicals with an adverse effect at any level, for which there is no harmless dose;*
2. *Chemicals that have to reach a threshold level before any adverse effects occurs;*
3. *Chemicals with no (known) health effect, or with a low intrinsic toxicity, or of low toxicological concern as used.*

### 1.4.1 Chemicals with an adverse effect at any level, for which there is no harmless dose

Some harmful effects on individuals, such as carcinogenic and genotoxic effects, appear to act through mechanisms that are independent from the dose of the substance that comes in contact with organism. Consequently, carcinogenic and genotoxic substances are assumed to have no threshold dose below which their toxic effect will not appear. In this case it is appropriate to estimate the probability of occurrence of the effect that is consequence of the dose to which the organism is exposed, and hence they are referred to as stochastic effects (UNEP/IPCS, 1999).

For these substances EFSA recommends the use of the Margin of Exposure (MoE) approach. The MoE approach uses a reference point, often taken from an animal study and corresponding to a daily dose causing a low but measurable increase in the incidence of response in animals. This reference point is then compared with the exposure estimates in humans. The MoE approach compares the margin between a dose or an exposure causing cancer in animals or humans with the estimated human exposure to that substance. In other words, the reference point is divided by the estimate of human exposure to the substance in order to obtain a dimensionless ratio that is the MoE (EFSA, 2005; ILSI, 2009).

It is recommended the use of the benchmark dose (BMD) to obtain the MoE. The BMD is a standardised reference point derived from the animal data by mathematical modelling within the observed range of experimental data. The BMD is the dose estimated to cause a predefined increase (e.g. 10%) in the background incidence of tumours or genotoxic effects in rodents. Furthermore it is recommends the use of the $BMDL_{10}$ (benchmark dose lower confidence limit 10%) which is an

estimate of the lowest dose that is 95% certain to cause no more than a 10% cancer incidence in rodents. The benchmark dose approach should also be applied to human data when available (EFSA, 2005; ILSI, 2009). Several chemical substances are part of this group , as for example aflatoxins, benzene, furan and acrylamide (Benford et al., 2010; Smith et al., 2010; Carthew et al., 2010; Michael Bolger et al., 2012)

### 1.4.2 Chemicals that have to reach a threshold level before any adverse effects occurs

Compounds, for which a threshold level has been established, are thought to be harmless at sufficiently low concentrations. However, at doses above the threshold level, increasingly severe effects will appear (UNEP/IPCS, 1999). Consequently it is necessary to establish the acceptable or tolerable intakes of substances that exhibit thresholds of toxicity.

In assessing an acceptable level of a particular substance, it is appropriate to identify the NOAEL (No-observed-adverse-effect level), which is an experimentally determined dose at which there are no statistically or biologically significant indications of the toxic effect in rodents. If several NOAELs have been estimated in different experiments, the regulatory focus is normally on the highest one, so that with the term NOAEL we refer to the highest experimentally determined dose without a statistically or biologically significant adverse effect. In cases in which a NOAEL has not been demonstrated experimentally, the LOAEL (lowest-observed-adverse-effect level) is used (Herrman and Younes, 1999)

The threshold values are then derived by dividing the appropriate NOAEL by a safety factor (SF). Generally, the SF consists of multiples of 10, each factor representing a specific area of uncertainty inherent in the available data. Namely, a factor of 10 is introduced to account for the possible differences in responsiveness between humans and animals (inter-species variability), and a second factor of 10 is used to account for variation in susceptibility among individuals in the human population (intra-species variability). Indeed a SF of 100 is used for most of the chemicals. For other chemicals, with limited amount of information (for example, those for which only the results of subchronic studies are available), an additional factor of 10 (leading to a SF of 1000) might be more appropriate (Herrman and Younes, 1999).

With regard to food additives with possible adverse effects, an Acceptable Daily Intake (ADI) is defined before the substance can enter the market. The ADI is an estimate of the amount of a food additive, expressed in concentration for body weight unit (e.g. ng/kg bw), that can be ingested daily over a lifetime without appreciable health risk. The ADI is used widely to describe “safe” levels of intake; indeed it must be ensured that the amounts of additives permitted in various foods will not

result in the consumer having a cumulative daily intake higher than the ADI (European Parlament 1994a, European Parlament 1994b, European Parlament 1995). Examples of additives that may induce health effects if ingested over are certain level, so additives for which an ADI has been established, are numerous: Sulphites, Benzoates, Parabenes, Nitrites, Nitrates, BHA, BHT, Aspartame and others.

The procedures used in the risk assessment of contaminants are essentially the same as those used for food additives. However, in this case a Tolerable Daily Intake (TDI) is established instead of an ADI. The term "tolerable" is used because it means permissibility rather than acceptability for the intake of contaminants unavoidably associated with the consumption of otherwise wholesome and nutritious foods (e.g. cadmium, arsenic, bisphenol A). Several chemical substances are slowly metabolized and cleared from the body, and tend to create a body burden (e.g., dioxins and dioxin-like chemicals); in these cases a Tolerable Weekly Intake (TWI) is calculated. For the TWI tolerable intakes are expressed on a weekly basis because the contaminants for which it is calculated may accumulate within the body over a period of time (Larsen, 2006).

Finally, it must be taken into account the Tolerable Upper Intake Levels (UL) referring to essential minerals and vitamins. When assessing micronutrients' intake the interest is in both the high and low extremes of the intake distribution. Indeed these components are essential but are not produced in the body, or not in sufficient quantities, and have to be obtained from food in an adequate quantity. Still, like other chemical substances, micronutrients may have adverse effects if consumed in excess. Consequently, when evaluating the intake of micronutrients it is necessary to take into account that, in contrast to non-essential chemical substances, there is both a lower level of intake below which risk of deficiency conditions or sub-optimal functioning arises, and a maximum level of total chronic daily intake of a nutrient over which adverse health effect may occur. Examples of micronutrient for which both a lower and an upper threshold value have been established are vitamin B, vitamin D, potassium, sodium and many others (EFSA, 2006; Howlett et al., 2012).

### 1.4.3 Chemicals with no (known) health effect, or with a low intrinsic toxicity, or of low toxicological concern as used

It is worth highlighting some critical issues relating to food additives that are judged with no health effect, or with a low intrinsic toxicity or of low toxicological concern as used. Indeed, if no adverse effects have been demonstrated in the safety evaluation, no numerical value for the ADI is specified and the additive may be used according to the principles of Good Manufacturing Practice (quantum satis) (Ilback and Busk, 2000). The EU Scientific Committee for Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) gave the following definition of "ADI not specified":

“ADI not specified is a term used when, on the basis of the available toxicological, biochemical and clinical data, the total daily intake of the substance, arising from its natural occurrence and/or its present use or uses in food at the levels necessary to achieve the desired technological effect, will not represent a hazard to health. For this reason, the establishment of a numerical limit for the ADI is not considered necessary for these substances. Any additive allocated as “ADI not specified” must be used according to good manufacturing practice, i.e. it should be technological efficacious, should be used at the lowest level necessary to achieve its technological effect, should not conceal inferior quality or adulteration, and should not create a nutritional imbalance” (European Commission, 1990). Recently EFSA suggested that adequate information on reported or analytical level of food additives with an ADI “not specified” should be collected in any case in order to be able to perform a dietary exposure assessment and re-evaluate the risk associated to these food additives using updated techniques (EFSA, 2014c).

## 1.5. Veterinary public health involvement in exposure assessment to chemical substances

Keeping in mind that veterinary public health was defined by the WHO in 1999 as “the sum of all contributions to physical, mental and social well-being of humans through an understanding and application of veterinary science”, it is easy to understand the key role of veterinarians in the context of exposure estimation (Tabbaa 2009).

Firstly, the figure of the veterinarian has key role in the framework of food safety in the broadest sense. Increasingly, the European Community is moving towards an integrated and multidisciplinary approach to food safety in which the veterinarian has a privileged point of view due to its heterogeneous and transverse training. Considering that the chemical risk is one of the biggest concerns of the modern society, studies that aim to assess the exposure to chemicals must be considered as a main core in food safety (Fabiansson and Vernazza, 2012). It should also be kept in mind that the methodological approaches summarized in the previous pages, with the appropriate modifications, could also be applied to the estimation of exposure to foodborne pathogens but also for the exposure estimation to radioactive material always via food.

Secondly, the veterinarian has an important role in proposing different methodological approaches to estimate the animals’ exposure to chemical substances. Indeed, animals, both domestic and wild, can play a role as "sentinels". In fact, animals can be more sensitive to the toxic effect of chemical substances and so act as an alarm bell that rings before the effects becomes visible in humans (van der Schalie et al, 1999). Animals can also play an important role because they can be “accumulators”, meaning that they can bioaccumulate the substance both for biological reason (e.g. lipophilic

substances) and for a major exposure to the chemical substance as a consequence of specific behaviours (e.g. graze, drinking ground water). These aspects are particularly important when considering that animals' biological specimens are normally introduced in control circuits and consequently can be used in exposure study without increasing too much the costs (Ravera O. 2001; Scaramozzino et al, 2012).

Furthermore, chemicals' exposure studies can contribute to evaluate possible pathological effects that chemicals can cause in the animal population itself. The exposure of animals to chemicals' toxic levels can lead to both a financial loss related to a decrease in productivity of the animals themselves, but can also damage the equilibrium of the ecosystem in which the animals live (Bellingham et al, 2012; Vos et al, 2000).

## 1.6. General aims of the thesis

This thesis focuses broadly on how human population exposure can be estimated considering the kind and nature of the chemical substances and the detail in the data available. As explained above, different methodological approaches can be used to estimate the chronic exposure of specific populations' subgroups to natural or antropogenic chemicals, and the choice of the "best" approach should be performend taking into account different factors, including what populations, information and quality of data are available. The idea behind this project is to provide examples of different methodological approaches highlighting their limitations and advantages and discuss the possible implications that the results can have in terms of risk assessment. In the following four chapters, different methodological approaches to estimate the exposure to chemicals have been chosen and implemented, with a specific focus on dietary exposure assessment and biomonitoring.

In the first chapter the objective was to estimate the intake of food additives using a tiered (stepwise) approach as recommended by the European Commission. Such approach, in which the initial steps rely on conservative screening methods, is commonly used to minimise estimation costs and focus resources on the most important issues for which there is a potential health concern. The stepwise approach in dietary exposure assessment is such that as the accuracy of dietary exposure assessments increases, the cost of collecting adequate data and human resources needed to undertake the assessments also increases. For this purpose seven food additives, considered of the highest priority, have been chosen as an example, namely benzoates (E 210 – E 213), parabenes (E 214 – E 219), nitrites (E 249 – E250), nitrates (E 251 – E 252), BHA (E 320), BHT (E 321) and aspartame (E 951). Children 1-36 months old that are considered more vulnerable to chemicals since exposure occurs during the development process and since they have a higher rate of consumption on weight compared to the rest of the population, were investigated.

The second chapter deals with the capacity of capturing chemicals' dietary usual intakes, defined as the long-run average of daily dietary intakes of a substance by an individual, using the so-called "Usual Intake Models". Considering the increasing tendency to use "Usual Intake Models" to estimate long-term dietary exposure to chemical substances, the main objectives of the study were to highlight the potential impact that usual-intake-models can have on exposure estimate and risk assessment and to point out which are the key aspects to be considered in order to properly run these models and be sure to correctly interpret the output. To achieve this goal, cadmium, acrylamide and sulphites have been chosen as examples representing three chemicals with different origin, toxicological characteristics and distribution pattern within food groups.

The aim of the third study was to characterize the exposure to inorganic arsenic and patterns of dietary intake of the general population living in a natural arsenic-rich area in Italy (Latium). There is concern related to the chronic exposure to low dose of Arsenic, but it is difficult to achieve correct estimates of global exposure when different routes are involved. To achieve this objective an integrated approach combining a survey of arsenic speciation in locally-grown vegetables, a study of the impact of arsenic-rich water in processed and cooked food, a survey of the water used for drinking and cooking by the local population, a duplicate diet study and a biomonitoring study based on speciated urinary arsenic, was implemented.

Finally, a biomonitoring study conducted in the framework of the Italian project PREVIENI (Study in model areas on the environmental and health impact of some emerging chemical contaminants (endocrine disrupters): living environment, reproductive outcomes and repercussions in childhood; http://www.iss.it/prvn, supported by the Italian Ministry of Environment) is presented in the last chapter of this thesis. This study differs from the previous ones because, measuring substances and metabolites in human specimens and not considering food contamination and intake, it estimates the total exposure level. More in details, the total exposure level to several substances considered potential endocrine disrupters, namely PFOA (perfluorooctanoic acid), PFOS (perfluorooctane sulfonate), DEHP (di-2-ethylhexyl phthalate) and BPA (bisphenol A) was assessed. The main aim was to highlight possible differences of exposure to PFOA, PFOS, DEHP and BPA among fertile and infertile women living in three Italian areas representing different living environment scenarios (rural, urban and metropolitan scenarios).

Each the chapters of this thesis is "self-conclusive", that is, intended to stand alone composed of a detailed introduction to the specific topic, the materials and methods used and the results obtained, as well as their discussion and conclusions drawn.

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# Chapter 2.

# Dietary exposure to Benzoates (E 210 – E 213), Parabens (E 214 – E 219), Nitrites (E 249 – E250), Nitrates (E 251 – E 252), BHA (E 320), BHT (E 321) and Aspartame (E 951) in children less than 3 years old in France.

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*Abstract*

This study aimed to estimate the exposure to seven additives (benzoates, parabenes, nitrites, nitrates, BHA, BHT and aspartame) in children aged less than 3 years old in France. A conservative approach, combining individual consumption data with maximum permitted levels, was carried out for all the additives. More refined estimates using occurrence data obtained from products' labels (collected by the French Observatory of Food Quality) were conducted for those additives that exceeded the acceptable daily intake (ADI). Information on additives' occurrence was obtained from the foods' labels. When the ADI was still exceeded, the exposure estimate was further refined using measured concentration data if available. When using the maximum permitted level (MPL), the ADI was exceeded for benzoates (1.94 mg/kg bw/day), nitrites (0.09 mg/kg bw/day) and BHA (0.39 mg/kg bw/day) in respectively 25%, 54% and 20% of the entire study population. The main food contributors identified with this approach were current foods as these additives are not authorized in specific infant food: vegetable soups and broths for both benzoates and BHA, delicatessen and meat for nitrites. The exposure estimate was significantly reduced when using occurrence data, but in the upper-bound scenario the ADI was still exceeded significantly by the age group 13-36 months for benzoates (2%) and BHA (1%), and by the age group 7-12 months (16%) and 13-36 months (58%) for nitrites. Measured concentration data were available exclusively for nitrites and the results obtained using these data showed that the nitrites' intake was below the ADI for all the population considered in this study.

These results suggest that refinement of exposure, based on the assessment of food levels is needed to estimate the exposure of children to BHA and benzoates for which the risk of exceeding the ADI cannot be excluded when using occurrence data.

## 2.1 Introduction

Food additives are widely used for various purposes such as conservation, coloring or sweetening. Their use improves the food in terms of taste, appearance and practicality, making it more attractive

for consumers. In the European Union, the authorization and use of additives are regulated by the Regulation (EC) 1333/2008, which takes over from Directives 94/36/EC on colors, 94/35/EC on sweeteners and 95/2/EC on food additives other than colors and sweeteners (European Parlament 1994a, 1994b, 1995, 2008).

Despite such a strict regulation, it seems that certain additives could pose potential risks to consumers' health: some are suspected of causing allergies, hyperactivity or being carcinogenic, neurotoxic or endocrine disruptors (Cardinale F et al 2009; Soffritti M et al 2010; Stevens LJ et al 2013; Towers CV et al 2014). For the additives that represent a concern for the consumers' health an ADI is established that is defined as the amount of a substance in food or drinking water, expressed on a body-weight basis that can be ingested daily over a lifetime without appreciable risk.

In recent years, the European regulation requires that member states to monitor the intake of food additives of the population in order to ensure that the ADI is not exceeded. For this purpose, the European commission recommended a stepwise method to estimate the additive intakes (European Commission 2001). The first tier uses the Budget method and concerns all the additives for which an ADI and maximum permitted level (MPLs) are established (Hansen S 1979; Machinski JM 1998). It estimates the theoretical maximum daily intake (TMDI) by combining the maximum quantity of food and drinks that an individual consumes with the MPLs of the additive. When the TMDI exceed the ADI, the second tier (Tier 2) is carried out by using actual national food consumption data and MPLs (Verger P et al 1998). For the additives that exceed the ADI, a third tier (Tier 3) is performed using individual food consumption data and additives' measured concentrations (Bemrah N et al 2008). Since measured concentration data are in many cases difficult to obtain, other authors have suggested assuming the presence of the additive at the MPL only when reported in the label of the food product and combining it with the actual national food consumption data. This approach is defined as the Tier 2a in this study (Diouf F et al 2014) (Figure 1).

Infants and children are considered more vulnerable to chemicals since exposure occurs during the development process (Makri A et al 2004; Landrigan PJ et al 2004; Sly PD and Flack F 2008; Diamanti-Kandarakis E et al 2009). Moreover, the rate of consumption on weight, used to calculate exposure, is higher in these populations. Considering their immature organ systems, rapid physical development and higher metabolic rates, infants and children must therefore be treated as separate sub-groups when assessing additive intake.

The objective of the current study is to estimate the intake of seven food additives, considered of the highest priority, in the French population that is 1-36 months old using MPLs, occurrence data and actually measured concentration data in order to estimate the risk for this population through the diet. Even if the seven additives included in the current study are not authorised for use in food intended for infants' consumption, it is important to consider that children can be exposed to these

additives through the consumption of current foods (vegetables, meats, sweets, drinks and others), especially knowing that fewer than 25% of French new-borns are still breastfed at the age of 6 months (French Ministry 2011; Salanave et al., 2014).

Namely the additives included in our study are as follows:

- Benzoates, for which an ADI of 5 mg / kg bw / day has been established since it appears that, under certain conditions, especially in the presence of ascorbic acid, benzoates can induce the formation of benzene which is carcinogenic to humans (Aprea E et al 2008).
- Parabens, which are suspected to interfere with the reproductive system and hormonal response, potentially causing fertility problems and for which the ADI has been established as 10 mg / kg bw / day (EFSA 2004; Tavares RS et al 2009; Vo TTB et al 2010).
- Nitrites and nitrates, which are widely used in the food chain as antimicrobial additives and colour stabilisers. The ADI for nitrite and nitrate is 0.07 mg / kg bw / day and 3.7 mg / kg bw / day respectively. The chronic toxic effects are due to the reaction of nitric oxide with amines (products derived from amino acid metabolism) that can lead to the formation of nitrosamines, which are carcinogenic (Fan AM and Steinberg VE 1996; Manassaram DM et al 2006).
- BHA and BHT for which EFSA established ADIs of 1mg/kg bw/day and of 0.25 mg/kg bw/day respectively based on the possible endocrine disruptors and carcinogenic effect of these two food preservatives (IARC 1987; Jos A et al 2005; EFSA 2012).
- Aspartame's ADI was set at 40 mg/kg bw/day based on the fact that it can exert neurotoxic effects when consumed in large quantities (Tsakiris S et al 2006; Simintzi I et al 2007).

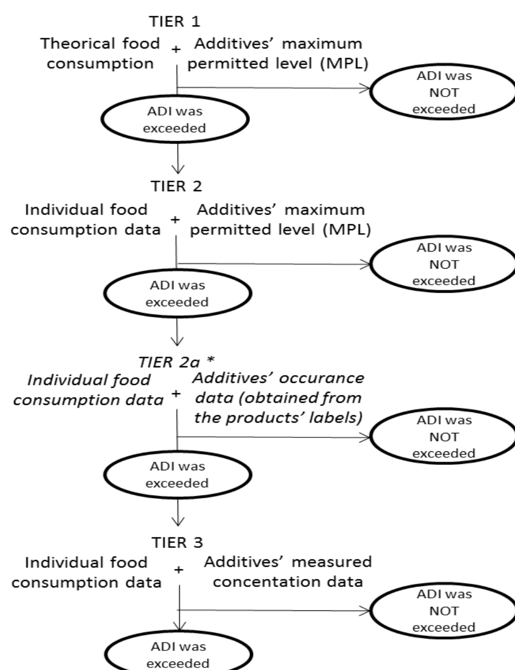


**Figura 1**: explanatory diagram of the Tier approach applied for the estimation of food additives' dietary exposure recommended by the European Commission (2001).
TIER 2a * refers to the tier applied in this article and in the that of Diouf F et al (2014) but not recommended by the European Commission

## 2.2 Material and Method

1. Food consumption data

Consumption data were collected from BEBE-SFAE dietary survey, the last published French survey on individual dietary consumption in children under 3 years old (Fantino M and Gourmet E 2008). The survey was conducted for the French Association for Children's Food (SFAE) over the period from 12 January to 10 March 2005 on a random sample of French children aged less than 3 years. Selection was made through a proportionate quota sampling based on the age of the children, the occupation of the mother and the family's socioeconomic category. Consumption data of all food (solid or liquid) and drinks ingested by the child were collected in a food diary during 3 consecutive days (usually including a weekend day because consumption is different from the other days of the week) by people taking care of children (their mother, father or carer). Breastfed infants at the time of the study were excluded because it would have been very difficult to properly quantify their consumption of breast milk properly; children attending a nursery or kindergarten were excluded as well from this survey because of the impossibility of collecting properly the consumption information. Information on each food intake, weight (or volume) of food ingested, the exact name of the product and the brand, the method of preparation and the detailed recipe of all foods prepared at home were reported in a questionnaire. For each food or drink, the amount consumed was estimated by weighing with a household scale using the available information on the packaging or picture-calibrated portions.

Dietary data were collected on 706 children categorized into four age groups: 1-4 months (n=124), 5-6 months (n=127), 7-12 months (n=196) and 13-36 months (n=259).

2. Theoretical concentration data

A database on food additives was developed under the European FACET research project (Flavourings Additives Contact materials Exposure Task) (Vin K et al 2013). This database contains the MPLs of additives in different food categories according to Regulation (EC) 1333/2008. From this database, MPLs of the corresponding additives were linked to the food products present in the BEBE SFAE 2005 dietary survey.

