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1 A BRIEF INTRODUCTION TO HUMAN BIOMONITORING

☑ Human biomonitoring makes pollution get personal

Environmental health sciences focus on the link between the presence of contaminants in the environment, and their relation with possible adverse health effects. The traditional way of describing these relationships is by estimating the concentration of chemicals in different environmental compartments using empirical or modeling efforts, taking into account human exposure estimates to quantify the dose. Recently, due to both an increase in analytical capacity and a change in social awareness towards pollution exposure, there has been a rapid increase in the development and application of human biomonitoring (HBM) as a tool to evaluate exposure (i.e. the use of human tissue or fluid samples to estimate exposure). In many cases, HBM data have been proven to be a valuable completion, or have even surpassed, estimates of exposure based on environmental measures (Bates et al 2005). HBM directly measures the amount of a chemical substance in a person's body, taking into account often poorly understood processes such as bioaccumulation, excretion, metabolism and the integrative uptake variability through different exposure pathways, rather than each individual exposure source (Hoppin et al 2000). Hence, these data are much more relevant for risk assessment than extrapolations from chemical concentrations in soil, air, and water. As was phrased by Stokstad (2004), "pollution gets personal" when HBM data are being collected. Not only does this pertain a change of philosophy in the general public, it also integrates environmental exposure in a way that is more likely to be consistent with health effects, as the causative compounds have actually entered the body and are still detectable (Hoppin et al 2000).

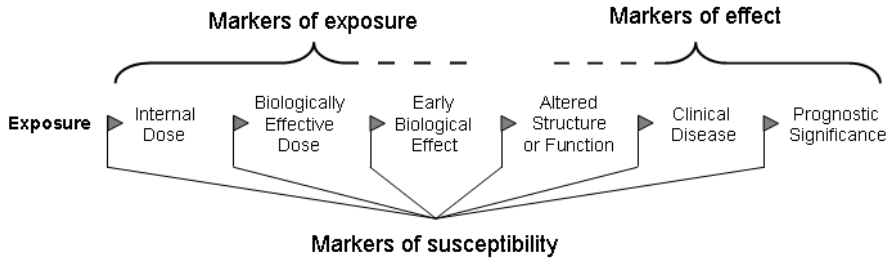
☑ Types of HBM

Biological indicators, or biomarkers, generally are biochemical, molecular, genetic, immunologic, or physiologic observations in biological systems. Traditionally, biomarkers have been classified as biomarkers of exposure, effects and susceptibility. Though this classification suggests the existence of clearly defined subgroups, biomarkers actually form a continuum between the external exposure to a chemical and

the internal clinical effect (Figure 1) (NRC 2006). According to the WHO (2001), biomarkers of exposure, effect, and susceptibility are defined as follows:

- **Biomarkers of exposure**: The chemical or its metabolite or the product of an interaction between a chemical and some target molecule or cell that is measured in a compartment in an organism
- **Biomarker of effect**: A measurable biochemical, physiological, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease.
- **Biomarker of susceptibility**: An indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific chemical substance.

Figure 1.1: Simplified flow chart of classes of biomarkers (NRC 1987, 2006)



This report mainly focuses on biomarkers of exposure, and aims at providing an overview of the major classes of biomarkers that are currently available, describes their use, indicates (if available) standardized methods and reference values, and tries to identify confounding factors. However, before going into detail, it is desirable to first briefly sketch the policy framework for the implementation of HBM in the European Environment and Health Action plan, and to provide the reader with a framework to integrate HBM in the current risk assessment paradigm.

☑ HBM and the European Environment & Health Action Plan 2004-2010

The "European Environment & Health Action Plan 2004-2010" originates from the concern of EU citizens on the well-being of individuals and the general population. Together with improving general public health, there are also indirect yet large benefits in terms of long-term economic growth and sustainable development, since the indirect

costs in productivity losses due to illness or premature death may be substantially larger than the cost for direct health care (EC, 2004a).

The Action Plan has three main themes:

1. Improving the information chain to understand the links between sources of pollution and health effects (Action 1-4);
2. Filling the knowledge gap by strengthening research and addressing the emerging issues on environment and health (Action 5-8);
3. Reviewing policies and improving communication (Action 9-13).

For each of these three themes, a number of actions were identified. The need for human biomonitoring is presented specifically under Action 3: *Develop a coherent approach to biomonitoring in Europe*. Within this Action 3, the need for integration with environmental and health data is specifically foreseen:

Biomonitoring is not an automatic instrument, which can be considered in isolation, but has to be integrated with environmental monitoring, toxicological and eco-toxicological data and especially with considerations related to analytical epidemiology. (EC, 2004b)

From this, it can be postulated that human biomonitoring (HBM) is not an island on itself, but should be considered a stepstone between environmental and health data. The final aim of this view should be an integrated and holistic systematic risk assessment paradigm where HBM serves as a pivotal point between environment and health, on the one hand leaning on environmental data to provide detailed information on the sources and pathways of pollutants that enter the human body, and on the other hand clarifying new and existing hypotheses on the relationship between environmental pollutants and the prevalence of diseases or the occurrence and identification of disease clusters (Hoppin et al 2000). Also, since most health responses are the result of long-term, chronic exposure to environmental pollutants, linking historical exposure to current response without looking at the integrating dose will overlook the history of individuals.

☑ A need for integration

Originally, HBM originates from occupational exposure, where often high exposure concentrations were causing (semi-)acute effects in industry workers, and the effects of

remediation actions were usually rapidly visible. The same was true for ecotoxicological testing, which was mainly based on non-ecological factors, such as convenience, availability of test organisms and const-efficiency rations. Ecological factors were generally irrelevant since water quality problems were acute and obvious , e.g. involving persistent mortality over large areas due to deoxygenation of surface waters (Chapman, 1995).

However, due to a better sanitary legislation, improved technical capacities and increasing awareness in the general population, consistent acute effects of environmental pollution are currently rare, or at least attributable to accidental exposures (Pew Environmental Health Commission, 2000).

With the acute effects mostly out of the way, scientists and policy makers however were faced with an even bigger challenge, being the lack of detailed and specific information that documented possible links between environmental hazards and chronic disease.

As already mentioned earlier, HBM provides excellent opportunities to work as a pivotal point between environment and health. In itself, HBM is confined to being a surveillance tool