For those additives (benzoate, parabens, aspartame) that are allowed only in a specific part of the food product, such as topping and / or filling we assumed that the percentage of filling was always equal to 70 % of the total weight of the food item considered, in order to be conservative.

3. Occurrence data

Occurrence data were obtained from the French Observatory of Food Quality database (Oqali), which centralises, at the branded product level, all nutritional data provided on labels (such as the ingredients list) to monitor processed food quality, over time (see www.oqali.fr) (Menard et al 2011).

The project also monitors ingredient occurrences, such as additives (Oqali 2012). To date, almost all processed products are followed by an Oqali food sector (with data collected between 2008 and 2011 for the present study). The estimated coverage of the Oqali database for the different food categories considered goes from 50% to more than 80% of the cumulative market share of the products on the market.

4. Measured concentration data

Measured concentration data were obtained by the second French Total Diet Study (TDS2) for nitrites. Samples of foodstuffs (n = 1319) were purchased from June 2007 to January 2009 in eight greater regions of the French metropolitan territory (when consumed in the region), and each food sample was collected, when possible, during two different seasons to take into account possible differences in chemicals' concentration. Samples were prepared as consumed by the population according to the cooking habits recorded in the second individual and national study on food consumption (INCA2). The corresponding sampling methodology has already been described in detail elsewhere (Sirot V et al 2009).

The analyses were performed by the laboratories of the Joint Service Laboratory (SCL) of the Directorate General for Competition, Consumer Affairs and Fraud Control (DGCCRF) and the Directorate General of Customs and Excise. Additional details on analytical methods are available elsewhere (Bemrah N et al 2012).

5. Exposure Assessment

A stepwise approach was used in order to calculate dietary exposure to the seven additives included in this study, as recommended by the European Union (European Commission 2001).

As individual food consumption data were available, a Tier 2 approach was directly performed by combining individual food consumption data with the MPLs (defined by Regulation 1333/2008) for the seven food additives. The intake was estimated by multiplying the average intake of food products over the 3 days of survey by the MPL of the additive in that food. Specifically the exposure was assessed individually according to the following formula:

$$E_{i,j} = \sum_{k=(1;n)} C_{i,k} * MPL_{k,j} / BW_i$$

Where $E_{i,j}$ is the exposure to the additive j for the subject $i$, $C_{i,k}$ is the consumption level of the food $k$ by the subject $i$, $MPL_{k,j}$ is the maximum permitted level of additive $j$ in the food $k$, $BW_i$ is the body weight of the subject $i$ and $n$ is the total number of food considered. This equation is used in all the tiers developed in this study.

For those additives that overstepped the ADI, the intake was reassessed by taking into account the data of occurrence (Tier 2a): for each food item for which the additive was authorised, the food additive was considered to be present at a concentration equal to the MPL according to the

occurrence data obtained from the label information. A random sample that reproduced the proportion of food items containing the additive within the food categories was carried out to reproduce with a probabilistic approach the reality of use of the additive among the foods on the market. The proportion of food products within the food category that reported the additive on the label was obtained through the Oqali database and used for the random sampling. For those food products for which no label information was available, we made two assumptions: first we assumed that the additive was present in 100% of the food products without label information (upper-bound scenario); and second, we assumed that the additive was present in 0% of the products without label information (lower-bound scenario).

Finally, in the Tier 3 the MPLs were replaced with the actual additives' concentration levels measured in the food items, when available from the French TDS2. The contribution of each food category was estimated as the percentage of intake attributed to the food category considered compared with the total intake of the additives taken into account in the three tiers. In Tier 2a the percentage of contribution of the different food categories to the total intake of benzoates, nitrites and BHA was calculated for both the lower- and for the upper-bound scenario.

The data presented in the context of this work are the mean, median and 90th percentile of the population considered, as recommended by EFSA due to the limited number of children in each age group (EFSA 2009).

Calculations were performed with SAS 9.3 software (Copyright © 2009, SAS Institute Inc., Cary, NC, USA).

## 2.3 Results

*Exposure calculated with MPLs (Tier 2)*

Exposure (mean, median, $90^{th}$ percentile and per cent of individuals exceeding the ADI) to the seven selected food additives per age group calculated using the MPLs for the whole population (consumers and non-consumers) is reported in table 1.

For benzoate, nitrites and BHA, the exposure was above the ADI for a significant percentage of individuals considered in this study.

For paraben, nitrates and BHT the exposure was below the ADI in all age classes, while aspartame exposure exceeded the ADI (40 mg/kg bw/day) only for less than 1% [95% CI 0-1.6%] of the age group 13-36 months, corresponding to one subject. In general for all additives the exposure increased with the age reaching the highest exposure levels in the age group 13-36 months. The age class 1-4 months had a mean exposure level equal to zero for all additives except aspartame, where the mean exposure was 0.20 mg/kg bw/ day.

Results will be presented in more detail for the three additives that exceed the ADI for some consumers. The main food categories that have been identified as contributors for the intake of the seven additives considered in this study are shown in Appendix A.

In all age groups except 1-4 months, a proportion of subjects had a benzoate exposure above the ADI (5 mg/kg bw/day). The class with the highest percentage of children for which the intake was above the ADI was the age group 13-36 months (35% [95% CI 31-39%]) (Table 1). Exposure to benzoate was mainly due to the consumption of "vegetables soups and broths" for children aged 5 to 36 months, with a maximum contribution of nearly 100% of the total intake among the age group 5-6 months. The 12% and 20% of benzoate's intake was ascribable respectively to "sweet or savory biscuits and bars" consumption in the ages groups 7-12 and 13-36 months. Other food categories that contributed to benzoate's intake in the age groups 7-12 and 13-36 months were "fish" and "cold drinks without alcohol" (see Appendix A).

Exposure to nitrites of the total population considered in this study was on average equal to 0.09 mg/kg bw/day. Among the population 7-12 months old and 13-36 months old, nitrites' exposure was significantly above the ADI (0.07 mg/kg bw/day) respectively for 23% [95% CI 16-31%] and 74% [95% CI 70-78%] of individuals (Table 1). "Delicatessen" and "Meat" were the main contributors for the age groups 7-12 and 13-36 months, while "Mixed dishes" represented nearly the 10% only in the age group 13-36 months (see Appendix A).

Concerning exposure to BHA, the ADI (1 mg/kg bw/day) was significantly exceeded for the age groups 7-12 months (13% [95% CI 7-19%]) and 13-36 months (26% [95% CI 22-30%]) (Table 1). The exposure to BHA for the total population considered in this study was on average 0.39 mg/kg bw/day. The main contributor of BHA's intake in all age groups was "vegetables soups and broths". The second most relevant contributor to BHA's intake in the age group 7-12 months was "rice and durum wheat". Among the age group 13-36 months, other food categories that contributed to the BHA intake were "condiments and sauces", "mixed dishes", "rice and durum wheat" and "delicatessen" (see Appendix A).

**Table 1**: Estimated exposure (mg/kg bw/day) and percentage of individuals exceeding the ADI of benzoate, parabene, nitrite, nitrate, BHA, BHT and aspartame per age group calculated using the maximum permitted levels (Tier 2).

| Additive | Age group (*N* subjects) | Mean (SD) | Median (min-max) | 90° percentile | Percentage *N*>ADI (95% CI) |
|---|---|---|---|---|---|
| Benzoates | 1-4 months (124) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 5-6 months (127) | 0.28 (0.79) | 0 (0-9.87) | 0 | 3.4% (0 - 9.1) |
| | 7-12 months (195) | 1.32 (2.37) | 0 (0-18.69) | 4.96 | **8.8% (3.7 - 14.0)*** |
| | 13-36 months (259) | 4.28 (5.55) | 3.525 (0-27.94) | 9.22 | **34.9% (30.6 - 39.2)*** |
| Parabens | 1-4 months (124) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 5-6 months (127) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 7-12 months (195) | 0.04 (0.10) | 0 (0-0.89) | 0.07 | 0 |
| | 13-36 months (259) | 0.35 (0.79) | 0.175 (0-4.50) | 0.90 | 0 |
| Nitrites | 1-4 months (124) | 0.00 (0.03) | 0 (0-0.61) | 0 | 0.4% (0 - 1.8) |
| | 5-6 months (127) | 0.01 (0.04) | 0 (0-0.60) | 0 | 3.3% (0 - 8.9) |
| | 7-12 months (195) | 0.04 (0.08) | 0 (0-0.52) | 0.18 | **23.4% (15.7 - 31.1)*** |
| | 13-36 months (259) | 0.20 (0.29) | 0.135 (0-1.11) | 0.51 | **74.3% (70.3 - 78.2)*** |
| Nitrates | 1-4 months (124) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 5-6 months (127) | 0.01 (0.00) | 0 (0 - 0.08) | 0 | 0 |
| | 7-12 months (195) | 0.01 (0.04) | 0 (0 - 0.31) | 0 | 0 |
| | 13-36 months (259) | 0.11 (0.21) | 0.048 (0 - 0.78) | 0.31 | 0 |
| BHA | 1-4 months (124) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 5-6 months (127) | 0.13 (0.33) | 0 (0-3.95) | 0 | 4.9% (0 - 11.6) |
| | 7-12 months (195) | 0.33 (0.88) | 0 (0-6.74) | 1.39 | **12.9% (6.8 - 19)*** |
| | 13-36 months (259) | 0.72 (1.57) | 0.15 (0-8.00) | 2.31 | **26.3% (22.3 - 30.3)*** |
| BHT | 1-4 months (124) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 5-6 months (127) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 7-12 months (195) | 0 (0) | 0 (0-0) | 0 | 0 |
| | 13-36 months (259) | 0.00 (0.01) | 0 (0-0.08) | 0 | 0 |
| Aspartame | 1-4 months (124) | 0.19 (1.12) | 0 (0-15.71) | 0 | 0 |
| | 5-6 months (127) | 2.14 (2.68) | 0 (0-24.61) | 8.58 | 0 |
| | 7-12 months (195) | 7.41 (6.50) | 4.109 (0-34.65) | 19.03 | 0 |
| | 13-36 months (259) | 14.11 (10.93) | 12.632 (0-58.84) | 23.93 | 0.8% (0- 1.6) |

* = Confidence Interval not including zero

*Exposure calculated with occurrence data (Tier 2a)*

Additives for which the estimated exposure significantly exceeded the ADI in the Tier 2 approach (benzoate, nitrite and BHA) were reassessed by using occurrence data. Occurrence data are presented in detail in Table 2.

Occurrence data for benzoates were available for 65% of the food products in which it is permitted and among these it was found on the labeling for 14% of them. As shown in Table 3, the mean and 90$^{th}$ percentile of exposure to benzoates were equal to zero for children from 1 to 6 months in both the upper- and lower-bound scenarios. When considering the age groups 7-12 and 13-36 months, the mean exposure in the upper-bound scenario was 0.14 mg/kg bw/day (90$^{th}$ percentile exposure: 0 mg/kg bw/day) and 0.79 mg/kg bw/day (90$^{th}$ percentile exposure: 3.03 mg/kg bw/day) respectively. In the lower-bound scenario the mean exposure was equal to 0 mg/kg bw/day (90$^{th}$ percentile exposure= 0 mg/kg bw/day) for the age group 7-12 months and to 0.02 mg/kg bw/day (90$^{th}$ percentile exposure: 0 mg/kg bw/day) for the age group 13-36 months. A significant excess of the ADI was reported only for the age group 13-36 months in the upper-bound scenario (1.9% [95% CI 0.7-3.2%]).

In the upper-bound scenario the food category identified as the main contributor to the benzoates intake in the age groups 7-12 and 13-36 months, was “Fish”. But when having a closer look at the occurrence data, for the “Fish” category no data on the occurrence of benzoates were available, so the 100% occurrence was assumed. In the lower-bound scenario the main contributor to the benzoates intake was in fact “Cold drinks without alcohol” for both age groups (see Appendix B).

Occurrence data for nitrites were available for 80% of the food products and the average occurrence was 88%. In the upper-bound scenario the average exposure varied from 0 mg/kg bw/day (90$^{th}$ percentile exposure= 0 mg/kg bw/day) in the age group 1-4 months to 0.18 mg/kg bw/day (90$^{th}$ percentile exposure= 0.45 mg/kg bw/day) in the age group 13-36 months, while in the lower-bound scenario it varied from 0 mg/kg bw/day (90$^{th}$ percentile exposure= 0 mg/kg bw/day) to 0.16 mg/kg bw/day (90$^{th}$ percentile exposure= 0.43 mg/kg bw/day) respectively in the age group 1-4 months and 13-36 mg/kg bw/day. Zero values for the 90$^{th}$ percentile are explained by a rate of exposed consumers lower than 10%. Nitrites’ ADI was exceeded significantly by 15.9% [95% CI 9.3-22.6%] of the individuals in the age groups 7-12 months and 57.6% [95% CI 53.2-62.1%] in the age group 13-36 months when considering the upper bound scenario, while 14.3% [95% CI 7.9-20.6%] and 51.9%[95% CI 47.4-56.4%%] in the lower-bound scenario (Table 3).

The food category that contributed the most in all age groups was “Delicatessen” in both scenarios hypothesized (see Appendix B).

Finally BHA’s occurrence data covered 83% of the food products in which they are permitted and the average of occurrence was 28%. In the lower-bound scenario the mean exposure of BHA was equal

to zero for all age groups except for the age group 13-36 months (mean exposure= 0.03, mg/kg bw/day; 90$^{th}$ percentile exposure= 0 mg/kg bw/day). When considering the upper-bound scenario, the mean exposure for the age groups 5-6, 7-12 and 13-36 months was 0.02 mg/kg bw/day (90$^{th}$ percentile exposure= 0 mg/kg bw/day), 0.02 mg/kg bw/day (90$^{th}$ percentile exposure= 0 mg/kg bw/day) and 0.03 mg/kg bw/day (90$^{th}$ percentile exposure= 0.01 mg/kg bw/day) respectively. A significant excess of the ADI was estimated only in the upper-bound scenario for the age group 13-36 months (1.1% [95% CI 0.2 - 2.1]) (Table 3). “Vegetables soups and broths” was the food category that contributed most to BHA intake, except for the age group 5-6 months where the main intake in was due to “Condiments and sauces”, more specifically to the food product “Meat bouillon cube”.

**Table 2**: Additives' percentage of occurrence in the food categories included in the current study calculated using the French Observatory of Food Quality (Oqali) data, separately under the upper- and lower-bound scenarios' assumptions.

| | Food categories | Number of products taken into account within the food category | Percentage of products containing the additive in the upper-bound scenario | Percentage of products containing the additive in the lower-bound scenario |
|---|---|---|---|---|
| Benzoates | Chocolate | 174 | 0% | 0% |
| | Cold drinks without alcohol | 1002 | 22.6% | 4.5% |
| | Condiments and sauces | 340 | 31.4% | 0.7% |
| | Crustaceans and molluscs | 0 | 100% | 0% |
| | Deserts and cakes | 223 | 33.3% | 0% |
| | Desserts, puddings and jellied milks | 432 | 0.3% | 0.3% |
| | Dried fruits and oilseeds | 0 | 100% | 0% |
| | Fish | 0 | 100% | 0% |
| | Hot drinks | 58 | 0% | 0% |
| | Ice cream and frozen desserts | 267 | 0% | 0% |
| | Mixed Dishes | 126 | 20.0% | 0% |
| | Pastries | 5 | 0% | 0% |
| | Pizzas, quiches and savoury pastries | 117 | 34.3% | 1.0% |
| | Potatoes and derivates | 0 | 100% | 0% |
| | Sandwiches and snacks | 68 | 0% | 0% |
| | Soups and broths | 358 | 0% | 0% |
| | Sweets and derivates | 886 | 23.1% | 0% |
| | Vegetables (excluding potatoes) | 169 | 33.3% | 0% |
| Nitrites | Delicatessen | 449 | 96.3% | 96.3% |
| | Meat | 0 | 100% | 0% |
| | Mixed Dishes | 132 | 65.4% | 65.4% |
| | Offal | 0 | 100% | 0% |
| | Pizzas, quiches and savoury pastries | 136 | 85.3% | 52.0% |
| | Sandwiches and snacks | 68 | 81.0% | 81.0% |
| BHA | Breakfast cereals | 329 | 0% | 0% |
| | Chocolate | 51 | 0% | 0% |
| | Condiments and sauces | 340 | 18.2% | 0% |
| | Delicatessen | 185 | 0% | 0% |
| | Dried fruits and oilseeds | 0 | 100% | 0% |
| | Dried vegetables | 40 | 0% | 0% |
| | Fish | 0 | 100% | 0% |
| | Fresh dairy products | 107 | 0% | 0% |
| | Ice cream and frozen desserts | 118 | 0% | 0% |
| | Mixed Dishes | 230 | 17.5% | 5% |
| | Pizzas, quiches and savoury pastries | 117 | 33.3% | 0% |
| | Potatoes and derivates | 59 | 50.0% | 0% |
| | Rice and durum wheat | 0 | 100% | 0% |
| | Sandwiches and snacks | 68 | 0% | 0% |
| | Soups and broths | 358 | 2.0% | 2.5% |
| | Sweet or savoury biscuits and bars | 517 | 5.0% | 5.0% |
| | Vegetables (excluding potatoes) | 40 | 50.0% | 0% |

**Table 3**: Estimated exposure (mg/kg bw/day) and percentage of individuals exceeding the ADI of benzoate, nitrite and BHA per age group calculated using the occurrence data under the upper- and lower-bound assumption (Tier 2a).

| Additive | | Age group (*N* subjects) | Mean (SD) | Median (min-max) | 90° percentile | Percentage N>ADI (95% CI) |
|---|---|---|---|---|---|---|
| Benzoates | Upper-bound | 1-4 months (124) | 0 | 0 | 0 | 0 |
| | | 5-6 months (127) | 0 | 0 | 0 | 0 |
| | | 7-12 months (195) | 0.14 (0.55) | 0 (0-6.35) | 0 | 1.1 (0 - 2.9) |
| | | 13-36 months (259) | 0.79 (1.99) | 0 (0-9.36) | 3.03 | 2.0 (0.7 - 3.2)* |
| | Lower-bound | 1-4 months (124) | 0 | 0 | 0 | 0 |
| | | 5-6 months (127) | 0 | 0 | 0 | 0 |
| | | 7-12 months (195) | 0 | 0 | 0 | 0 |
| | | 13-36 months (259) | 0.02 (0.25) | 0 (0-2.46) | 0 | 0 |
| Nitrites | Upper-bound | 1-4 months (124) | 0.00 (0.03) | 0 (0- 0.61) | 0 | 0.4 (0 - 1.9) |
| | | 5-6 months (127) | 0.01 (0.02) | 0 (0- 0.28) | 0 | 1.7 (0 - 5.7) |
| | | 7-12 months (195) | 0.03 (0.06) | 0 (0- 0.50) | 0.17 | 15.9 (9.3 - 22.6)* |
| | | 13-36 months (259) | 0.18 (0.28) | 0.11 (0- 1.12) | 0.45 | 57.6 (53.2 - 62.1)* |
| | Lower-bound | 1-4 months (124) | 0.00 (0.03) | 0 (0-0.61) | 0 | 0 |
| | | 5-6 months (127) | 0.01 (0.04) | 0 (0-0.60) | 0 | 2.4 (0-7.2) |
| | | 7-12 months (195) | 0.03 (0.05) | 0 (0-0.44) | 0.13 | 14.3 (7.7-20.7)* |
| | | 13-36 months (259) | 0.16 (0.25) | 0.11 (0-1.05) | 0.43 | 51.9 (47.4-56.4)* |
| BHA | Upper-bound | 1-4 months (124) | 0 | 0 | 0 | 0 |
| | | 5-6 months (127) | 0.02 (0.14) | 0 (0 - 2.71) | 0 | 0.9 (0-3.7) |
| | | 7-12 months (195) | 0.02 (0.24) | 0 (0 - 6.02) | 0 | 0.3 (0-1.2) |
| | | 13-36 months (259) | 0.03 (0.20) | 0 (0 - 1.24) | 0.01 | 1.1 (0.2 - 2.1)* |
| | Lower-bound | 1-4 months (124) | 0 | 0 | 0 | 0 |
| | | 5-6 months (127) | 0.0 (0.1) | 0 (0-2.3) | 0 | 0.9 (0 -3.7) |
| | | 7-12 months (195) | 0 | 0 | 0 | 0 |
| | | 13-36 months (259) | 0.0 (0.6) | 0 (0-6.8) | 0 | 0.8 (0 - 1.5) |

* = Confidence Interval not including zero

*Daily intake estimated with actual measured concentration data (Tier 3)*

Actual measured concentration data were available exclusively for nitrites for which the ADI was significantly exceeded both in the upper- and lower-bound scenarios within Tier 2a. As shown in table 4, exposure to nitrite, estimated using the measured concentration data, is below the ADI in all four age groups considered in this study. The highest mean exposure is about 0.01 mg/kg bw/day observed in the 13-36 months age group, which is more than 10 times lower than the nitrite's ADI (0.07 mg/kg bw/day). Also considering high consumers of the most exposed age group ($90^{th}$ percentile of the 13-36 months age group), the exposure level is equal to 0.01 mg/kg bw/day, which is five times lower than the ADI.

**Table 4**: Estimated exposure (mg/kg bw/day) and percentage of individuals exceeding the ADI of Nitrites, per age group calculated using the concentration values measured in foods (Tier 3).

| Age group (*N* subjects) | Mean (SD) | Median (min-max) | 90° percentile | Percentage *N*>ADI (95% CI) |
|---|---|---|---|---|
| 1-4 months (124) | 0 (0) | 0 (0-0.01) | 0 | 0 |
| 5-6 months (127) | 0 (0) | 0 (0-0.01) | 0 | 0 |
| 7-12 months (195) | 0.00 (0.00) | 0 (0-0.41) | 0.00 | 0 |
| 13-36 months (259) | 0.01 (0.01) | 0 (0-0.03) | 0.01 | 0 |

## 2.4 Discussion and conclusion

In this study a stepwise approach was used to determine the exposure to seven additives in the French population of 0-3 years old. First of all, the Tier 2 conservative approach– using individual consumption data and MPLs of additives - showed that the ADI for benzoate (E210-E213), nitrites (E249-E250) and BHA (E320) was exceeded in 25%, 54% and 20% of the population taken into account respectively. Conversely, for parabens (E214-E219), nitrates (E251-E252), BHT (E321) and aspartame (E951) the ADI was not exceeded in any age class. Based on the MPLs, the main contributors to the benzoates were "Vegetables soups and broths". The "Delicatessen" were the major source of nitrites, while concerning BHA, the food category "Vegetables soups and broths" was the major contributor to exposure.

Because food products in which additives are permitted, do not always contain them, a more refined intake estimate for the sole additives that overstepped the ADI was conducted including information on additives' occurrence (Tier 2a). From the Oqali database, occurrence data for benzoate, nitrites and BHA were available for 65%, 80% and 83% of the food products in which their use is permitted respectively. In the Tier 2a upper-bound scenario (assuming a presence of additive in 100% of the products for which occurrence data were not available) the estimated exposure to the three

additives resulted significantly lower than in the Tier 2 for all age groups, but still the ADI was significantly exceeded for benzoate, nitrites and BHA. A progressive decrease of the estimated exposure has been achieved in the Tier 2a lower-bound scenario (assuming that the additive was not present in those food products for which no occurrence data were available) where the ADI was not overstepped for benzoates and BHA, but a significant percentage of the population still had an intake above the ADI for nitrites.

When looking at the main contributors in Tier 2a, no large difference was highlighted for nitrites and BHA for which, respectively, “Delicatessen” and “Vegetables soups and broths” represented the major source of exposure both in the upper- and lower-bound scenario, just as in Tier 2. Different were instead the results obtained for benzoates: as said above, in the Tier 2 the food category “Vegetables soups and broths” was identified as the main contributor, while in the Tier 2a this food category was not present since the additive actually never occurred. Furthermore in the upper-bound scenario, benzoates’ main contributor was “Fish”, for which no occurrence data were available and 100% of presence was assumed, while in the lower-bound scenario “Cold drinks without alcohol” contributed the most, probably reflecting a more realistic condition.

Finally a Tier 3 exposure assessment was performed for nitrites, for which actual measured concentration data were available. In this case the estimated exposure to nitrites resulted much lower than in Tiers 2 and 2a and in no age class the ADI was overstepped.

The results of our study are not easily comparable to others due to different dietary behaviors in different countries, data availability, the targeted population, the studied food group etc.

Indeed according to our study the risk correlated to parabens, nitrates, BHT and aspartame can be excluded, since the ADI is not overstepped not even at Tier 2.

A study conducted in the United Sates estimated the daily intake of parabens in infants and toddlers as 940 and 879 ng/kg bw/day respectively (much lower than the ADI of 10 mg/kg bw/day) using the mean concentration value measured in foods and the mean daily ingestion rates of food items (Liao C et al 2013). A study concerning children 1-6 years old living in Estonia reported a mean daily intake of nitrates of 1.7 mg, and no subject exceeding the ADI when using actual concentration data (Reinik M et al 2005). Soubra and colleagues estimated using actual concentration data an intake of BHT above the ADI for 10% of students aged between 9 and 18 years old. This result may appear in contrast with what we found in our study, but this discrepancy is probably due to the populations examined, which belong to two very different age groups and therefore are characterized by different eating patterns (Soubra L et al 2007). As mentioned above, it is difficult to compare our findings with those of other studies because the population that we have taken into account (0-3 years) is characterized by very particular eating habits that differ from those of other age groups and there are almost no other studies for this age group. According to our results, aspartame’s exposure

in the French population aged 0-3 years is far below the ADI. This result is in accord with the results obtained from the European project FACET for which in the Tier 2 exposure estimation the aspartame's mean intake of children 1–4 years old was equal to 30% of the ADI (Vin K et al 2013).
Looking at our results, we cannot exclude the risk of exceeding the ADI for benzoates since a 2% of the age group 13-36 months had an exposure above 5 mg/kg bw/day in the upper-bound scenario of Tier 2a. Similar results were obtained by Bilau and colleagues for which 6% of the preschool children (aged 2-6 years old) had an exposure to benzoates higher than the ADI when multiplying individual consumption data with the MPLs for food groups (Bilau M et al 2008). In any case our results must be interpreted with cautions since for 35% of the food products the occurrence data were not available and we assumed that benzoates were used in all of them. It was not possible to carry out a Tier 3 to obtain a more realistic estimation.
Always the FACET project estimated that children's (1–4 years) intake of nitrites accounted for 103% of the ADI at Tier 2, but when using the real concentration data at Tier 3 a significant reduction of the potential intake was achieved and no intake value exceeding the ADI (Vin K et al 2013). These results are similar to those obtained in our study; in fact the risk of exceeding the nitrites ADI in children aged 0-3 years old could be excluded only when using actual concentration data (Tier 3).
According to our findings the risk of exposure to BHA above the ADI cannot be excluded for the age group 13-36 months. At our knowledge no studies on the exposure to BHA in children in pre-school age are available at this time. Soubra et al. (2007) estimated with a Tier 3 that the BHA's ADI was exceeded by fewer than 1% of children between 9 and 18 years old (Soubra L et al 2007). In our study for BHA it was not possible to perform a Tier 3 exposure assessment, which instead would be strongly recommended.
Bearing in mind that the seven additives investigated are not authorized in food intended for infants' consumption, the results of our study confirm that children aged less than 4 months are not usually fed with current food and this explains why the intake of the seven additives is much lower in this age group compared with the other age groups. The additives intake increases with age and this is due to the fact that current food becomes the main source of nutrition in older children.
We are aware of several limits of our study. The first point concerns the consumption data available for estimating additives intake. The BEBE-SFAE survey is based on a 3 days of food diary, so it does not reflect consumption over the long-term. In fact, this method does not take into account intra-individual variability in the long term, since we assume that the three days food consumption reflect the consumption pattern over the long run. It would therefore be necessary to repeat the survey over a wider time window including non-consecutive survey days and to analyze the data with more adequate models (usual intake models) (Hoffmann K et al 2002). Moreover the consumption data were collected in 2005, so it would be useful to collect updated consumption data.

The second point to consider is that consumers' brand loyalty may have an impact on exposure. Studies have shown that consumers tend always to buy products of a given brand, and some brands may have a higher concentration of additives than others (Leclercq C, et al 2000). This limits concerns the Tier 3 for nitrites since the concentration has been obtained by pooling together food products of different brands. Therefore a mean intake was obtained without taking into account brand loyalty: people who eat only food products without additive from those who always consume food products of a brand with high nitrite concentration levels were not differentiated.

Furthermore in our study only additives deliberately added to the normal diet were taken into account, while dietary supplements and natural sources have not been included in the exposure estimate. Indeed nitrites and nitrates are naturally present in some foods, especially in vegetables grown in greenhouses. Furthermore additives such as aspartame may be added to food integrators in order to make them more appetizing. However as we considered children under 3 years old, the use of dietary supplements is limited. It would be interesting to take into account all routes of exposure in the calculations in order to have a more comprehensive view of the exposure (for instance dermal exposure with cosmetic products).

Finally actual concentration data were not available for BHA and benzoates, for which the risk of exceeding the ADI by the French population aged 0-3 years could not be excluded with our study. Therefore, in the future survey, for all those additives that present a risk, occurrence data should be collected as well as the actual measured concentration levels of additives in foods in case the risk cannot be excluded in the previous step.

In conclusion our results confirm that Tier 2 can be considered a conservative approach since the actual occurrence of the additive may range from less than 30% to more than 80% of the food products in which it is allowed; the use of occurrence data may lead to a more realistic estimate of exposure to additives (Gilsenan MB et al 2003). On the other hand, the different results obtained with the lower- and the upper-bound scenarios of Tier 2a, especially for benzoates, emphasises the importance of collecting occurrence data on the greatest number of food products. Moreover the availability of occurrence data permits one to identify more realistically the food categories that contribute most to the exposure and on which eventually it is necessary to intervene. Furthermore the results obtained in Tier 3 for nitrites underline the importance of collecting actual measured concentration data for those additives for which the exposure is estimated above the ADI in Tier 2a. Even if less conservative than Tier 2, Tier 2a still assumes that the additive, when reported in the label, is used at a concentration equal to the MPL and this does not correspond to reality. Further refined exposure assessment is still needed.

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**Appendix A**: Food categories divided in food items with the percentage contribution to the intake of benzoate, parabene, nitrite, nitrate, BHA, BHT and aspartame in the French population aged less than 3 years old, estimated in Tier 2.

| | Age group | Food categories | Percentage contribution |
|---|---|---|---|
| Benzoates | 5-6 months | Hot drinks | 0,21% |
| | | Vegetables soups and broths | 99,79% |
| | 7-12 months | Desserts, puddings and jellied milks | 0,01% |
| | | Pizzas, quiches and savoury pastries | 0,02% |
| | | Crustaceans and molluscs | 0,37% |
| | | Chocolate | 0,67% |
| | | Hot drinks | 0,67% |
| | | Condiments and sauces | 0,89% |
| | | Mixed Dishes | 1,01% |
| | | Deserts and cakes | 1,36% |
| | | Cold drinks without alcohol | 4,09% |
| | | Fish | 9,75% |
| | | Sweet or savoury biscuits and bars | 11,63% |
| | | Vegetables soups and broths | 69,54% |
| | 13-36 months | Dried fruits and oilseeds | 0,01% |
| | | Potatoes and derivates | 0,03% |
| | | Sandwiches and snacks | 0,05% |
| | | Desserts, puddings and jellied milks | 0,10% |
| | | Hot drinks | 0,93% |
| | | Pizzas, quiches and savoury pastries | 1,11% |
| | | Crustaceans and molluscs | 1,14% |
| | | Vegetables (excluding potatoes) | 1,23% |
| | | Mixed Dishes | 2,81% |
| | | Sweets and derivates | 2,91% |
| | | Chocolate | 3,05% |
| | | Pastries | 3,30% |
| | | Condiments and sauces | 4,72% |
| | | Deserts and cakes | 5,62% |
| | | Cold drinks without alcohol | 10,87% |
| | | Fish | 11,07% |
| | | Sweet or savoury biscuits and bars | 19,04% |
| | | Vegetables soups and broths | 32,03% |
| Parabenes | 7-12 months | Cold drinks without alcohol | 0,03% |
| | | Chocolate | 4,87% |
| | | Deserts and cakes | 9,93% |
| | | Sweet or savoury biscuits and bars | 85,17% |
| | | Ice cream and frozen desserts | 0,01% |

| | | | |
|---|---|---|---|
| | 13-36 months | Breakfast cereals | 0,02% |
| | | Cold drinks without alcohol | 0,07% |
| | | Mixed Dishes | 0,40% |
| | | Dried fruits and oilseeds | 0,55% |
| | | Pizzas, quiches and savoury pastries | 1,03% |
| | | Sweets and derivates | 5,08% |
| | | Chocolate | 7,54% |
| | | Pastries | 8,16% |
| | | Deserts and cakes | 13,92% |
| | | Delicatessen | 14,42% |
| | | Sweet or savoury biscuits and bars | 48,80% |
| Nitrites | 1-4 months | Delicatessen | 100,00% |
| | 5-6 months | Delicatessen | 100,00% |
| | 7-12 months | Pizzas, quiches and savoury pastries | 0,18% |
| | | Meat | 14,00% |
| | | Delicatessen | 85,82% |
| | 13-36 months | Offal | 0,32% |
| | | Sandwiches and snacks | 0,51% |
| | | Pizzas, quiches and savoury pastries | 1,56% |
| | | Mixed Dishes | 7,48% |
| | | Meat | 14,44% |
| | | Delicatessen | 75,68% |
| Nitrates | 5-6 months | Cheese | 100,00% |
| | 7-12 months | Pizzas, quiches and savoury pastries | 1,26% |
| | | Mixed Dishes | 1,82% |
| | | Delicatessen | 22,72% |
| | | Cheese | 74,19% |
| | 13-36 months | Potatoes and derivates | 0,06% |
| | | Sweet or savoury biscuits and bars | 0,30% |
| | | Sandwiches and snacks | 0,64% |
| | | Mixed Dishes | 4,00% |
| | | Pizzas, quiches and savory pastries | 4,06% |
| | | Delicatessen | 16,05% |
| | | Cheese | 74,89% |
| BHA | 5-6 months | Condiments and sauces | 0,59% |
| | | Vegetables soups and broths | 99,41% |
| | 7-12 months | Potatoes and derivates | 0,03% |
| | | Fresh dairy products | 0,11% |
| | | Breakfast cereals | 0,13% |
| | | Condiments and sauces | 0,64% |
| | | Mixed Dishes | 0,70% |
| | | Rice and durum wheat | 2,43% |
| | | Vegetables soups and broths | 95,96% |

| | | | |
|---|---|---|---|
| | 13-36 months | Ice cream and frozen desserts | 0,00% |
| | | Sandwiches and snacks | 0,06% |
| | | Dried vegetables | 0,08% |
| | | Potatoes and derivates | 0,14% |
| | | Dried fruits and oilseeds | 0,18% |
| | | Vegetables (excluding potatoes) | 0,27% |
| | | Sweet or savoury biscuits and bars | 0,70% |
| | | Pizzas, quiches and savoury pastries | 0,84% |
| | | Delicatessen | 1,09% |
| | | Rice and durum wheat | 1,72% |
| | | Mixed Dishes | 3,20% |
| | | Condiments and sauces | 5,37% |
| | | Breakfast cereals | 9,15% |
| | | Vegetables soups and broths | 77,20% |
| BHT | 13-36 months | Mixed Dishes | 14,92% |
| | | Delicatessen | 85,08% |
| Aspsrtame | 1-4 months | Fresh dairy products | 14,44% |
| | | Cooked fruit puree | 85,56% |
| | 5-6 months | Chocolate | 0,03% |
| | | Hot drinks | 0,11% |
| | | Cooked fruit puree | 0,80% |
| | | Vegetables soups and broths | 3,29% |
| | | Desserts, puddings and jellied milks | 5,12% |
| | | Fruit | 20,09% |
| | | Fresh dairy products | 70,56% |
| | 7-13 months | Chocolate | 0,23% |
| | | Deserts and cakes | 0,24% |
| | | Vegetables soups and broths | 0,89% |
| | | Sweet or savoury biscuits and bars | 1,38% |
| | | Vegetables soups and broths | 2,72% |
| | | Cold drinks without alcohol | 2,92% |
| | | Desserts, puddings and jellied milks | 6,45% |
| | | Fruit | 8,45% |
| | | Cooked fruit puree | 9,59% |
| | | Fresh dairy products | 67,12% |
| | | Mixed Dishes | 0,03% |
| | | Dried fruits and oilseeds | 0,05% |
| | | Pizzas, quiches and savoury pastries | 0,08% |
| | | Ice cream and frozen desserts | 0,32% |
| | | Breakfast cereals | 0,44% |
| | | Milk | 0,48% |
| | | Pastries | 0,67% |
| | | Sweet or savoury biscuits and bars | 0,73% |

| | | |
|---|---|---|
| | Hot drinks | 1,12% |
| | Deserts and cakes | 1,30% |
| | Vegetables soups and broths | 2,17% |
| | Chocolate | 3,34% |
| | Sweet or savoury biscuits and bars | 3,87% |
| | Cooked fruit puree | 7,77% |
| | Cold drinks without alcohol | 13,80% |
| | Desserts, puddings and jellied milks | 13,91% |
| | Fruit | 16,53% |
| | Fresh dairy products | 33,39% |

**Appendix B**: Food categories divided in food items with the percentage of contribution to the intake of benzoate, nitrite and BHA in the French population aged less than 3 years old, estimated in the Tier 2a lower- and upper-bound scenarios.

| | Age group | Food categories | Percentage contribution in the upper-bound scenario | Percentage contribution in the lower-bound scenario |
|---|---|---|---|---|
| Benzoates | 7-12 months | Cold drinks without alcohol | 1,47 | 100% |
| | | Crustaceans and molluscs | 3,61 | |
| | | Fish | 94,92 | |
| | 13-36 months | Dried fruits and oilseeds | 0,07 | |
| | | Vegetables (excluding potatoes) | 0,07 | |
| | | Potatoes and derivates | 0,14 | |
| | | Mixed Dishes | 0,87 | |
| | | Pizzas, quiches and savoury pastries | 2,25 | 14,90% |
| | | Condiments and sauces | 2,8 | 2,90% |
| | | Crustaceans and molluscs | 6,13 | |
| | | Cold drinks without alcohol | 6,44 | 82,20% |
| | | Deserts and cakes | 9,47 | |
| | | Sweets and derivates | 11,11 | |
| | | Fish | 59,7 | |
| Nitrites | 1-4 months | Delicatessen | 100 | 100% |
| | 5-6 months | Delicatessen | 100 | 100% |
| | 7-12 months | Meat | 14,75 | |
| | | Delicatessen | 85,25 | 100% |
| | 13-36 months | Offal | 0,35 | |
| | | Sandwiches and snacks | 0,45 | 0,60% |
| | | Pizzas, quiches and savoury pastries | 1,27 | 1,70% |
| | | Mixed Dishes | 5,92 | 7,40% |
| | | Meat | 15,62 | |
| | | Delicatessen | 76,39 | 90,30% |
| BHA | 5-6 months | Condiments and sauces | 100 | |
| | 7-12 months | Vegetables soups and broths | 100 | |
| | 13-36 months | Mixed Dishes | 0,02 | 0,20% |
| | | Fish | 0,06 | |
| | | Sweet or savoury biscuits and bars | 0,29 | |
| | | Potatoes and derivates | 0,43 | |
| | | Pizzas, quiches and savoury pastries | 0,78 | |

| | | | |
|---|---|---|---|
| | Condiments and sauces | 1,63 | |
| | Dried fruits and oilseeds | 2,52 | |
| | Vegetables (excluding potatoes) | 3,34 | |
| | Rice and durum wheat | 24,62 | |
| | Vegetables soups and broths | 66,32 | 99,80% |

# Chapter 3.

# Use and impact of usual intake models on dietary exposure estimate and risk assessment of chemical substances: a practical example for cadmium, acrylamide and sulphites.

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*Abstract*

Intake data collected for a short period are limited estimator of long-term usual intake; indeed the within-person variability inflates the intake distribution leading to biased estimation of extreme percentiles. Statistical models, named usual-intake-models, that separate the within-person variability from the between-persons variability have been implemented. Our objectives were to highlight the impact that usual-intake-models on exposure estimate and risk assessment and to emphasize the aspects to consider when running these models and correctly interpret the output.
We used the consumption data obtained by the French survey INCA2 and the concentration data of the French TDS2, using MonteCarlo Risk Assessment (MCRA) software, release 8.0.
For the substances considered (cadmium, acrylamide, sulphites) upper percentiles' exposure were significantly reduced when using usual-intake-models compared to the observed-individual-mean models, even if in terms of risk assessment the impact of usual-intake-models was limited. Aspects to consider when using usual-intake-models are: the normality of the log-transformed intake distribution, the contribution per single food group to the total exposure, the independency of food consumption data on multiple days.
In conclusion, usual-intake-models may have an impact on exposure estimates although, referring to our results, it didn't bring to changes in terms of risk assessment but further investigations are needed.

## 3.1 Introduction

When assessing dietary intake among populations or individuals, investigators are often interested in capturing usual intakes, which is defined as the long-run average of daily intakes of a substance by an individual. Estimation of the usual intake distribution can be considered less complicated than that of acute intake distribution, because fluctuations in the chemical concentration in food can be assumed to average out in the long run. Hence, the chemical's concentration in foods can be represented by the mean. The difficulty of usual intake estimation is that it is not feasible to collect individuals' food consumption data over long periods of time (De Boer et al., 2009). Two to seven 24-h dietary recalls

(24-HDR) per individual is the most frequently employed tool in food surveys. The 24-HDR provides rich details about dietary intake for a given day, but collecting more than seven 24-HDRs per individual is impractical (Tooze et al., 2006; Thompson and Subar 2013).

Most of the time, intake distribution are calculated by multiplying food concentration data with the average food intake over a small number of 24-HDR data, this models are named Observed Individual Mean models (OIM). These models do not separate the between-individual variability from the within-individual variability. Consequently OIM models do not adequately represent individual usual intakes since the total variance is inflated by the day-to-day variation, resulting in misleading estimates of the prevalence of low or high intakes (Mackerras et al., 2005; Dodd et al., 2006; Van Klaveren et al. 2012).

Therefore, to estimate usual dietary intake it is necessary to use statistical methods that separate the day-to-day variation (intra-individual variation) from the between individuals variation (inter-individual variation), whereas for long-term exposure modelling only the second source of variation is relevant (Beaton et al., 1979; Boon et al., 2011). All the usual intake models have the common idea to eliminate the intra-individual variability of the short-term measurements or at least to negate its impact on the estimated usual intake distribution since in the long-run the variation between days of the same individual will level out (Hoffmann et al., 2002; Dodd et al., 2006; Roodenburg et al., 2013). Examples of usual intake models are the betabinomial-normal (BBN) and the lognormal-normal model (LNN) (Tran et al., 2004;Dodd et al., 2006; De Boer et al., 2009; Huybrechts et al., 2011). Both the BBN and the LNN are two-part models, these models are based on the principle of separately modelling intake frequencies (the probability of consuming a food) and intake amounts (the usual amount consumed), followed by an integration step. In summary:

Step 1 - a betabinomial or logistic normal distribution is fitted to the number of days with consumption per individual;

Step 2 - the positive daily intake distribution is transformed into a more normal distribution using a logarithmic or a power function. A normal-distribution based variance components model is fitted to remove the intra-individual variation;

Step 3 - the resulting normal intake distribution is back-transformed and combined with the exposure frequency distribution (Monte Carlo integration).

There are basically two approaches that can be applied to the BBN and the LNN models. The first is the Add-then-model approach, where the chemical intake is calculated per food for each person on each day, then summed over the foods and finally the statistical model is applied. The second approach is the Model-then-add. In this approach the statistical model (BBN or LNN) is applied to every single foods or food groups, and then the resulting usual intake distributions per food or food group are summed to obtain an overall usual intake distribution. When using the Model-then-add

approach the foods that contribute the most to the total chemical intake must be identified and modelled separately (Van der Voet et al., 2014). Independently from which approach is used, the chemical positive daily intake distribution is assumed to be approximately normal after a suitable transformation. Non normality, especially bi- or multimodality, can arise when the contribution of one food to the total intake of a chemical substance is considerably higher compared to the rest of the diet. Indeed, the main difference between the two approaches presented above, is that the chemical intake via separate food groups may show a better fit to normal distribution than via all foods together, so the Model-then-add approach can better handle anormality (Goedhart et al., 2012; Van der Voet et al., 2014).

When running a usual intake model, multiple 24-HDRs with no correlation between days, so defined as independent, per every individual included in the study, in order to measure and eliminate the intra-individual variability (Van Klaveren et al.,2012). Since it has been demonstrated that estimated percentiles of usual intake are similar using 2 or 7 survey days, more than two repetitions per individual seem to be superfluous, as far as the total number of sampling days is sufficiently large (Hoffmann et al., 2005). Furthermore, it has been reported by other authors that consecutive-day intakes are more highly correlated than non-consecutive-day intakes (Hartman et al., 1990; Van der Voet et al., 2014). As well as to assess the normality of the chemical intake distribution, when using usual-intake-models, is important to ensure that consumption data have been collected in an appropriate way.

Considering the increasing tendency to use usual-intake-models to estimate long-term exposure to chemical substances, the main objective of our study is to highlight their potential impact on chemical exposure estimates and risk assessment. Furthermore, we want to point out which are the key aspects to be considered in order to properly run these models and be sure to correctly interpret the outputs, taking into account the constraints created by the assumptions underlying the models themselves.

## 3.2 Materials and methods

The consumption data were obtained from the French dietary survey INCA2 conducted in 2006-7 and concentration data were those collected during the Second French Total Diet Study (TDS2) carried out from 2007 to 2009 (Sirot et al., 2009; Dubuisson et al., 2010).

In this study, we focused on three substances: cadmium, acrylamide and sulphites. These substances have been selected among those for which, in the TDS2, it was not possible to exclude a health risk for the population (ANSES, 2011a; ANSES, 2011b). These three substances represent three examples

of chemicals with different origin, toxicological characteristics and distribution pattern within food groups. Briefly:

- Cadmium is a heavy metal found as an environmental contaminant, both through natural occurrence and from industrial and agricultural sources. Cadmium is primarily toxic to the kidney, but it has been associated with increased risk of cancer in the lung, endometrium, bladder, and breast (EFSA, 2009). The Eurpean Food Safety Authority (EFSA) confirmed a tolerable weekly intake (TWI) for cadmium equal to 7 μg/kg/week (EFSA, 2011).
- Acrylamide is a chemical that typically forms in food products during high-temperature cooking, including frying, baking and roasting. Acrylamide showed neurotoxic, reproductive, genotoxic and carcinogenic properties. The Joint FAO/WHO expert committee on food additives (JECFA) identified 2 $BMDL_{10}$ (0.18 and 0.31 μg/kg bw/day) based on Harderian gland tumour and mammary tumour, and recommend to calculate a margin of exposure (MoE) to assess the risk (JECFA, 2011).
- Sulphites are widely used as preservative and antioxidant additives in food. Exposure to sulphites has been reported to induce a range of adverse clinical effects in sensitive individuals (Vally et al. 2012). The JECFA established an acceptable daily intake (ADI) for sulphites of 0.07 mg/kg/day (JECFA, 2008).

Briefly, consumption data were available for 3362 individuals (1918 adults aged 18–79 years and 1444 children aged 3–17 years) recruited in the framework of the INCA2 survey and asked to complete a 7-day food record (Dubuisson et al., 2010). In the current study we considered only the adult population. Based on these data, core foods (n=212) that covered about 90% of the whole diet of adults and children were identified and sampled in the French total diet study TDS2 (Sirot et al., 2009). Food items known or assumed contributors of the substances considered in the study were also included. To account for the potential regional and seasonal variations in occurrence, the sampling was performed in eight great metropolitan regions, and each food collected in a region was sampled during two different seasons. All in all, 20 280 different food products were purchased, prepared "as consumed" by the population (peeled, cooked...) to make up the 1352 composite samples of core foods to be analysed. The chemicals were analysed in known contributors or in foods where these substances are thought to be detected. Additional details on analytical methods are available elsewhere (Sirot et al., 2012; Arnich et al., 2012; Bemrah et al., 2012).

*Usual intake modelling*

To estimate usual intake the Monte Carlo Risk Assessment (MCRA) software, release 8.0 was used. Both the BBN and the LNN models are implemented in MCRA (de Boer et al., 2013).

Data were entered in MCRA and as first thing the OIM model was ran estimating the 50th, 75th, 95th and 99th percentile and percentage of subjects exceeding the threshold value or above the MoE, for

the three substances taken into account. The OIM model is comparable to the model used in the TDS2 study. The TDS2 results could not be directly used since regional differences were taken into account, but in our study we did not consider regional differences in the food concentration of the substances considered. Moreover the TDS2 report does not specify the 75th and 99th percentiles of exposure, which are needed for the purpose of comparison in the current study.

BBN and LNN models were ran for the three substances considered in this study. For all substances both the Add-then-model and the Model-then-add approach were applied to the usual-intake-models: the results (50th, 75th, 95th and 99th percentile and percentage of subjects exceeding the threshold value or MoE calculated on the 95th percentile exposure) of the usual-intake-models ran with the two approaches were compared to each other and with the results of the OIM model.

*Normality, main contributors and best approach*

To establish if the assumption of normality was respected, a visual inspection of the log-transformed intake distribution of the three substances was carried out and the normality was judged. Moreover the percentage of contribution of the several food groups to the total intake of the substances was calculated in order to picture the different distribution pattern within food groups. Food groups that contributed for more than 5% of the total intake of the substance were defined as main contributors. Finally, the most appropriate approach to use for the estimate of usual intake was defined for the three substances taken into account in this study.

*Correlation between consumption days*

Since the consumption data collected in the INCA2 survey are on seven consecutive days, we calculated the correlation among consumption data collected on consecutive days for those food groups that were identified as main contributors to the intake to at least one substance included in the study. A dataset was generated with the individual consumption of food items (grouped in food groups) per day. For each food group, we classified consumers in two categories: “large consumers” (subjects who for more than 4 days out of the seven days of the survey had eaten at least one food belonging to the food group considered) and “not large consumers” (those who had eaten for 4 days or less foods belonging to the food group considered). Non-consumers (those who had never eaten foods belonging to the food group considered during the seven days of survey) were excluded from the analysis. For each food group considered, the following logistic regression model was performed:

$$\mathbf{Pr(Cons_{Day\ x;F}=1|\ LargeCons_F,\ Cons_{Dayx\text{-}1;F}) = F(\beta_0+\beta1(Cons_{Dayx\text{-}1;F})+\beta2(LargeCons_F))}$$

We estimated if the probability of eating a food included in the food group F on Day x was significantly higher if a food item included in the same food group F had been eaten the day before

(day x-1) and taking into account the consumer category, so considering if the subject was a big consumer for the food group considered (Large Cons F).

The same probability was then calculated performing a random sampling of two non consecutive days per individual within the 7 days of the survey and repeating the logistic regression model for the two non consecutive days in order to compare the correlation among non consecutive days to the correlation among consecutive days. The analysis described above were performed with SAS 9.3 software (Copyright © 2009, SAS Institute Inc., Cary, NC, USA).

Finally, with the same subset of two non consecutive survey days we ran again the BBN and LNN models with MCRA 8.0, using the more appropriate approach for each substance, and compared the results with those obtained previously.

## 3.3 Results

Since we did not have any significant difference in the results of the two models (BBN and LNN), we present only the results obtained with the BBN model.

*Usual intake modelling*

The results obtained with the OIM model and those obtained with both the add-then-model approach and with the model-then-add approach are showed in table 1.

Cadmium's usual intake estimated with the add-then-model approach was +6.7% for the 50th percentile but the 95th percentile was -6.7% compared to the estimates obtained with the OIM model, and none of the subjects exceeded the TWI.

The cadmium's exposure modelled with the model-then-add approach showed an increase of the 50th percentile estimates of +23.4% while the 95th percentile decreased of -0.7% compared to the result of the OIM model. Overall, the estimate of the exposure obtained with the model-then-add approach were higher than those obtained with the add-then-model-approach. With the model-then-add approach the percentage of subjects that exceeded the TWI was 0.1% of the population.

Modelling acrylamide's exposure with the add-then-model approach led to higher estimates of the usual intake compared to the result obtained with the OIM model, indeed exposure estimate of the 50th and the 95th percentiles increased of +8.6% and +10.1%, respectively.

The 50th percentile of the usual intake distribution estimated with the model-then-add approach was +32.7% compared to result obtained with the OIM model, but the 95th percentile decreased -6.5%. Furthermore, the MOEs calculated for the 95th percentile of exposure to acrylamide in the OIM model were respectively 316 and 184 for both $BMDL_{10}$ (0.31 and 0.18 mg/kg bw/day). According to the results obtained applying the add-then-model approach the MOEs calculated for the 95th

percentile of exposure for both $BMDL_{10}$ were respectively 287 and 166, while applying the model-then-add approach they were respectively 338 and 196.

Considering sulphites, the add-then-model approach provided an over-estimation of the exposure that ranged from +8.4% for the 50th percentile to +50.5% for the 95th percentile in comparison to the OIM estimate. The percentage of subjects that exceeded the ADI calculated with the add-then-model approach was equal to 8.2%. On the other hand, the results obtained by the model-then-add approach provided a higher estimate of the 50th percentile (+15.4%) compared with the OIM model, but a lower estimate of the 95th percentile (-3.9%), which led to a 0.5% decrease of subjects that exceed the ADI, corresponding to 2.3% of subjects with sulphites' intake above the threshold value.

**Table 1**: Comparison of the sulphites', cadmium's and acrylamide's long-term exposure percentiles and percentage of subjects exceeding the threshold value or MoE for the adult French population according to the Observed Individual Mean (OIM) and to the BetaBinomial-Normal (BBN) models run both with the add-then-model approach and with the model-then-add approach, and difference (in %) between the estimates obtained with the OIM model and those obtained with the usual-intake-models.

| Substance | percentiles of exposure | OIM | BBN add-then-model | | BBN model-then-add | |
|---|---|---|---|---|---|---|
| | | µg/kg bw/day | µg/kg bw/day | difference % with TDS2 | µg/kg bw/day | difference % with TDS2 |
| Cadmium | 50th | 0,113 | 0,12 | 6,5 | 0,139 | 23,4 |
| | 75th | 0,149 | 0,151 | 1,6 | 0,17 | 13,9 |
| | 95th | 0,224 | 0,209 | -6,7 | 0,222 | -0,7 |
| | 99th | 0,294 | 0,257 | -12,6 | 0,285 | -3,3 |
| | *%>TWI* | 0.4% (95% CI 0.1-0.7) | 0% (95%CI 0-0.1) | | 0.1% (95%CI 0-0.2) | |
| Acrylamide | 50th | 0,347 | 0,377 | 8,6 | 0,461 | 32,7 |
| | 75th | 0,569 | 0,623 | 9,5 | 0,624 | 9,6 |
| | 95th | 0,98 | 1,079 | 10,1 | 0,916 | -6,5 |
| | 99th | 1,419 | 1,526 | 7,5 | 1,307 | -7,9 |
| | *MOE (310 µg/kg bw/day)* | *316 (95% CI 338-294)* | *287 (95% CI 306-273)* | | *338 (95% CI 352-306)* | |
| | *MOE (180 µg/kg bw/day)* | *184 (95% CI 196-171)* | *166 (95% CI 178-158)* | | *196 (95% CI 203-178)* | |
| Sulphites | 50th | 95,6 | 103,6 | 8,4 | 110,3 | 15,4 |
| | 75th | 226,4 | 286,6 | 26,6 | 232,7 | 2,8 |
| | 95th | 603,6 | 908,7 | 50,5 | 579,8 | -3,9 |
| | 99th | 870,6 | 1303 | 49,7 | 803 | -7,8 |
| | *%>ADI* | *2.8% (95% CI 1.9-3.7)* | *8.2% (95% CI 6.6-9.9)* | | *2.3% (95% CI 1.6-3)* | |

*Normality, main contributors and best approach*

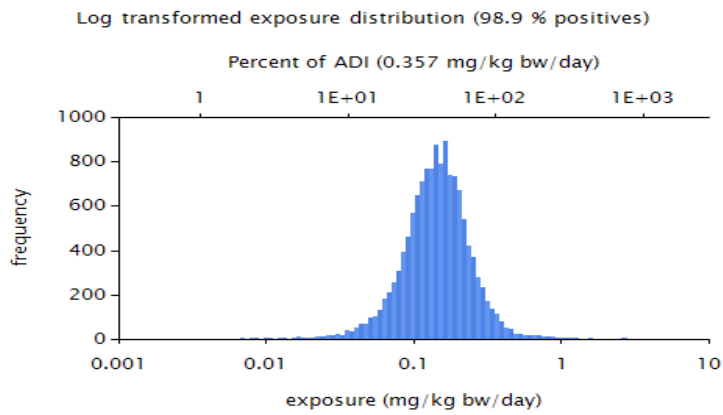
The log transformed intake distribution for cadmium was assumed as normal (figure 1-A). Moreover, when looking at the food groups' contribution to the total cadmium intake, six food groups that contributed for 5% or more to the total intake were identified, accounting overall for the 60% of the intake: “bread and dried bread products” (22%), “potatoes and potato products” (12%),” vegetables excluding potatoes” (10%), “rice and wheat products” (6%), “crustaceans and molluscs” (5%) and “mixed dishes” (5%). The “rest food group” consisted of 27 different food groups that contributed for the residual 40% of the total intake (figure 1-B). Finally, the add-then-model approach was considered the most appropriate to estimate exposure to cadmium because the lognormality hypothesis was well respected and the results of the model were in adequation with the theoretical expectation of a shrinked distribution.

For acrylamide, although the log transformed intake distribution of was not far from a normal distribution (figure 2- A), most of the total intake (89%) was due to only four food groups, that contributed to the total intake for 5% or more (“sautéed potatoes or chips” 45%, “coffee” 29%, “sweet and savoury biscuits” 9%, “bread and dried bread products” 6%). The “rest food group” was represented by the remaining 12 food groups that contributed individually for less than 5% and globally for 11% (figure 2-B). The model-then-add approach has been considered more appropriate for the estimation of acrylamide’s usual intake.

Looking at the log transformed sulphites’ intake distribution, it appeared clear that the assumption of normality was not satisfied; indeed the log transformed intake distribution was bimodal with the second peak much wider and higher than the first one (figure 3- A). Furthermore, we observed that the intake of sulphites was mainly due to “alcoholic beverages” and “sugar products” groups; in fact more than 90% of the intake was explained by the consumption of these two food groups. The “rest food group” included the remaining other 15 food groups that contributed individually to the total intake for less than 5% (figure 3-B). In this case the use of model-then-add approach was judged more appropriate to estimate sulphites usual intake.

**Figure 1:** A - Log-transformed intake distribution via all food to cadmium in the French population 18-79 years old;
B - Contribution to the total exposure to cadmium for food groups in the French population 18-79 years old.

**A**




**B**

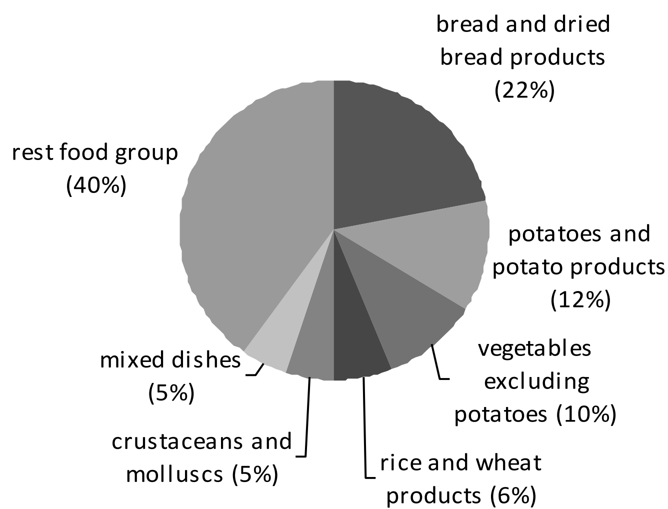
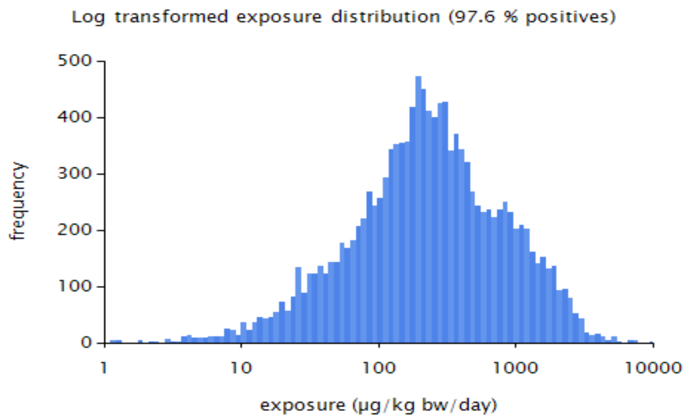


**Figure 2:** A - Log-transformed intake distribution via all food to acrylamide in the French population 18-79 years old;
B - Contribution to the total exposure to acrylamide for food groups in the French population 18-79 years old.

**A**




**B**

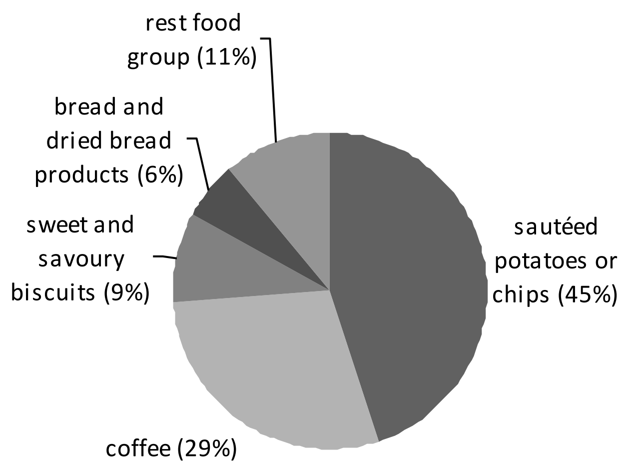
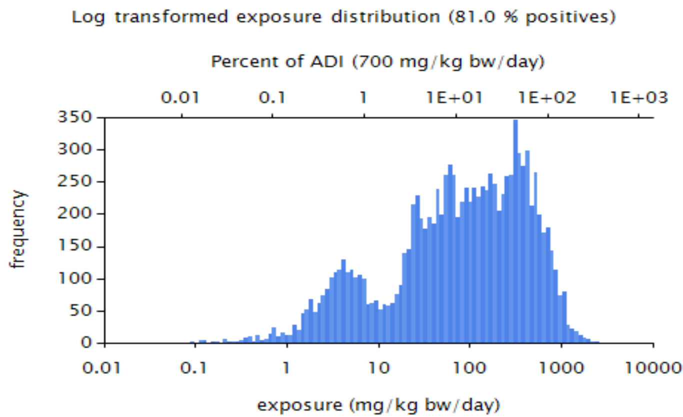
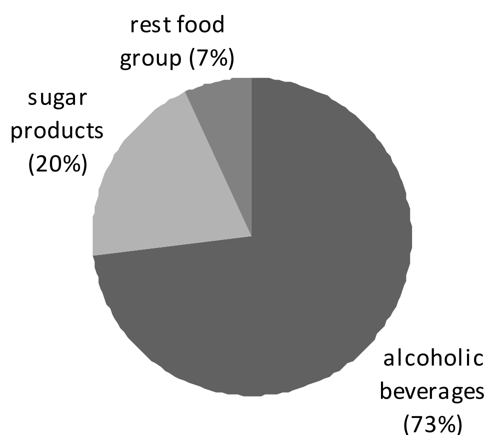


**Figure 3:** A - Log-transformed intake distribution via all food to sulphites in the French population 18-79 years old;
B - Contribution to the total exposure to sulphites for food groups in the French population 18-79 years old.

**A**




**B**




*Correlation between food consumption on consecutive days of the survey*

The existence of correlation among food consumption on consecutive survey days has been investigated for the following food groups: ”alcoholic beverages”, “sugar products”, “bread and dried bread products”, “potatoes and potato products”, “vegetables excluding potatoes”, “rice and wheat products”, “crustaceans and molluscs”, “mixed dishes”, “sautéed potatoes or chips”, “coffee” and finally “sweet and savoury biscuits”.

The results of the logistic regression models obtained using both consecutive days of survey and two random non consecutive days are summarized in table 2.

When considering the results obtained using all the seven days of the survey, the results were extremely variable among the food groups considered. The consumption on consecutive days was

positively correlated for “sugar products” (OR 1.58, 95% CI 1.34-1.86), “coffee” (OR 2.35, 95% CI 1.93-2.87), “alcoholic beverages” (1.38, 95% CI 1.21-1.58) and “bread and dried bread products” (OR 1.51, 95% CI 1.29-1.77), while “crustaceans and molluscs” (OR 0.65, 95% CI 0.51-0.83), “sautéed potatoes and chips” (OR 0.65, 95% CI 0.57-0.75), “vegetables excluded potatoes” (OR 0.88, 95% CI 0.79-0.98) and “potatoes and potato products” (OR 0.75, 95% CI 0.68-0.82) where negatively correlated. Finally, for the remaining categories (“sweet and savory biscuits”, “rice and wheat products” and “mixed dishes”) no significant correlation was found.

When two random non consecutive days of survey were considered, for all the food groups considered the consumption was significantly negatively associated between the two days, except for “sugar and sugar products” and “bread and dried bread products” for which no significant association was identified.

In table 3 are summarized the long-term exposure percentiles of the three substances included in this study according to the BBN model, ran with the most appropriate approach for the specific substance, using both seven consecutive days and two random non consecutive days of survey.

For sulphites, when using data from two non consecutive survey days, the estimates of exposure and the percentage of subject that exceeded the ADI were significantly reduced compared to the estimates obtained using all seven days of survey. Indeed exposure estimate of the 50th percentile increased of +1.8% but the 95th percentile decreased of -11.1%.

Considering cadmium, the exposure estimates using two non consecutive survey days were slightly lower for the 50th (-1.7%) and for the 95th percentile (-2.9%) compared to the results of the seven consecutive survey days. It is worth notice that since the exposure estimate of the 99th percentile increased of +3.9%, the percentage of subject exceeding the ADI was slightly higher when using only two non consecutive survey days. Acrylamide’s exposure estimate using two non consecutive days of survey was higher both at 50th percentile (+10.0%) and at the 95th percentile (+3.8%) while was lower at the 99th percentile (-8.2%).

In any case, for both cadmium and acrylamide the differences between the results of the two models were not significant.

**Table 2**: Correlation between consumption of food belonging to the food groups that represented the main contributors for the intake of sulphites, cadmium and acrylamide in logistic regression models using both seven consecutive days and two random non consecutive days of the survey and taking into account the consumers' category.

| Substances for which the food group is the main contributor | Food group (sum of individual day consumption)* | 7 survey days | | | | 2 random survey days | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | OR | 95% CI | | | OR | 95% CI | |
| Cadmium | crustaceans and molluscs (n=3102) | day before | 0,65 | 0,51 | 0,83 | other day | 0,32 | 0,18 | 0,57 |
| | | big consumers category | 27,75 | 3,21 | 240,22 | big consumers category | 7,06 | 0,39 | 127,92 |
| Cadmium | rice and wheat products (n=7110) | day before | 0,91 | 0,8 | 1,04 | other day | 0,64 | 0,5 | 0,81 |
| | | big consumers category | 11,27 | 7,99 | 15,91 | big consumers category | 18,37 | 9,57 | 35,27 |
| Cadmium | mixed dishes (n=7374) | day before | 0,91 | 0,81 | 1,03 | other day | 0,71 | 0,58 | 0,88 |
| | | big consumers category | 10,7 | 6,47 | 17,7 | big consumers category | 6,34 | 2,93 | 13,72 |
| Cadmium | vegetables excluding potatoes (n=10980) | day before | 0,88 | 0,79 | 0,98 | other day | 0,64 | 0,53 | 0,77 |
| | | big consumers category | 8,37 | 7,56 | 9,26 | big consumers category | 10,28 | 8,55 | 12,36 |
| Cadmium | potatoes and potato products (n= 10080) | day before | 0,75 | 0,68 | 0,82 | other day | 0,75 | 0,64 | 0,87 |
| | | big consumers category | 6,69 | 5,69 | 7,86 | big consumers category | 6,76 | 5,1 | 8,95 |

| Cadmium and Acrylamide | bread and dried bread products (n=10920) | day before | **1,51** | **1,29** | **1,77** | other day | 0,98 | 0,74 | 1,29 |
|---|---|---|---|---|---|---|---|---|---|
| | | big consumers category | 20,33 | 17,57 | 23,51 | big consumers category | 24,21 | 18,6 | 31,52 |
| Acrylamide | sautéed potatoes or chips (n=7434) | day before | 0,65 | 0,57 | 0,75 | other day | 0,62 | 0,49 | 0,78 |
| | | big consumers category | 10,14 | 6,91 | 14,87 | big consumers category | 5,13 | 2,81 | 9,36 |
| Acrylamide | Coffee (n=8550) | day before | **2,35** | **1,93** | **2,87** | other day | 0,36 | 0,23 | 0,58 |
| | | big consumers category | 34,7 | 28,57 | 42,14 | big consumers category | 171,4 | 106,22 | 276,58 |
| Acrylamide | sweet and savoury biscuits (n=2026) | day before | 1,08 | 0,95 | 1,23 | other day | 0,68 | 0,54 | 0,86 |
| | | big consumers category | 12,83 | 10,35 | 15,89 | big consumers category | 13,22 | 9,2 | 18,99 |
| Suphites | sugar products (n=9474) | day before | 1,58 | 1,34 | 1,86 | other day | 0,88 | 0,65 | 1,18 |
| | | big consumers category | 37,88 | 31,82 | 45,1 | big consumers category | 57,13 | 41,4 | 78,85 |
| Suphites | alcoholic beverages (n=7044) | day before | 1,38 | 1,21 | 1,58 | other day | 0,69 | 0,53 | 0,88 |
| | | big consumers category | 21,62 | 18,22 | 25,65 | big consumers category | 38,64 | 27,87 | 53,58 |

* Overall sum of the number of days in which food items (grouped in the food groups considered) have been declared to be consumed by each individual. This information provides the frequency of consumption. With regard to the models, for each food group, the denominator was calculated by multiplying the number of consumers (both big and non big consumers) for the seven days of the survey. For the two non consecutive days, the denominator was the number of consumers (both big and non big consumers) multiplied by the two random selected non consecutive days

**Bold** = significant positive correlation;

Underlined = significant negative correlation.

**Table 3**: Sulphites', cadmium's and acrylamide's long-term exposure percentiles for the adult French population according to the Betabinomial-Normal (BBN) model run with the most appropriate approach using seven consecutive days of survey and two non consecutive days of survey.

| Substance and approach used | percentiles of exposure | 7 consecutive days | 2 non consecutive days | % difference |
|---|---|---|---|---|
| Cadmium | | | | |
| add-then-model | 50th | 0,12 | 0,118 | -1,70% |
| | 75th | 0,151 | 0,148 | -2,00% |
| | 95th | 0,209 | 0,203 | -2,90% |
| | 99th | 0,257 | 0,267 | 3,90% |
| | *%>ADI* | *0% (95% CI 0-0.1)* | *0.1% (95% CI 0-0.3)* | |
| Acrylamide | | | | |
| model-then-add | 50th | 0,461 | 0,507 | 10,00% |
| | 75th | 0,624 | 0,653 | 4,60% |
| | 95th | 0,916 | 0,951 | 3,80% |
| | 99th | 1,307 | 1,2 | -8,20% |
| | *MOE (310 µg/kg bw/day)* | *338 (95% CI 352-306)* | *326 (95% CI 346-296)* | |
| | *MOE (180 µg/kg bw/day)* | *196 (95% CI 203-178)* | *189 (95%CI 201-172)* | |
| Sulphites | | | | |
| model-then-add | 50th | 110,3 | 112,3 | 1,80% |
| | 75th | 232,7 | 234,9 | 0,90% |
| | 95th | 579,8 | 515,5 | -11,10% |
| | 99th | 803 | 699,2 | -12,90% |
| | *%>ADI* | *2.3% (95% CI 1.6-3)* | *1% (95% CI 0.5-1.6)* | |

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## 3.4 Discussion

In this study the long-term dietary exposure to cadmium, acrylamide and sulphites of the adult French population was calculated applying usual-intake-models with different approaches.

The main aim of this study was to highlight the potential impact of the use of usual-intake-models on chemical exposure estimates and risk assessment. The results of our study confirm that usual-intake-models give less conservative and probably more realistic estimates of usual intake, compared to the traditional OIM model (classical deterministic approach), that does not manage the intra-individual variability. If the proper approach is applied, the effect of the usual-intake-model is mainly on the highest percentiles of exposure. Indeed, in our study the intake estimated with the usual-intake-models for the 95th percentile was reduced of nearly -7% for cadmium and acrylamide and around -4% for sulphites compared to the results of the OIM model with 7 days. This is related to the fact that the usual intake distribution estimated by the average intake from 24-HDR (OIM), tend to be

rightly skewed, thus can produce overestimate of the long run intakes especially for the subject in the upper tail of the distribution (Van Klaveren et al., 2012). However, from our result it didn't arise any significant difference in terms of percentage of population exceeding the threshold value when comparing the results of usual-intake-models with the OIM model. Usual-intake-models reduced the exposure estimates, but such reduction didn't affect the risk assessment performed comparing the exposure results with the health-based guidance values. This observation should be compared with results obtained from future studies that take into account different substances possibly with higher concentration values so to evaluate the impact of usual-intake-models in different scenarios.

We confirm that it is crucial to choose the most appropriate approach for the usual-intake-models. Indeed we observed that if the hypotheses of the models are not respected, the usual-intake-models provide strongly biased results and a significant over estimation of the intake for the extreme percentiles so that the theoretical expected shrinkage of the distribution is not observed.

The main criteria that drive the choice of the approach are the features of the substance's log-transformed intake distribution and the number of food groups that contribute to the total substance intake. If the log transformed intake distribution can be defined as "normal", an add-then-model approach can be used, as we did for the estimate of the cadmium. On the other hand, if the substance's intake is mainly ascribable to one (or few) food group, as we observed for both acrylamide and sulphites, it is worthwhile to apply the model-then-add approach, even if more time consuming.

When using usual-intake-models, independence of food consumption data collected on multiple days of survey should be guaranteed in order to strengthen the correlation with true usual intake (Hoffmann et al., 2002). When focusing on food consumption patterns, it must be taken into account that people tend to diversify and alternate the foods eaten during a short period of time (for example during 1 week). On the other hand, there is also the habit of consuming "leftovers" from the day before. These two habits generate correlation among food consumption data, but this correlation tends to be negative when considering for two non consecutive days within one week, while it is reduced or becomes positive when two consecutive days are considered ("leftovers effect"). Based on our results, independency of food consumption data is not ensured for data collected on consecutive days nor on non consecutive days within one week. The recently published EFSA Guideline for EU menu methodology (Efsa, 2014) recommends to collect two 24-HDR with a time distance of at least seven days, but, as far as we know, little research has been done to determine the distance required between two survey days so that they can be considered independent.

Concerning the lack of independency between days of survey within one week (both consecutive and non consecutive), we evaluated if this condition influenced the usual–intake-model estimates. Only

for sulphites we observed significantly different results when the intake was calculated with data from consecutive and non consecutive survey days. This difference is probably due to the fact that the two food groups that contribute to the intake are both characterized by a positive correlation when considering consecutive days and no correlation for two non consecutive days. On the contrary, the non homogenous correlation pattern showed by food groups that contribute to acrylamide's and cadmium's intake did not significantly change the results obtained with data collected on consecutive or non consecutive survey days.

We are aware of some limitation of our study. We have included three susbstances only that we considered representative of the distribution of contaminants in foods, choosing among those with different patterns, but since the great variability of substances and food items contaminated, our results cannot be representative of all the possible patterns of contamination/consumption.

Furthermore, we did not include information from Food Frequency Questionnaires (FFQ). The FFQ may provide additional information on the usual food consumption frequency, useful to adjust the estimate, for instance, in case of episodically eaten foods (Goedhart et al. 2012)

Lastly, INCA2 consumption data included only seven days, so it was not possible to evaluate the minimum distance needed between survey days in order to ensure independency.

In conclusion, our study suggests that in terms of risk assessment, the usual-intake-models provide a more accurate estimate of the exposure compared to OIM model. Although their impact on the risk assessment based on cut-off values seems to be limited, a more accurate exposure assessment is important at population level, especially in association with epidemiological or biomonitoring studies. From the methodological point of view, in order to obtain the proper estimate using usual-intake-models, three key aspects should be carefully considered: 1) the normality of the log-transformed intake distribution, 2) the contribution per single food group to the total exposure, 3) the independency of food consumption data on multiple days.

To better understand the advantages and disadvantages of these models and to improve their use further studies comparing the impact of usual-intake-models in exposure estimate according to the substances' patterns of contamination and to the different consumption data available are needed.

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# *Chapter 4.*

# Exposure to inorganic arsenic in an area with high environmental arsenic concentrations in Italy.

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*Manuscript in preparation*

*Abstract*

In Latium (central Italy), arsenic concentrations in groundwater from three provinces encompassing a large area of volcanic origin exceed the regulatory limit of 10 µg/L for drinking water. The aim of the present study was to estimate the intake of iAs in a population living in this area taking into account the contribution of the whole diet (water and food) and to estimate the correlation between concentration of UAs (sum of iAs and As metabolites in the urine) and the iAs in drinking and cooking water and the intake of iAs due to water and food.
Overall, the concentration of iAs in drinking water ranged from 0.1 µg/L to 35.2 µg/L (median 4.4 µg/L), while in cooking water the iAs concentration ranged from 0.2 µg/L to148.9 µg/L (median 14.4 µg/L). The overall intake of iAs was on average 0.22 µg/kg bw/day (S.D. 0.16 µg/kg bw/day): the solid diet component contributed for 64.4%, the liquid diet component for 17.3% and the drinking water for 18.3%. The proportion of iAs intake from the solid and liquid components was inversely related to the intake contribution from drinking water. The median concentration of UAs in urine samples was 11.5 µg/L (range, 1.7-75.5 µg/L). The concentration of UAs in urine was significantly correlated with the iAs concentration in cooking water and in drinking water (coef.=0.01, 95% CI=0.01-0.02 and coef.=0.03, 95% CI=0.02-0.04 respectively), and with the iAs intake attributable to solid diet component (coef.=1.2, CI95%=0.1-2.2) and to drinking water (coef.=3.2, CI95%=0.1-6.3).
Duplicate diet study is useful to estimate the dietary exposure to iAs and to identify which diet components contribute the most to the total intake. The UAs in urine provides a reliable estimate of the iAs dietary exposure and the use of this biomarker is important to define the population exposure to iAs and address specific protective measures.

## 4.1 Introduction

Arsenic is a metalloid that exists in the environment and living organisms in a variety of naturally-occurring chemical species. Organic species include methylated arsenic compounds (mono- to tetra-substituted), arsenosugars, and arsenolipids, whereas inorganic arsenic (iAs) species consist in the oxyanions arsenite and arsenate, which are generally found as thio complexes or bound to thio groups in peptides or proteins (EFSA, 2009). Whereas organoarsenic compounds have generally negligible to low toxicity - even though some species such as arsenolipids are still insufficiently characterized - iAs is a well-established human carcinogen and chronic oral exposure is associated

with skin, lung, bladder, liver, and kidney cancer (EFSA, 2009; FAO & WHO, 2011; Young-Seoub et al, 2014).

Arsenic contamination of groundwater due to the release of the element from arsenic-rich rocks and sediments is a major public health issue affecting tens of millions of people in many countries worldwide. Arsenic in water occurs as iAs and the use of contaminated groundwater leads to dietary exposure to the toxic species of this element via drinking water, food preparation and cooking as well as in consequence of its entry in the food chain through crop irrigation (EFSA, 2009; Kurzius-Spencer et al, 2014; Huq et al, 2006). When the arsenic concentration in water is high, drinking water tends to become the major source of exposure to iAs. On the other hand, when the arsenic concentration in water is at background levels, food is the major contributor to the intake of iAs in the general population (EFSA, 2014; Xue et al, 2010) .

Whereas early awareness of the toxic and carcinogenic effects of iAs emerged from epidemiological studies in areas with very high levels of arsenic in groundwater (>100 μg/L), there is increasing evidence of adverse effects at moderate to low exposure levels such as those resulting from water arsenic concentrations below 50 μg/L (Leonardi et al, 2012; García-Esquinas et al, 2013; Moon et al, 2013; Zheng et al, 2013). In particular, the WHO has provided clear information on the toxicity of the iAS in drinking water and indicated as acceptable only temporarily, the value from 1 to 10 μg/L of iAs in water intended for human consumption, while established as auspicable values between 0 and 5 μg/L, given the uncertainty concerning the risk to human health associated with exposure even at very low concentrations.

The iAs is absorbed in the intestine and undergoes a first methylation in the liver leading to the formation of monometilarsonato (MMA). A second methylation follows which transforms the MMA in dimethylarsinate (DMA). The methylation of iAs to MMA and DMA facilitates the urinary excretion of As, the main pathway for the elimination of arsenic from the human body (Le et al., 1994). The sum of iAs, MMA and DMA species in urine is considered the biomarker of choice for assessing recent exposure to iAs (Cubadda et al, 2012). The total urinary As as indicator of exposure to inorganic As is influenced by the organic arsenical Arsenobetaine, that can be found in certain seafood, and it is rapidly excreted unchanged in urine following ingestion increasing considerably the concentration of total As in urine (Navas-Acien et al, 2011).

The European Directive 98/83/EC, since 2003 has significantly lowered the acceptable value of iAs in drinking water from 50 to 10 μg/L. In the period 2003-2009, Italy obtained two derogations from the European Commission on the basis of the particular geological conditions present in different areas of the country which determine the natural presence of iAs in aquifers. To date, the goal of 10 μg/L of iAs in water for human use has not yet been reached in all Italian regions. Particularly in the Lazio Region 91 municipalities are above the treshold of iAs in water, 22 in the Province of Rome, 60 in the

Province of Viterbo and 9 in the Province of Latina. (Angelone et al, 2009;Euro-lex, 1998). Institutions and citizens in these areas have put in place intervention strategies (alternative supply of water for human consumption, filtering system etc) to reduce the As water intake. Such a situation doesn't allow a precise estimate of the exposure to As of human population residing in these areas by the evaluation of iAs concentration in the water system, to obtain such estimate other apporaches are required.

The aims of the study were:

1. to estimate the intake of iAs in a population living in the areas with iAs natural contamination in water, taking into account the contribution of the whole diet (water and food)
2. to estimate the correlation between UAs concentration in urine (sum of iAs and As metabolites, considered suitable biomarker of exposure), and the iAs in water and in the other diet components.

## 4.2 Materials and methods

*Study population and sample collection*

Between November 2010 and March 2011, we enrolled 267 subjects aged between 1 and 88 years old residing in areas of the provinces of Viterbo (138 subjects), Rome (14) and Latina (115) where a derogation on the use of tap water was in place, due to the iAs concentration above the threshold values (10 µg /L as temporary acceptable value and 5 µg/L as desirable value) (FAO & WHO, 2011; EuroLex, 1998).

For the purpose of the analysis the subject residing in the Provinces of Rome and Latina were grouped in the same group due to the similarity in the hydrogeological characteristics and for the vicinity between these areas. Consequently for the comparison between areas two areas have been identified: Latina-Rome and the Province of Viterbo.

From all the subjects enrolled we collected a sample of urine and information on dietary habits, type of water used for drinking and for cooking (bottled or tap water) by submitting to them a questionnaire. Information on demographic, morphometric and lifestyles were also collected with the same questionnaire. First morning voided urine samples were collected in polypropylene tubes and transported at ambient temperature to the laboratory where they were stored at –80 °C until analysis.

Samples of drinking and cooking water (when different from the drinking water) used by each subject were collected. Moreover, a sample of tap water from every house, regardless from the use, was also collected.

All subjects enrolled in the study were asked not to eat fish or other seafoods during the three days immediately preceding the urine samples collection, as these foods have naturally high levels of organic As (arsenobetaine) that can overrate the urinary As concentration.

Of the 267 participants, 26 volunteers joined the duplicate diet study. They were asked to provide a duplicate serving of the food consumed within the 24 hours of three consecutive days. Participants were instructed to save duplicate portions from each meal in polypropylene resealable bags, which were kept refrigerated until processing.

The food was collected separating in solid and liquid components (different from drinking water) to determine the relative contribution of water, solid and liquid diet components to the total intake of iAs. In the laboratory, the portions from each participant were weighed and homogenized into a 3-d composite sample, using a blender for solid foods. Homogenized samples were aliquoted into polypropylene tubes and frozen at –80°C pending analysis.

*Chemical analysis*

Total arsenic determination in water was carried out by directly injecting the acidified samples (1% v/v HNO3) in the sample introduction system of the inductively coupled plasma- mass spectrometry (ICP-MS).

Urine specimens for total arsenic determination were diluted with 1% v/v $HNO_3$, filtered through 0.22 µm PVDF syringe filters and analyzed by inductively coupled plasma-dynamic reaction cell-mass spectrometry (ICP-DRC-MS). For arsenic speciation analysis of urine samples, 25 µl of 0.22 µm PVDF-filtered urines were injected on a ICSep ION-120 anion exchange column (Transgenomics, San Jose, CA) using gradient elution. 14 Arsenic speciation analysis of food samples was performed with a PRP-X 100 anion exchange column (Hamilton Company, Reno, Nevada, USA), using isocratic elution. For the conditions used and more details on analytical measurements see the Supporting Information (SI) (Cubadda et al, 2012).

Food samples (duplicate diets) were analyzed for total arsenic after closed-vessel microwave-assisted digestion by a Milestone Ethos E microwave labstation (FKV, Bergamo, Italy) using HNO3 and H2O2 as reagents. For speciation analysis, iAs and other water-soluble species were solubilised using microwave-assisted extraction. Samples (0.35 g) were added with 10 mL of 1% (v/v) HNO3 and 1% (v/v) H2O2 and left to stand overnight. After microwave irradiation, the extracts were centrifuged (10 min, 8000 rpm, 4 °C) and the supernatants filtered (0.22 µm). With the extraction procedure used, As(III) is quantitatively converted to As(V), which appears as a well separated peak in the anion exchange HPLC-ICP-MS chromatogram.

*Arsenic concentration in water and foods.*

Arsenic concentration levels in all the matrices analyzed in this study have been summarized with mean, standard deviation, median and minimum and maximum values (range).

The percentage of tap water samples, collected regardless from the use, with iAs concentration above the established threshold values for potable water was calculated.

The study population was divided into three groups according to concentration of iAs in the water used for drinking and cooking (the water could be either tap or bottled):

Group 0: subjects with iAs concentration <10µg/L in both drinking and cooking water;

Group 1: subjects with iAs concentration >10µg/L only in cooking water and <10µg/L in drinking water;

Group 2: subjects with iAs concentration >10µg/L in both drinking and cooking water.

The concentration levels of iAs in drinking and cooking water were compared between the two residential areas (Viterbo province vs Latina and Rome provinces) with the Mann-Whitney test. iAs concentration in the solid and liquid diet components were compared between the two residential areas (Mann-Whitney test) and between the three groups defined above (Kruskal-Wallis test).

*Arsenic daily intake estimate*

The iAs daily intake (g/kg bw/day) from the different diet components was calculated as follows:

iAs intakes (g/kg bw/day) from solid and liquid diet components: [iAs] in 1gr of homogenate multiplied by 1/3 of the total weight of the servings provided for the 3 days and divided by the subject's body weight.

iAs intake from drinking water was calculated using the mean water consumption of the Italian population per age and sex (Picinelli et al, 2011): [iAs] in 1ml of drinking water multiplied by the mean water consumption and divided by the subject's body weight.

The total exposure was calculated as the sum of the contribution of the solid and the liquid diet components, and the drinking water. The relative contribution of the three components to the iAs total intake was calculated and expressed as percentage. Differences in the relative contributions to the total intake of the three diet components among groups were tested using the Kruskal-Wallis test.

*Internal levels of arsenic: biomarkers in urine*

Concentration of UAs (sum of iAs and As metabolites) in urine was compared between the subjects from the two residential areas (Mann-Whitney test ) and between the three groups (Kruskal-Wallis test).

For the concentration of UAs in urine we defined two threshold values: 15µg/L (upper limit of the reference concentration value proposed for the Italian population (SIVR 2011)) and twice this value (arbitrary choice). We used these threshold values to compare the proportion of subjects that exceeded the thresholds in the three groups (Kruskal-Wallis test).

Two different linear regression models were set up to analyze the correlations between the concentration of UAs in urine (dependent variable) with the iAs concentrations in drinking and cooking water and the iAs intake attributable to the different diet components (solid, liquid and water). The UAs concentrations were log-transformed, and adjustment for age, sex, body mass index (BMI) and area of residence was performed in each model. The outliers were excluded from both the linear regression models and the graphics.

Statistical analysis was performed with STATA 11.2 (StataCorp, 4905 Lakeway Drive, College 17 Station, Texas, USA) setting significance at $P<0.05$.

## 4.3 Results

*Study population and concentration of arsenic in drinking and cooking water and in the diet.*

Demographic, morphological and life styles characteristics of the study population are summarized in Table 1. The study population included 116 males and 151 females, with median age of 46 years (range 1-88 years, mean 42.4, SD 23.0), while the subgroup of 26 subjects that participated to the duplicate diet study was composed of 8 males and 18 females, with a median age of 56.5 years (range 12-83, mean 56.1, SD 18.4).

The iAs concentration of water supply of the households ranged from 0.2 to 148.9 µg/L (median 17.1 µg/L); about 87% of the samples were above 5 µg/L and about 76% above 10 µg/L (Table 2).

Overall, the concentration of iAs in drinking water ranged from 0.1 µg/L to 35.2 µg/L (median 4.4 µg/L), while in cooking water the iAs concentration ranged from 0.2 µg/L to148.9 µg/L (median 14.4 µg/L). We did not find statistically significant differences in the concentration of iAs in drinking and cooking water in the two areas (Latina-Rome vs Viterbo).

**Table 1**. Demographic, morphological and lifestyles variables of the 267 subjects participating at the entire study and of the 26 subjects participating at the total diet study.

| | | 267 subjects of the entire study | 26 subjects of the TDS |
|---|---|---|---|
| Sex | Male | 116 | 8 |
| | Female | 151 | 18 |
| Age | 0-17 | 60 | 1 |
| | 18-65 | 163 | 17 |
| | 65< | 44 | 8 |
| BMI | <18.5 | 43 | 1 |
| | 18.5-25 | 107 | 12 |
| | 25< | 117 | 13 |
| Number of cigarettes smoked per day | 0 | 236 | 19 |
| | <10 | 17 | 3 |
| | 10< | 14 | 4 |
| Consumption frequency of alcoholic beverages | Never | 142 | 11 |
| | Rare | 106 | 10 |
| | Frequent | 19 | 5 |
| Type of water used | Use of drinking and cooking water with concentration of As <10µg/L (Group 0) | 80 | 12 |
| | Use of drinking water with concentration of As<10 mg/L and of cooking water with concentration of As>10µg/L (Group 1) | 116 | 7 |
| | Use of drinking and cooking water with concentration of As> 10µg/L (Group 2) | 71 | 7 |
| Area of residence | Viterbo province | 138 | 12 |
| | Latina and Rome provinces | 129 | 14 |

**Table 2.** Distribution of the concentration of iAs in the water supplied to households by public water network, independently from the use.

| | iAs in tap water (µg/L) | | | |
|---|---|---|---|---|
| | Median (min-max) | Mean (S.D) | % samples >5 µg/L | % samples >10 µg/L |
| Total | 17.1 (0.2 - 148.9) | 18.8 (15.5) | 86.9% | 75.7% |
| Viterbo province | 26.6 (0.4 - 56.5) | 21.2 (14.0) | 87.5% | 77.7% |
| Latina and Rome provinces | 14.1 (0.3 - 148.9) | 16.0 (16.6) | 86.2% | 73.4% |

The median concentration values (minimum and maximum value) of iAs in drinking water were: in group 0 2.8 µg/L (0.1-9.8 µg/L), in groups 1 and 2, respectively 1.6. µg/L (0.1-9.7 µg/L) and 17.0 µg/L (10.2-35.2 µg/L). The median concentration value (minimum and maximum) of iAs in cooking water in group 0 was 4.2 µg/L (0.2-9.8 µg/L), whereas in groups 1 and 2, the medians were 18.2 µg/L (10.1-148.9 µg/L) and 21.5 µg/L (10.4-65.2 µg/L).

iAs average concentration in the solid diet component was 10.1 ng/g (DS 8.0 ng/g, range 4.9 - 45.5 ng/g), while in the liquid diet was 5.8 µg/L (SD 3.5 µg/L, range 1.1-15.8 µg/L). The concentration of iAs in the different diet components (solid and liquid) did not vary significantly neither among the two residence areas nor among the three groups.

*Arsenic intake estimate*

The overall intake of iAs (sum of the intake attributable to solid and liquid diet component, and to drinking water) was on average 0.22 µg/kg bw/day (S.D. 0.16 µg/kg bw/day; range 0.07–0.90 µg/kg bw/day). In particular, the solid diet component contributed for 64.4% of the total intake (average 0.14 µg/kg bw/day; SD 0.12 µg/kg bw/day) the liquid diet component for 17.3% (average 0.03 µg/kg bw/day, SD 0.02 µg/kg bw/day) and the drinking water for 18.3% (average 0.05 µg/kg bw/day, SD 0.05 µg/kg bw/day).

The proportion of iAs intake due to the solid diet component was significantly higher (test Wilcoxon-Mann-Whitney, $p<0.05$) in groups 0 (68.1%) and 1 (70.2%) compared to group 2 (51.5%), that instead was characterized by a significantly higher proportion of iAs intake related to drinking water (42,3% versus 14,7% for group 0 and 15.6% for group 1) (Figure 1). The proportion of iAs intake from the solid and liquid components was inversely related to the intake contribution from drinking water: indeed, as the arsenic level in drinking water increases both the total intake of iAs and the proportion of the intake due to drinking water increase.

*Internal levels of arsenic: biomarker in urine*

Overall the median concentration of UAs in urine samples was 11.5 µg/L (range, 1.7-75.5 µg/L; mean 14.2; SD 9.9).

Individuals from the Province of Viterbo did not have a concentration of UAs in urine significantly different from the subjects from the area of Latina-Rome, (15.5 µg/L and 12.8 µg/L, respectively)

According to the type of water used, the median concentration (range) of UAs in urine was 8.9 µg/L (3.8-29.6 µg/L) in group 0, 11.0 µg/L (1.7-75.7 µg/L) in group 1 and 18.5 µg/L (5.3-72.4 µg/L) in group 2 (Figure 2)

Significant differences were highlighted for the UAs concentration in urine among groups: in particular UAs concentration was higher in subjects from group 2 with respect to both the other groups, and in group 1 compared to group 0 (Wilcoxon-Mann-Whitney test, $p<0.001$).

Overall 35% and 8% had urine UAs concentration respectively higher than 15 µg/L and 30 µg/L.
Given these threshold values, in group 0 12.5% had UAs concentrations levels in urine above 15 µg/L and 1.2% above 30 µg/L. UAs concentration levels in urine for groups 1 and 2 were above 15 µg/L in 27.6% and 70.8% of subjects, and above 30 µg/L in 6.9% and 16.7% of subjects respectively.
The multivariate linear regression models highlighted that the log-transformed concentration of UAs in urine was significantly correlated with the iAs concentration in cooking and drinking water (coef.=0.01, 95% CI=0.01-0.02 and coef.=0.03, 95% CI=0.02-0.04 respectively). No significant correlations were observed with the other variables considered (age, BMI index, gender, area) (Table 3).
The log-transformed concentration of UAs in urine showed a linear correlation with the intake of iAs attributable to solid diet component (coef.=1.2, CI95%=0.1-2.2) and to drinking water (coef.=3.2, CI95%=0.1-6.3). No other significant correlations were detected (Table 4).

**Figure 1.** Proportion of the contribution of the solid diet, liquid diet and drinking water to the total intake of iAs by group defined as follows:
Group 0: (N= 12) subjects with iAs concentration <10µg/L in both drinking and cooking water;
Group 1: (N=9) subjects with iAs concentration >10µg/L only in cooking water and <10µg/L in drinking water;
Group 2: (N=5) subjects with iAs concentration >10µg/L in both drinking and cooking water.

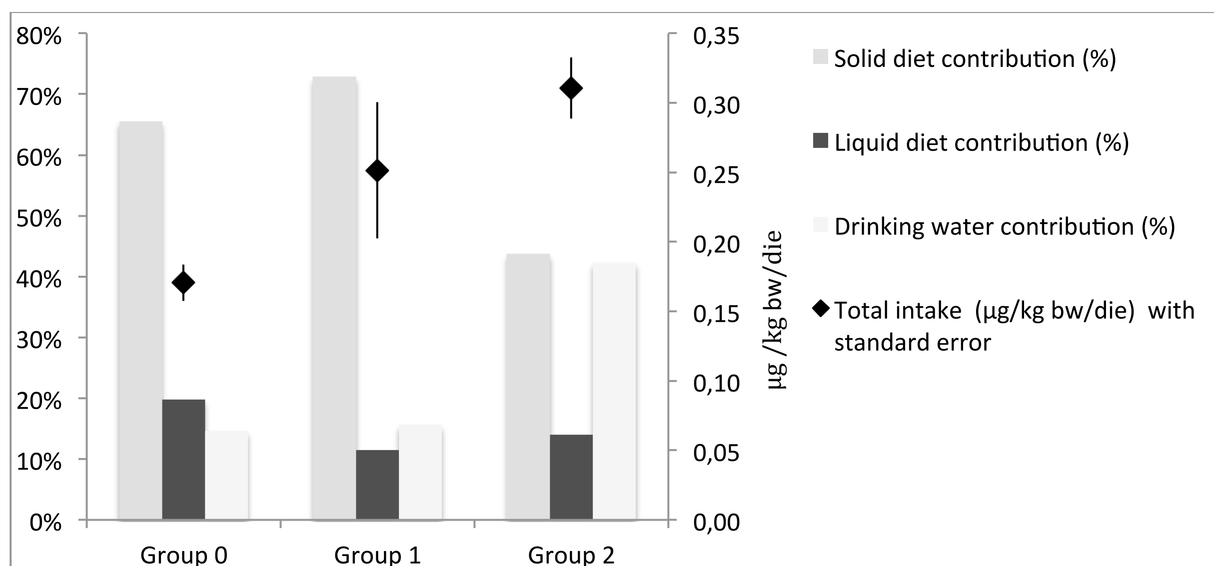

**Figure 2.** Distribution (median, interquartile range, minimum, maximum and extreme values) of the concentration of UAs in urine (µg/L) in subjects by group defined as following;
Group 0: subjects with iAs concentration <10µg/L in both drinking and cooking water;
Group 1: subjects with iAs concentration >10µg/L only in cooking water and <10µg/L in drinking water;
Group 2: subjects with iAs concentration >10µg/L in both drinking and cooking water.

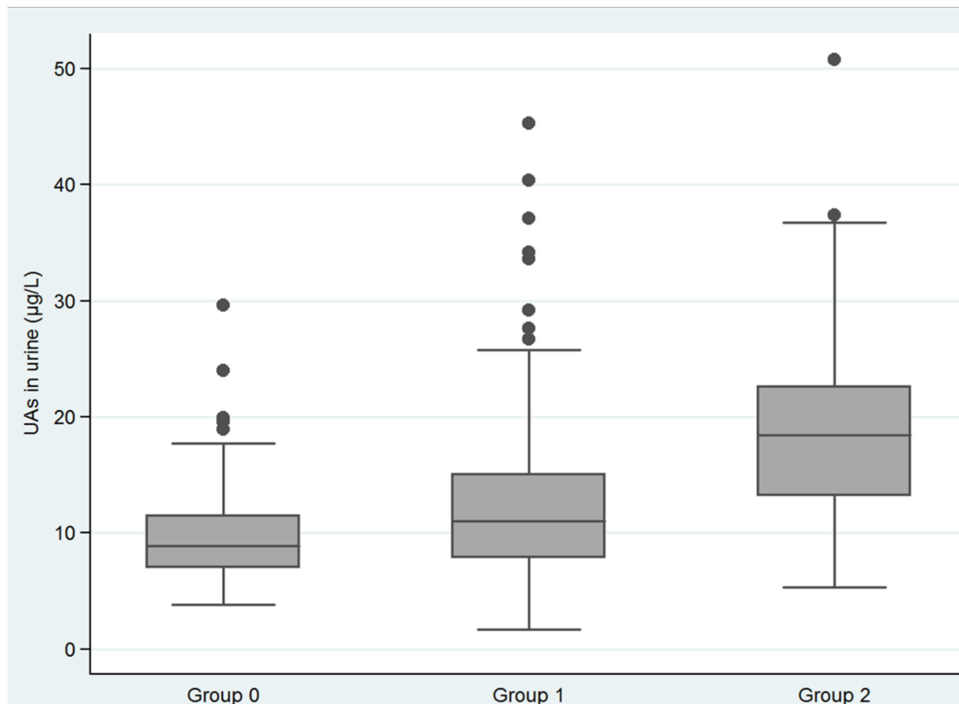


**Table 3.** Estimate of the correlation between the log-transformed concentration of UAs in urine with the log-transformed concentration of iAs in the drinking and cooking water, adjusted for age, sex, BMI, and area of residence.

| **Log- transformed Concentration of UAs in urine** (N=267) | | | |
|---|---|---|---|
| | Coef. | 95% CI | |
| iAs in drinking water (µg/L) | 0.03* | 0.02 | 0.04 |
| iAs in cooking water (µg/L) | 0.01* | 0.01 | 0.02 |
| age | 0.00 | 0.00 | 0.00 |
| female | -0.04 | -0.17 | 0.10 |
| Body mass index (BMI) | -0.01 | -0.03 | 0.01 |
| province of Viterbo | 0.09 | -0.04 | 0.23 |
| *Costant* | *2.2* | *1.8* | *2.6* |
| $R^2$ | *0.29* | | |

* values are statistically significant ($p$<0.05)

**Table 4** Estimate of the correlation between the log-transformed concentration of UAs in urine with the intake of iAs due to the solid diet component, the liquid diet component and drinking water adjusted for age, sex, BMI, and area of residence in the subpopulation of 26 subjects.

| **Log- transformed Concentration of UAs in urine** (N=26) | | | |
|---|---|---|---|
| | Coef. | 95% CI (%) | |
| iAs intake (µg /kg $_{body\ weight}$ /die) due to the solid portion | 1.07* | 0.01 | 2.12 |
| iAs intake (µg /kg $_{body\ weight}$ /die) due to the liquid portion | -4.58 | -12.33 | 3.17 |
| iAs intake (µg /kg $_{body\ weight}$ /die) due to the drinking water | 3.14* | 0.12 | 6.17 |
| Females vs males | -0.12 | -0.46 | 0.21 |
| Age | 0.00 | -0.01 | 0.00 |
| Body mass index (BMI) | 0.04 | -0.00 | 0.09 |
| Costant | 1.49 | 0.16 | 2.8 |
| $R^2$ | *0.43* | | |

* statistically significant values ($p$<0.05)

## 4.4 Discussion

The current study investigated the exposure to iAs of the population residing in an arsenic-rich area in Italy (Latium region). In the study area, arsenic-rich groundwater from a large volcanic aquifer is in both the public water supply and in a multitude of wells in rural areas. Public awareness of the problem grew during the second half of 2010 and led to widespread use of bottled water for drinking, whereas the water of the public water supply and private wells was devoted mainly to other household uses including food preparation and cooking. Indeed, this study highlighted that the iAs concentration in water supply was above the thresholds in most of the households confirming that the area can still be considered at risk for iAs exposure.

In this study an integrated approach was adopted by combining the concentration of iAs concentration in water for drinking and cooking and in duplicate diets with speciated urinary arsenic as biomarker of exposure.

UAs concentration in urines, meaning the sum iAs and its metabolites (MMA and DMA), was positively and linearly correlated with the concentration of iAs in drinking water, confirming what already reported by the scientific literature (Calderon et al, 1999; Hughes, 2006; Normandin et al, 2014). Interestingly UAs concentration had a significant linear positive correlation also with the iAs concentration in water used for cooking, supporting the hypothesis that iAs can migrate from water to food and *vice versa* during the cooking process (D'Amato et al, 2013; Perelló et al, 2008; Raab et al, 2009; EFSA, 2009). This aspect highlights the importance of cooking water and not only drinking

water as source of iAs, and the importance to intervene on the water supply as a whole in order to reduce the overall population exposure to iAs.

The duplicate diet study allowed an accurate estimate of the dietary iAs intake. The overall iAs intake estimates ranged from 0.07 to 0.90 µg/kg bw/day. Our results are similar to those estimated in the second total diet study in France and in the EFSA report. In the French study the mean iAs intake for the adult population was 0.24 µg/kg bw/day for the low speciation assumptions and 0.28 µg/kg bw/day for high speciation assumptions (ANSES, 2011). EFSA estimated the chronic dietary exposure to iAs based on the EFSA Comprehensive European Food Consumption Database using 28 surveys from 17 European countries and the mean dietary exposure in the adult population ranged from 0.09 to 0.38 µg/kg b.w. per day (min LB- max UB). In that report, EFSA specifies that the most important sources of uncertainty were the heterogeneity of the food consumption data, the conversion of total As into iAs and the treatment of the left censored occurrence data, and underlines the need of more analytical data in order to reduce such uncertainty (EFSA, 2014). However, since we directly measured food quantity and food composition (the subjects saved duplicate portions from each meal consumed in 24H), and the arsenic speciation (we measured the different arsenic species in food samples) without making any assumption on them, direct comparison between our results and those from the other studies mentioned above are not straightforward. Indeed, the similarity of our results with the French and the EFSA studies is probably related to the overestimation of the exposure that was made in the two studies in order to be more cautious.

The contribution of drinking water to the total iAs intake is predominant when iAs concentration in drinking water is above the threshold (in our study >10µg/L), while if drinking water has low iAs (in our study below 10µg/L), the solid food component becomes the main source of iAs intake. Moreover, the proportion of iAs intake due to the solid component is inversely related to the intake contribution from drinking water. Focusing on this result, it appears clear the contribution of food to the total exposure to iAs, especially in case the iAs water levels are low (in our study below 10µg/L), such source should be taken into account when considering the policy to reduce the overall iAs exposure (Xue et al, 2010; Roychowdhury et al, 2003; EFSA, 2009).

Finally the concentration of UAs in urine showed a linear correlation with the intake of iAs attributable to solid diet component and with the iAs intake through drinking water. These results confirm that UAs provides a reliable estimate of the iAs dietary exposure and can be used in population studies to estimate the iAs exposure even in context where food is the main contributor to the total iAs intake.

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# Chapter 5.
# Exposure to Endocrine Disrupters and Nuclear Receptor Gene Expression in Infertile and Fertile Women from Different Italian Areas.

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*Abstract*

Within the PREVIENI project, infertile and fertile women were enrolled from metropolitan, urban and rural Italian areas. Blood/serum levels of several endocrine disrupters (EDs) (perfluorooctane sulfonate, PFOS; perfluorooctanoic acid, PFOA; di-2-ethylhexyl-phthalate, DEHP; mono-(2-ethylhexyl)-phthalate, MEHP; bisphenol A, BPA) were evaluated concurrently with nuclear receptors (NRs) gene expression levels (ERa, ERb, AR, AhR, PPARg, PXR) in peripheral blood mononuclear cells (PBMCs). Infertile women from the metropolitan area displayed significantly higher levels of: BPA compared to fertile women (14.9 vs. 0.5 ng/mL serum); BPA and MEHP compared to infertile women from urban and rural areas; enhanced expression levels of NRs, except PPARg. Infertile women from urban and rural areas had PFOA levels significantly higher than those from metropolitan areas. Our study indicates the relevance of the living environment when investigating the exposure to EDs and the modulation of the NR panel in PBMC as a suitable biomarker of the effect, to assess the EDs impact on reproductive health.

## 5.1 Introduction

Infertility is defined as "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild et al. 2009). This condition affects millions of women of reproductive age worldwide; the prevalence depends on the residing geographic area, pointing to the role of environmental factors (Mascarenhas et al, 2012). Several toxicological studies identified associations between exposure to endocrine disrupters (EDs) and women's reproductive problems leading to infertility (Caserta et al, 2008; Caserta et al, 2011). In addition, an increasing

number of human biomonitoring (HBM) studies shows that the general population is exposed to persistent and bioaccumulating EDs (Bonde et al, 2008; Wojtyniak etal, 2010). On the contrary, limited HBM data still exist on several EDs that are widely present in foods, the living environment and consumer products, such as perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), di-2-ethylhexyl phthalate (DEHP), as well as bisphenol A (BPA).

PFOS and PFOA are still widely used in paper, food packaging contact materials and textiles (Benford et al, 2008), and since 2010, they have been included among persistent organic pollutants (POPs) (UNEP, 2010). Human exposure occurs mainly through the diet, especially fish, but neither compound is routinely monitored in foods in Europe; indoor dust is also important for PFOA exposure (EFSA, 2011; EFSA, 2012). PFOS and PFOA exposures have been associated with reduced fertility (Governini et al, 2011), longer time to pregnancy (Fei et al, 2009)[12], as well as endometriosis (Louis et al, 2012). Both chemicals enhance the activity of different nuclear receptors (NRs), including the estrogen receptor (ER) (Benninghoff et al, 2011), the peroxisome proliferator-activated receptors (PPARs) (Bjork et al, 2009; Takacs et al, 2007) and the pregnane X receptor (PXR) (Ren et al, 2009).

DEHP is a common plasticizer used primarily in soft polyvinyl chloride; its presence in several consumer products (building materials, floorings, clothing, furnishings, food contact materials) leads to a widespread human exposure, even though DEHP is not considered a persistent compound (Latini, 2005).

Due to toxicological evidence, DEHP use has been recently restricted (EU, 2011). Upon intake, DEHP is quickly metabolized to its major toxic metabolite, mono-(2-ethylhexyl) phthalate (MEHP), representing the toxicologically relevant biomarker of DEHP exposure. DEHP is an agonist of PPARs and PXR, also altering the biosynthesis of estrogens and androgens. Epidemiological studies suggest a possible association with endometriosis, albeit the results are not univocal (Cobellis et al, 2003; Itoh et al, 2009; Kim et al, 2011), whereas toxicological studies on rodents exposed to DEHP/MEHP point out impaired female reproductive function with decreased aromatase and estradiol levels (Lovekamp-Swan et al, 2003).

BPA is extensively used as a monomer in polycarbonate plastics and in epoxy resins, representing one of the world's highest production volume chemicals. A recent, comprehensive assessment by the European Food Safety Authority, currently available as draft, identifies food contact items, followed by thermal paper, as the main determinants of human exposure (Ritter, 2011; EFSA, 2014). The adverse health effects of BPA are still a matter of intense debate. In women, BPA internal levels have been positively correlated with infertility-related conditions (Ehrlich et al, 2012; Cobellis et al, 2009; Kandaraki et al, 2011). BPA is considered mainly as an ERα and ERβ agonist, but it can also affect other endocrine pathways, e.g., by acting as an antagonist of the androgen receptor (AR) or as an

agonist of the aryl hydrocarbon receptor (AhR), involved in cross-talk processes with ERs, AR and other NRs and agonist of PXR (Rubin , 2011; Sui et al, 2012).

PFOS, PFOA, DEHP and BPA are the target EDs of the project, PREVIENI (Study in model areas on the environmental and health impact of some emerging chemical contaminants (endocrine disrupters): living environment, reproductive outcomes and repercussions in childhood; http://www.iss.it/prvn, supported by the Italian Ministry of Environment). The project's first results pivoted on the possible relationship between EDs and reproductive health status. The PREVIENI cohort of Italian infertile women had a higher presence of detectable BPA serum levels, as well as enhanced expression of ERα, ERβ, AR and PXR in peripheral blood mononuclear cells (PBMCs) in comparison with fertile controls: significant correlations were also observed between ERα, ERβ, AR, AhR and PXR expression levels and BPA, MEHP concentrations and between AhR and PFOA (Caserta et al, 2013). Within PREVIENI, fertile and infertile women were enrolled in three Italian areas representing different living environment scenarios, which may be related to different EDs exposure patterns: Roma (Lazio, Central Italy), with all of the features of a metropolitan environment and lifestyle; Ferrara (Emilia-Romagna, Northern Italy), a medium-sized town amid a prosperous area with many farms and small- or medium-sized industries; Sora (Lazio, Central Italy), a rural municipality characterized by intensive agricultural activities. Therefore, the goal of the present study is to assess whether the area of residence can be related to any difference in serum ED concentrations and gene expression levels of NRs in women with different reproductive health status.

## 5.2 Materials and methods

*Areas under Study*

Three different geographic areas were considered in this study: a metropolitan area (Rome, Lazio Region, Central Italy, approximately 2,700,000 residents); a medium-sized urban area (Ferrara, Emilia-Romagna Region, Northern Italy, approximately 130,000 residents); and a rural area (Sora, Lazio Region, Central Italy, approximately 26,000 residents). In order to characterize the areas under study, territorial, demographic and productive indicators were chosen for their potential contribution to the environmental contamination as regards the EDs considered in this study. Data on the selected indicators for each area were obtained for the year 2011 from the Italian National Institute of Statistics (ISTAT, http://www.istat.it).

*Study Subjects*

From January 2009, to December 2011, on a voluntary basis, 110 infertile and 43 fertile women were enrolled in the following medical centers per area:

- n = 49 infertile and n = 13 fertile women in the Department of Women Health and Territorial Medicine of “Sapienza” University “Sant’Andrea” Hospital, Rome;
- n = 38 infertile and n = 22 fertile women in the Department of Biomedical Sciences and Advanced Therapies, Section of Obstetrics and Gynaecology, University of Ferrara;
- n = 23 infertile and n = 8 fertile women in the Infertility Center S.T.S. (Sterility Therapy and Study) of Sora.

The fertile women were selected among those with a regular menstrual cycle who obtained a spontaneous pregnancy in the last year and stopped breastfeeding at least six months before starting the study. The infertile women, selected among those with a diagnosis of primary infertility (tubal infertility, endometriosis, anovulation, immunological factors) or unexplained infertility, were enrolled in the study before starting the infertility treatment. Inclusion criteria were: residing in the municipalities included in the area, age from 18 to 40 years, body mass index (BMI) <30 and PBMCs levels within the range of normal values for age and sex.

The exclusion criteria, which included the main confounders, were: occupational exposure to the selected EDs (plastic, housewares or textile industries), smoking habit, vegetarian diet, BMI >30 and the evidence of inflammatory or infectious diseases. The study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Approval from the Ethical Committees of the responsible structures of the IVF centers were obtained before the beginning of this study, and all enrolled women gave informed consent to study inclusion.

*Collection and Storage of Samples*

All samples obtained from infertile women were collected before hormonal stimulation. Glass vials were used in order to avoid the possible release of DEHP or BPA from plastics. Three aliquots of venous blood were collected from each woman. For ED level determination, 5 mL of heparin-treated whole blood and 10 mL centrifuged blood to obtain serum were sampled and sent to the Environment Science Department “G. Sarfatti” (now the Department of Physical, Earth and Environmental Sciences) of the University of Siena.

For NR gene expression, blood samples were collected in PAXgene Blood RNA Tubes (PreAnalitiX, Plymouth, U.K.) and frozen until use. All samples were sent to the Food and Veterinary Toxicology Unit (Istituto Superiore di Sanità, Roma). Twelve samples from infertile subjects (eight from Roma, three from Ferrara and one from Sora) and two samples of fertile women from Ferrara were not analyzed for NR gene expression, due to delivery problems.

*Chemical Analysis of Biomarker of Exposure*

Based on established literature methods (see below), BPA, DEHP and MEHP were measured in serum and PFOS and PFOA in whole blood. All analyzed EDs were extracted using a liquid-liquid separation

procedure and measured using high performance liquid chromatography with electrospray ionization tandem mass spectrometry (LC-ESI-MS).

- PFOS/PFOA

For analysis of PFOS/PFOA, an extraction was performed according to the analytical procedure previously described (Governini et al, 2009). Briefly, the samples were extracted with methyl tert-butyl ether (MTBE, J.T. Baker). The solvent was evaporated under nitrogen and replaced with methanol (J.T. Baker). Twenty µL were injected into HPLC (equipped with Betasil© C18 column, Thermo Electron Corporation) interfaced to a mass spectrometer at linear triple quadrupoles, by an electrospray ionization (ESI) source, working in negative ion mode (Finnigan LTQ Thermo Electron Corporation, San Jose, CA). The limit of detection (LOD) for both PFOS and PFOA was 0.4 ng/mL, corresponding to the value of the compounds in the blanks +3 SD.

- DEHP/MEHP

The analytical procedure for the extraction of DEHP and MEHP from serum samples has been previously described (Caserta et al, 2013). Briefly, 0.5 g of each thawed sample were added to 4 mL of acetone (J.T. Baker), sonicated for 2 min and centrifuged for 15 min at 3000 rpm for two times. Supernatants were evaporated in a centrifugal evaporator (Thermo Scientific) and suspended with 0.5 mL of deionized water and 4 mL of acetic acid (J.T. Baker). After adjusting the volume to 0.5 mL, 5 µL of the sample were injected into the LC-ESI-MS system. A reverse phase HPLC column (Wakosil3C18, 2.0 × 100 mm, 3 µm; Wako Pure Chemical Industries Ltd.) was used. ESI-MS was operated in negative or positive ion mode depending on the analyte. The LODs were 2 ng/mL for MEHP and 10 ng/mL for DEHP.

- BPA

Total BPA (free plus glucuronated) in serum was analyzed according to the procedure previously described (Caserta et al, 2013). Each aliquot of 0.5 mL of serum was incubated with 2 µL/mL of the enzyme I glucuronidase (Sigma-Aldrich) at 37 °C for 12 h. Subsequently, the sample was added to 3 mL of ethyl ether (J.T. Baker), shaken for 30 minutes and centrifuged at 4,000 rpm for 5 minutes. The procedure was repeated three times. The collected supernatants were then evaporated and reconstituted in 0.5 mL of methanol. Twenty microliter of sample were injected into a Betasil C18 column 50 × 2.1 mm at a flow rate of 250 µL/min in the HPLC-ESI-MS instrument. The negative ion for the identification of BPA was obtained by fragmentation of the ion 227 with collision energy of 35 and production of the ion (m/z) 212. The ESI source was set at a voltage of 5 kV and to a rush of 3 µA. The LOD was 0.5 ng/mL.

- Data Quality Assurance and Quality Control

The analytical protocol comprised measures to avoid contamination from plasticizers in test materials, which included, besides the use of metal needles and glassware vials for collection and storage of samples, the use of glass labware rinsed by acetone and hexane to remove potential contaminants and the assessment of method blanks (Vandenberg et al, 2014).

In addition, in order to monitor and evaluate any possible contamination of samples, data quality assurance and quality control protocols were performed, including matrix spikes, laboratory blanks and continuing calibration verification. In particular, blanks were analyzed with each set of five samples to check possible iatrogenic contamination and interferences: levels of chemicals in such samples resulted in being below the limit of detection for each compound.

*Gene Expression Analysis of Nuclear Receptors*

Blood samples collected in PAXgene Tubes were extracted for their RNA content by the PAXgene Blood RNA Kit (Qiagen). Total RNA was quantified by NanoDrop (Thermo Scientific Wilmington, DE, USA) and assessed for its quality by 1% agarose gel electrophoresis; all of the samples were optimal to be further analyzed. For each sample, 1 µg of RNA was reverse transcribed to cDNA by the cDNA Synthesis Kit (Quantace, London, UK), according to the manufacturer's protocol.

Gene expression analysis was performed by quantitative real-time PCR using the Sensi Mix SYBR Kit (Quantace), with GAPDH as the reference gene. Specific primers for the selected NRs and GAPDH were designed using the Primer-BLAST web application (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in Table 1.

**Table 1.** Primers sequences, accession numbers and amplicon lengths for reference (GAPDH) and nuclear receptors (NR) genes.

| Gene | RefSeq Accession | | Sequence 5' to 3' | Amplicon Length (bp) |
|---|---|---|---|---|
| GAPDH | NM_002046.4 | forward | ACTCCTCCACCTTTGACGCT | 273 |
| | | reverse | CTTCAAGGGGTCTACATGGC | |
| ERα | NM_000125.3 | forward | ACTGCGGGCTCTACTTCATC | 275 |
| | | reverse | GGCTGTTCCCAACAGAAGAC | |
| ERβ | NM_001040275.1 | forward | CTCTTTTGCCTGAAGCAACG | 269 |
| | | reverse | CTGGGCAGTTAAGGAGACCA | |
| AR | NM_000044.3 | forward | CCCATCTATTTCCACACCCA | 259 |
| | | reverse | GCAAAGTCTGAAGGTGCCAT | |
| PPARγ | NM_138712.3 | forward | GATGACAGCGACTTGGCAAT | 269 |
| | | reverse | AGGAGCGGGTGAAGACTCAT | |
| AhR | NM_001621.4 | forward | TTCCACCTCAGTTGGCTTTG | 233 |
| | | reverse | GGACTCGGCACAATAAAGCA | |
| PXR | NM_003889.3 | forward | GGCCACTGGCTATCACTTCA | 343 |
| | | reverse | GGTTTTCATCTGAGCCTCCA | |

Amplification efficiencies, cDNA input dilution and primer concentrations were optimized by the standard curve method. Real-time PCR reactions were run on a Stratagene MP3005P Thermocycler. Experiments were performed in duplicate on 96-well PCR plates. The thermal program was as follows: 1 cycle at 94 for 10 min; 40 cycles at 94 °C for 10 s, 58 °C for 10 s and 72 °C for 10 s; and 1 dissociation cycle from 55 to 94 °C to verify amplification products. Data are expressed as $2^{-DCt}$ ($DCt = Ct_{TG} – Ct_{RG}$), with $Ct_{TG}$ as the threshold cycle of the target gene and $Ct_{RG}$ as the threshold cycle of the reference gene.

*Statistical Analysis*

We performed statistical descriptive and comparative analysis using non-parametric tests. We decided to limit the statistical inference to single variable analyses (univariate statistics) stratifying by areas to provide unbiased results, taking into account the number of samples.

The concentration of each ED below the respective LOD has been considered as “<LOD”. For the inferential analyses and comparisons, the values below LOD have been replaced by half the LOD value (medium-bound) (EFSA, 2010).

Dichotomous variables for concentrations of the EDs (0 if ≤ LOD and 1 if > LOD) were created. ED concentrations and NR expression values were not normally distributed, and the log transformation did not normalize the distributions. Therefore, differences between infertile and fertile women resident in the same area and across the areas were assessed with the Wilcoxon–Mann–Whitney test, adjusting for multiple comparisons using the Bonferroni procedure for correcting the p-value.

The risk of infertility in relation to the ED concentration was calculated stratifying by area of residence using univariate analysis and the chi-square test.

Statistical analysis was performed with STATA 11.2 (StataCorp, 4905 Lakeway Drive, College 17 Station, TX, USA) setting significance at $p < 0.05$.

**Table 2.** Distribution of a set of territorial, demographic and productive indicators in the study areas. Data from the Italian National Institute of Statistics (ISTAT).

| Areas | Metropolitan (Rome) | | Urban (Ferrara) | | Rural (Sora) | |
|---|---|---|---|---|---|---|
| Indicators | 1-10 employees | >10 employees | 1-10 employees | >10 employees | 1-10 employees | >10 employees |
| Agricultural enterprises | 393 | 17 | 1684 | 10 | 4 | 0 |
| Textile industries | 206 | 9 | 40 | 2 | 4 | 0 |
| Petroleum refinery | 16 | 12 | 0 | 1 | 0 | 0 |
| Manufactures of chemicals | 121 | 43 | 12 | 8 | 4 | 0 |
| Manufactures of articles of rubber | 0 | 0 | 0 | 0 | 0 | 0 |
| Manufacture of articles of plastics | 0 | 0 | 0 | 0 | 0 | 0 |
| Sanitation and waste management | 39 | 6 | 2 | 0 | 0 | 0 |
| Population | 2,724,347 | | 134,464 | | 26,542 | |
| Surface (km$^2$) | 1307.71 | | 404.36 | | 71.82 | |
| Population density (inhabitants/ km$^2$) | 2083.30 | | 332.54 | | 369.56 | |

## 5.3 Results

*Areas Characterization*

Considering the territorial, demographic and productive indicators, differences were evidenced in the three areas in the number and percentage of industries by category of production per km$^2$. In particular, the metropolitan area was characterized by a high population density with about three million people and by the presence of agricultural and industrial enterprises. However, considering the population density, the highest proportion of enterprises with more than 10 employees was observed in the urban area. In the rural area, neither factories nor farms with more than 10 employees were reported (Table 2).

*Biomarkers of Exposure*

PFOS, PFOA, MEHP and BPA blood/serum levels in the women enrolled are summarized in Table 3. The results expressed as mean, median and interquartile range (25th–75th percentile) values are provided for both fertile and infertile groups by area. Since DEHP was found above the

LOD only in one infertile woman in the metropolitan area (72.25 ng/mL) and in three infertile women in the rural area (range 10.03–25.33 ng/mL), it was excluded from the analysis.

**Table 3.** Analytical values of PFOS, PFOA (ng/mL blood), MEHP and total BPA (ng/mL serum) in enrolled women grouped by area of residence and subject group.

| | Chemicals | PFOS | | PFOA | | MEHP | | BPA | |
|---|---|---|---|---|---|---|---|---|---|
| Areas | | infertile | fertile | infertile | fertile | infertile | fertile | infertile | fertile |
| Total | mean | 3.5 | 2.2 | 1.8 | 1.7 | 37.9 | 13.1 | 10.6 | 4.8 |
| (110 infertile; | median | <0.4 | <0.4 | <0.4 | <0.4 | 8.3 | 3.3 | <0.5 | <0.5 |
| 43 fertile) | 25th p # | <0.4 | <0.4 | <0.4 | <0.4 | <2 | <2 | <0.5 | <0.5 |
| | 75th p | 0.86 | <0.4 | 3.7 | 3.3 | 26.8 | 11.3 | 9.4 | <0.5 |
| | %>LOD | 30.00% | 20.90% | 40.90% | 34.90% | 62.70% | 58.10% | 41.80% | 23.30% |
| Metropolitan | mean | 6.9 | 4.5 | 0.6 | <0.4 | 75.3 | 32.3 | 19.5 | 7.3 |
| (49 infertile; | median | <0.4 | <0.4 | <0.4 [a] | <0.4 [a] | 23.1 [a] | 12.1 [a] | 14.9 [a,*] | <0.5 * |
| 13 fertile) | 25th p | <0.4 | <0.4 | <0.4 | <0.4 | <2 | <2 | <0.5 | <0.5 |
| | 75th p | 2.9 | <0.4 | <0.4 | <0.4 | 127.4 | 18.2 | 25.8 | <0.5 |
| | %>LOD | 30.6% | 7.7% | 10.2% | 0.0% | 69.4% | 69.2% | 71.4% | 23.1% |
| Urban | mean | 0.8 | 1.3 | 3.2 | 2.6 | 8.4 | 6.2 | 1.7 | 2.2 |
| (38 infertile; | median | <0.4 | <0.4 | 3.6 [b] | 1.1 [b] | 4.4 [b] | 3.7 [a] | <0.5 [b] | <0.5 |
| 22 fertile) | 25th p | <0.4 | <0.4 | <0.4 | <0.4 | <2 | <2 | <0.5 | <0.5 |
| | 75th p | 0.6 | <0.4 | 4.9 | 5.2 | 9.4 | 5.6 | 3.8 | 5.4 |
| | %>LOD | 26.3% | 22.7% | 71.1% | 50.0% | 73.7% | 72.7% | 26.3% | 27.3% |
| Rural | mean | 1 | 1.2 | 2.1 | 1.9 | 7.2 | <2 | 6 | 7.8 |
| (23 infertile; | median | <0.4 | <0.4 | 2.2 [b] | 1.6 [b] | <2 [b] | <2 [b] | <0.5 [b] | <0.5 |
| 8 fertile) | 25th p | <0.4 | <0.4 | <0.4 | <0.4 | <2 | <2 | <0.5 | <0.5 |
| | 75th p | 0.9 | 1.6 | 3.7 | 3.2 | 18.6 | <2 | <0.5 | <0.5 |
| | %>LOD | 34.8% | 37.5% | 56.5% | 50.0% | 30.4% | 0.0% | 4.4% | 12.5% |

LOD = 0.4 ng/mL for PFOS and PFOA; 2 ng/mL for MEHP; 0.5 ng/mL for BPA. * indicates statistically significant different values between fertile and infertile women in the same area of residence (Mann-Whitney test corrected with the Bonferroni procedure). [a,b] Different superscript letters indicate statistically significant different values between areas within subjects of the same group (Mann-Whitney test corrected with the Bonferroni procedure). # 25th and 75th p indicate percentile values.

The percentage of subjects exposed to each specific ED (levels > LOD), as well as the corresponding concentrations were different in the three study areas. BPA was significantly more prevalent in the metropolitan area, with a significantly higher level in infertile women. MEHP was detected in over 65% of the women from both the metropolitan and urban areas, but levels were significantly higher in women residing in the metropolitan area; in the rural area, MEHP was found in about 22% of women, with significantly lower levels compared to the other areas. BPA was detected in over 60%, 25% and 6% of the women from metropolitan, urban and rural areas, respectively. PFOS was detected in about 30% of the subjects in each area without differences in concentration. PFOA was the only ED significantly more prevalent in the urban and rural areas: it was detected in over 50% of

the women from the urban and rural areas, but in less than 10% of those from the metropolitan area, where levels were significantly lower than in the other two areas.

The comparison of ED levels between infertile and fertile women for each area revealed that in the metropolitan area, infertile women had significantly higher BPA levels than fertile women (median values 14.9 vs. 0.5 ng/mL serum).

The comparisons of ED concentrations between fertile women by area of residence showed no significant difference for BPA or PFOS concentrations. MEHP concentration was significantly lower in the rural area, while no difference was found between the urban and the metropolitan area. Regarding PFOA, fertile women in the urban and rural areas had significantly higher levels than those in the metropolitan area.

Similar to fertile women, the comparison between infertile women by area of residence showed no significant difference for PFOS internal levels. On the other hand, in the metropolitan area, significantly higher concentrations of MEHP and BPA and significantly lower concentrations of PFOA were found compared to the urban and rural areas, while no significant differences were highlighted between the rural and urban areas. By comparing the proportion of infertile and fertile women with EDs concentration >LOD in univariate analysis, a significant association with infertility was observed for BPA in the metropolitan area (OR = 8.3; 95% CI = 1.7–52.1) (Table 4).

**Table 4.** Infertility risk factors associated with endocrine disrupters (ED) exposure (serum concentration >LOD) in enrolled women grouped by area of residence.

| Chemicals | Total (n = 153) | | | Metropolitan area (n = 62) | | | Urban area (n = 60) | | | Rural area (n = 31) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI | | OR | 95% CI | | OR | 95% CI | | OR | 95% CI | |
| PFOS | 1.6 | 0.7 | 4.3 | 5.3 | 0.7 | 241.1 | 1.2 | 0.3 | 5.3 | 0.9 | 0.1 | 7.3 |
| PFOA | 1.3 | 0.6 | 2.9 | ND | - | - | 2.5 | 0.7 | 8.4 | 1.3 | 0.2 | 8.9 |
| MEHP | 1.2 | 0.6 | 2.6 | 1.0 | 0.2 | 4.4 | 1.1 | 0.3 | 3.9 | ND | - | - |
| BPA | 2.4 * | 1.0 | 5.9 | 8.3 * | 1.7 | 52.1 | 1.0 | 0.3 | 3.8 | 0.3 | 0.0 | 28.5 |

* Indicates a statistically significant value; ND indicates not determinable.

*Nuclear Receptors Gene Expression*

NRs gene expression values (mean, median and interquartile range) in infertile and fertile women by area are summarized in Table 5.

**Table 5.** Gene expression values of NRs in enrolled women grouped by area of residence and subject group. Data are expressed as $2^{\Delta Ct}$ values with GAPDH as the reference gene.

| Nuclear Receptors | | ERα | | ERβ | | AR | | PPARγ | | AhR | | PXR | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Areas | | infertile | fertile | infertile | fertile | infertile | fertile | infertile | fertile | infertile | fertile | infertile | fertile |
| Total | mean | 0.1138 | 0.0151 | 0.0937 | 0.0122 | 0.1086 | 0.0142 | 0.0003 | 0.0008 | 0.0256 | 0.0047 | 0.1015 | 0.0122 |
| (98 infertile ; | median | 0.0082 | 0.0007 | 0.0081 | 0.0017 | 0.0087 | 0.001 | 0.0001 | 0.0002 | 0.0021 | 0.0012 | 0.0067 | 0.0004 |
| 41 fertile) | 25th p [#] | 0.0005 | 0.0003 | 0.0011 | 0.0009 | 0.0008 | 0.0005 | 0.0001 | 0.0001 | 0.0009 | 0.0007 | 0.0002 | 0.0002 |
| | 75th p | 0.0636 | 0.0058 | 0.0413 | 0.0071 | 0.0636 | 0.0099 | 0.0003 | 0.0002 | 0.0081 | 0.0022 | 0.0998 | 0.0043 |
| Metropolitan | mean | 0.2582 | 0.0282 | 0.2143 | 0.0216 | 0.2439 | 0.0232 | 0.0004 | 0.0003 | 0.0589 | 0.0068 | 0.2231 | 0.0195 |
| (41 infertile ; | median | 0.0647 [a,*] | 0.0007 [a,*] | 0.0591 [a,*] | 0.0013 [*] | 0.0593 [a,*] | 0.0008 [*] | 0.0002 | 0.0002 | 0.009 [a,*] | 0.0013 [*] | 0.0998 [a,*] | 0.0004 [a,*] |
| 13 fertile) | 25th p | 0.0256 | 0.0004 | 0.0132 | 0.0008 | 0.0186 | 0.0005 | 0.0000 | 0.0001 | 0.0041 | 0.0007 | 0.0170 | 0.0003 |
| | 75th p | 0.2963 | 0.0059 | 0.2398 | 0.0098 | 0.2707 | 0.0099 | 0.0003 | 0.0002 | 0.0265 | 0.0039 | 0.2698 | 0.0043 |
| Urban | mean | 0.0158 | 0.0125 | 0.0107 | 0.0105 | 0.0177 | 0.0137 | 0.0002 | 0.0013 | 0.0022 | 0.0048 | 0.0228 | 0.0124 |
| (35 infertile ; | median | 0.0012 [b] | 0.0014 [a] | 0.0017 [b] | 0.0022 [a] | 0.0015 [b] | 0.0022 [a] | 0.0001 | 0.0002 | 0.0016 [b] | 0.0013 [a] | 0.0006 [b] | 0.0007 [a] |
| | | | | | | | | | 0.000 | | | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rural | mean | 0.0004 | 0.0003 | 0.0011 | 0.0011 | 0.0009 | 0.0007 | 0.0002 | 0.0001 | 0.0009 | 0.0008 | 0.0003 | 0.0001 |
| (22 infertile ; | median | 0.0004 c | 0.0004 b | 0.0010 c | 0.0010 b | 0.0008 c | 0.0006 b | 0.0001 | 0.0001 | 0.0008 b | 0.0006 b | 0.0002 c | 0.0001 b |
| 8 fertile) | 25th p | 0.0003 | 0.0002 | 0.0006 | 0.0009 | 0.0005 | 0.0004 | 0.0001 | 0.0001 | 0.0004 | 0.0004 | 0.0001 | 0.0001 |
| | 75th p | 0.0006 | 0.0004 | 0.0015 | 0.0012 | 0.0011 | 0.0008 | 0.0002 | 0.0002 | 0.0011 | 0.0014 | 0.0002 | 0.0002 |

* Indicates statistically significant different values between infertile and fertile women in the same area of residence (Mann-Whitney test corrected with the Bonferroni procedure). [a,b,c] Different superscript letters indicate statistically significant different values between areas within women of the same group (Mann-Whitney test corrected with the Bonferroni procedure).

[#] 25th and 75th p indicate percentile values.

The mRNAs of the selected NRs were detected in all samples examined, therefore confirming the suitability of the NRs panel in PBMCs. Expression levels were comparable for ERα, ERβ, AR and PXR, while AhR and PPARg were expressed at lower levels.

Comparisons between infertile and fertile women within the areas showed that in the metropolitan area, the expression of ERα, ERβ, AR, AhR and PXR was approximately ten-fold ($p < 0.01$) higher in infertile women; on the contrary, in the other areas, no significant differences were found.

The comparisons of NR expression levels between fertile women by area of residence showed that women from the metropolitan and urban areas had a comparable expression of ERα, ERβ, AR, AhR and PXR. Fertile women from the rural area had significantly lower expression of all these NRs compared to the urban area and of ERα and PXR compared to the metropolitan area. No difference was detected for PPARγ expression.

The comparisons of NRs expression levels between infertile women by area of residence showed a different picture. Infertile women from the metropolitan area displayed significantly higher expression levels of all NRs, but PPARγ, when compared to infertile women from the other two areas ($p < 0.01$ for both comparisons. Mean expression levels in the metropolitan area were about 10-(PXR) to 25-fold (AhR) higher than in the urban area and about 70-(AhR) to 900-fold (PXR) higher than in the rural area).

## 5.4 Discussion

Our study, in the frame of the Italian project, PREVIENI, indicates the relationship between the residential area and biomarkers, serum/blood concentrations of some EDs and gene expression levels of a panel of NRs, in infertile and fertile women. In particular, BPA and MEHP levels, as well as expression of ERα, ERβ, AR, AhR and PXR were more prevalent in infertile women from the metropolitan area. In this area, BPA serum levels were also significantly higher in infertile women compared to fertile women. In the urban area, there was a noticeable increase of PFOA blood levels compared to the metropolitan area. This increase was detected also in the rural area, albeit to a lower extent; otherwise, women from the rural area overall showed the lowest values of MEHP and BPA serum concentrations, as well as of ERα, ERβ, AR, AhR and PXR expression. The presence of detectable PFOS blood levels was quite prevalent in the enrolled subjects, about 30%, but no significant relationship with the residential area was observed. Overall, the differences related to the residential area appeared more enhanced when comparing the infertile, rather than the fertile, groups.

The three areas represented quite distinct living environment scenarios according to selected territorial, demographic and productive indicators. The significantly higher levels of BPA (median

levels and % >LOD) and MEHP (median levels) in women from the metropolitan area may reflect both the greater presence of economic activities employing these chemicals, as well as characteristic usage patterns of food commodities and consumer products. Moreover, in our study, BPA was the only ED specifically associated with infertility: the significant association between detectable BPA levels and infertility was confined to the metropolitan area, with an OR = 8.3. Furthermore, BPA levels in infertile women were about 30-fold higher than in controls of the same area.

In our study, we sampled BPA and MEHP in serum, so as to establish a more direct correlation with the biomarkers of effect measured in PBMCs. Indeed, we aimed at studying in the same matrix (i.e., blood) both biomarkers of exposure and NR expression identified as a potential, and toxicologically relevant, biomarker of effect for EDs (Wens et al, 2013). Whereas blood is considered the matrix of choice for bioaccumulating compounds, like PFOS and PFOA, BPA and phthalates are regarded as EDs undergoing quick metabolism (Koch and Calafat, 2009). Nevertheless, several recent studies, using proper quality control analysis, have measured circulating levels of BPA and/or MEHP, as a toxicologically relevant DEHP metabolite, in humans, giving consistent evidence of their presence into the bloodstream. Studies using BPA and/or MEHP measurements in serum mainly address possible environment-health associations, such as the potential relationships with reproductive disorders (Lathi et al, 2014; Specht et al, 2014), chronic diseases (Lind et al, 2012; Olsén et al, 2012), breast cancer risk (Sprague et al, 2013), as well as the risk estimation of BPA mother-fetus transfer (Aris, 2014).

In our study, we observed a widespread presence of BPA and MEHP in the bloodstream with significant differences related to the selected residence areas. The adopted protocols and devices for sampling and analysis allowed us to rule out any external contamination from plastics, lending further support to the meaningful correlation of the detected BPA and MEHP internal levels with the living environment in women with different reproductive health statuses (Vandenberg et al, 2014; Ye et al, 2013).

We chose to determine total serum BPA, i.e., free together with glucuronated BPA, the major circulating metabolite (Völkel et al, 2002). We selected this approach taking into account the uncertainties on BPA metabolic fate in humans, recently discussed by Vandenberg et al. (2013). The consistent presence of detectable serum levels of BPA suggests a repeated and continuous uptake of the compound from aggregate exposure through both dietary and non-diet sources (EFSA, 2014; Stahlhut et al, 2009). Concentrations detected in infertile women in the metropolitan area were higher compared to serum levels found in other HBM studies analyzing total BPA, mainly by ELISA methods (Vandenberg et al, 2010), while those from the urban area were comparable to the reported ranges. A higher BPA exposure in the metropolitan area may also be consistent with the reported BPA increase in outdoor air in relation with the extent of urbanization (Fu and Kawamura, 2010).

Our findings are in agreement with studies demonstrating an association between BPA exposure and conditions associated with female infertility, such as polycystic ovary syndrome (Kandaraki et al, 2011; Takeuchi et al, 2004) and implantation failure in women undergoing IVF (Ehrlich et al, 2012; Ehrlich et al, 2012a). The available reports on endometriosis showed no significant correlation with urinary BPA (Itoh et al, 2007), while a significant association with serum BPA is described (Cobellis et al, 2009; Caserta et al, 2013). Overall, our study points to BPA as a pollutant of concern in metropolitan scenarios, in terms of both exposure and reproductive health.

Results on MEHP indicate a generalized and continuous human exposure to DEHP, rapidly metabolized to MEHP (Koch et al, 2003). Noteworthy, MEHP exposure was definitely higher (by an approximate 10-fold factor) in the metropolitan scenario compared to the other areas, both in fertile and infertile women. In our study, such generalized exposure was not associated with an increased risk of being infertile, also in the metropolitan area; however, we cannot conclude whether exposure levels were too low to show a detectable effect. Available data on MEHP serum levels in women of fertile age are limited: the values detected in our study were higher than in a Swedish population of women at delivery and lower than in an Italian group of endometriotic women (Högberg et ail, 2008; Cobellis et al, 2003). Epidemiological evidence on DEHP/MEHP reproductive effects in women is not univocal (Cobellis et al, 2003; Itoh et al, 2009; Kim et al, 2011), differently from in vivo studies demonstrating MEHP's adverse effects on fertility (Lovekamp-Swan et al, 2003; Reinsberg et al, 2009; vet al, 2010).

Women from the urban area, characterized also by a large presence of farm factories, had higher PFOA internal levels. Subjects from the rural area showed generally lower median concentrations for all of the EDs analyzed, which was expected from the characteristics of the territory; interestingly, however, 50% of women showed detectable PFOA levels, comparable to the urban areas. Eschauzier et al. (2013) point out that perfluorinated alkylated acids, such as PFOA, are a contaminant of groundwater and water surface from different sources, hinting to a possible relationship with the water sources used in local agricultural activities. In fact, higher PFOA levels in the selected urban area of Ferrara may reflect a site-specific problem: PFOA pollution has been reported in the major water basin nearby the Ferrara area, the Po River, probably deriving from industrial sources (Loos et al, 2008). Exposure to PFOS showed no difference among the areas, as well between infertile and fertile women. Unlike PFOA, PFOS exposure is almost completely related to food chain contamination (Benford et al, 2008; EFSA, 2012). Therefore, our findings may indicate a comparable PFOS dietary exposure in the selected population. PFOS and PFOA concentrations observed in our study were, on average, lower than estimated reference values for the German population, but comparable to levels found in Catalonia and Italy (Wilhelm et al, 2009; Ericson et al, 2007; Ingelido et al, 2010)[61].

Similarly to biomarkers of ED exposure, the area-stratified analysis showed significant differences as regards the gene expression levels of the NR panel, except for PPARγ, which did not differ either in relation to the health status or to the area of residence. Interestingly, women from the rural area generally presented significantly lower gene expression levels, with some difference between fertile and infertile women: although no conclusion can be made based on the present data, it is noteworthy that this finding seems to parallel the overall lower EDs exposure level in the same area.
In infertile women, the NR gene expression significantly differed among areas, with the highest levels in women from the metropolitan area followed by those from urban and rural area. Indeed, infertile women from metropolitan area presented a significant 10-fold increase, with respect to fertile women, in the gene expression levels of five out of six analyzed NRs, namely ERα, ERβ, AR, AhR and PXR. These NRs were all positively correlated, indicating a common responsiveness.
In our previous study, we showed a positive correlation between such NRs and BPA internal levels (Caserta et al, 2013). Since BPA serum levels were markedly higher in infertile women from the metropolitan area, it is plausible that this ED could contribute to the observed NRs increase in this group. Furthermore, MEHP showed a positive correlation with the same NR panel (Caserta et al, 2013). Even though MEHP internal levels showed no significant relationship with the fertility status, the internal levels in infertile women from the metropolitan area were significantly higher compared to the other areas. Therefore, it is plausible that MEHP might have added up to the BPA effect on NR expression in the study group of infertile women of the metropolitan area. The possible impact, if any, of an MEHP-related effect on NR expression in women remains to be ascertained.
We recognize that our data do not provide direct evidence of a causal link between ED serum levels and NR expression in PBMCs, including a possible combined effect of the simultaneous exposure to the EDs investigated. The metropolitan living environment is both associated with higher serum levels of BPA and MEHP and with an inducing effect on ERs and AR expression in infertile women. BPA is considered an ER-agonist, as well as an AR antagonist (Rubin et al, 2011); MEHP may display anti-estrogenic and anti-androgenic activities acting on steroid synthesis rather than on ERs or AR (Lee et al, 2003). Indeed, NR expression may be modulated by direct, as well as cross-talk or feed-back mechanisms (Guo et al, 2010). The observed increase of AhR and PXR is consistent with the available evidence on both BPA and MEHP mechanisms (Lovekamp-Swan et al, 2003; Rubin, 2011; Sui et al, 2012; Hurst et al, 2004; Mnif et al, 2007). However, due to the lack of information on the expression of NRs in PBMCs to compare with, we may only hypothesize that the increase may become evident only when exposure to expression-inducer compound(s) exceeds a certain threshold.
It is noteworthy that the more persistent substances, PFOS and PFOA, showed no association with fertility status in our study, contrary to the findings of some previous studies (Governini et al, 2011;

Fei et al, 2009; Louis et al, 2012). As previously noted, based on the limited data available, PFOS and PFOA internal levels were comparable to those found in the general population of Mediterranean Europe and lower than reference values for the Germany population (Ericson et al, 2007; Ingelido et al, 2010; Wilhelm et al, 2009). Therefore, our negative findings may simply reflect the lack of effect by a baseline exposure level.

While acknowledging the unavoidable limitations of cross-sectional studies, our results clearly point out the characteristics of the area of residence as a relevant factor when investigating environmental exposures, such as EDs. In the present study, the metropolitan area emerged as a hotspot for BPA and MEHP exposure, BPA being significantly associated with an increased risk of being infertile in women. Moreover, our results point out a panel of NRs (ERα, ERβ, AR, AhR and PXR) induced at the same time in PBMCs of infertile subjects. PBMCs are responsive to estrogens, natural ligand of ERs, which mediate the immune cells response and PBMC infiltration in tissues. Indeed, cardiovascular disorders and autoimmune diseases are associated with the presence of PBMCs in tissues. Notably, in reproductive female organs, PBMCs regulate the extracellular matrix and are implicated in several functions, such as ovulation, menstruation and implantation (Stygar et al, 2006). Thus, investigations in reproductive target tissues may be warranted. The identified panel of NRs can be further developed as a suitable biomarker of effect when evaluating ED exposure in relation to reproductive health.

Our study reinforces the concept that humans are continuously exposed to several EDs, still widely present in consumer goods, and that this may represent a risk factor for woman's fertility in relation to areas and living environment scenarios. Further research is warranted on the potential interactions of the internal burdens of EDs acting on similar pathways/targets, also expanding the range of EDs under scrutiny.

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# *Chapter 6.*

# General Conclusions

This thesis provides examples of different methodological approaches that can be used to estimate population exposure to chemical substances. Each chapter presents a different methodological approach that has been chosen and implemented. The main focuses are on dietary exposure assessment and biomonitoring, and their limitations and advantages have been highlighted.

From the results obtained in the different studies collected in this thesis, we point out that when selecting the approach to use for the estimate of exposure to chemicals, several different aspects must be taken into account.

Firstly, the choice of which approach depends on the nature of the chemical substance under study; indeed the knowledge available on the pathway and route of exposure are crucial in the choice of the methodology to use, as well as the knowledge on the capacity of the organism to metabolize or bioccumulate the substance.

Secondly, the choice should be driven taking into account the population of interest. The assessor must be sure of correctly characterize and identify the (sub)population to include in the study in order to be able to answer the main research question.

When conducting an exposure estimate, it is also important to clarify if the objective of the study is to assess chronic or acute exposure, since the approach used will be different. Moreover, it is important to distinguish between studies aimed at estimating the percentage of population with an exposure above or below a certain threshold value or at describing the range of exposure of the general population. In the first case we can accept a high degree of uncertainty for the range of exposure below the threshold, while in the second case the uncertainty should be quantified to correctly interpret the results.

Finally, an important aspect to consider is the kind, quality and quantity of data available that is closely interconnected with all the aspects listed above. Indeed, the data influence the choice of the method, and the amount of uncertainty that will be incorporated in the estimates. It is important to fully specify the assumptions and uncertainties inherent in the risk assessment to place the risk estimates in proper perspective. The characterization of uncertainty is also needed for identifying areas where additional data collection might significantly improve the reliability of the estimate and consequently help to identify possible actions to reduce potentially harmful exposures.

The assessment of human exposure to chemicals present in the diet, or more generally in the environment, is a rapidly developing discipline. Undoubtedly it had made great steps forward in

terms of quality and quantity of food consumption and chemicals' concentration data in different food and environmental matrices and, finally, in terms of increasing awareness of the toxicological behavior of these substances. Now it is possible to measure almost any chemical present in biologic matrices, so that biomonitoring studies can directly measure concentration of chemicals and metabolites present in the organisms (Luetzow, 2003; Paustenbach and Galbraith, 2006; Fryer et al, 2006). Such availability of new and more precise data permit to do more accurate estimation of exposure to chemicals. However, the work of the assessors is complicated by the facts that:

- exposures are typically at low concentrations,
- chemicals are present in mixtures and provide multiple simultaneous exposures,
- exposure periods are usually long,
- multiple exposure routes are involved.

Thus, more sofisticated techniques need to be constantly developed to account for those conditions (Villanueva et al, 2013).

Exposure information is the central step of the risk assessment, that is aimed at determining if a chemical can pose a risk to humans, animals or the environment. During the last several decades, risk assessment, risk management and risk communication have been formalized and incorporated into the process of risk analysis. This new approach links information on chemicals' hazards to data on human health in terms of risks and provides a science-based approach to improve decision-making processes. In this way risk analysis contributes to a reduction in the incidence of disease linked to chemicals' exposure and to maintain consumer confidence, as well as to enable the elaboration of appropriate response measures when necessary (FAO and WHO, 2005).

It appears clear that exposure science in the broadest sense needs to be constantly implemented to be update with new technologies. Furthermore, methodological approaches that have been emplemented with the primary purpose of estimating human exposure to chemical substances can be readapted in order to estimate animals' exposure in a prospective of food safety and of animal health and welfare.

In a world where old and new chemical substances are ubiquitously present in the environment, it is necessary to gain interaction among all stakeholders involved in public health in order to achieve a successful approach to a globalized and complex interlinked environment.

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## General Abstract

The public awareness that chemical substances are present ubiquitously in the environment, can be assumed through the diet and can exhibit various health effects is very high in Europe and Italy. National and international institutions are called to provide figure on the magnitude, frequency, and duration of the population exposure to chemicals, including both natural or anthropogenic substances, voluntarily added to consumers' good or accidentally entering the production chains.

This thesis focuses broadly on how human population exposure to chemicals can be estimated, with particular attention to the methodological approaches and specific focus on dietary exposure assessment and biomonitoring. From the results obtained in the different studies collected in this thesis, it has been pointed out that when selecting the approach to use for the estimate of the exposure to chemicals, several different aspects must be taken into account: the nature of the chemical substance, the population of interest, clarify if the objective is to assess chronic or acute exposure, and finally, take into account the quality and quantity of data available in order to specify and quantify the uncertainty of the estimate.

## Abstract in Italiano

La consapevolezza che le sostanze chimiche sono presenti ubiquitariamente nell'ambiente, che possono essere assunte attraverso la dieta e che sono in grado di causare svariati effetti negativi sulla salute è molto alta nelle popolazione Europea ed Italiana. Le istituzioni nazionali e internazionali, sono chiamati a fornire stime dell'entità, della frequenza e della durata dell'esposizione della popolazione alle sostanze chimiche, includendo sostanze chimiche sia naturali che di origine antropica, sia volontariamente aggiunte che accidentalmente presenti nella catena di produzione.

Questa tesi si concentra sostanzialmente su come può essere stimata l'esposizione della popolazione umana, con particolare attenzione agli approcci metodologici e con un focus specifico sulla valutazione dell'esposizione alimentare e biomonitoraggio.

Dai risultati ottenuti nei diversi studi raccolti in questa tesi, si evince che quando si seleziona il metodo da utilizzare per la stima di esposizione ad uno o più sostanze chimiche, diversi aspetti devono essere presi in considerazione: la natura della sostanza chimica, la popolazione inclusa nello studio, è necessario chiarire se l'obiettivo è quello di stimare l'esposizione cronica o acuta, infine, deve essere valutata la qualità e la quantità dei dati disponibili al fine di specificare e quantificare l'incertezza delle stime.

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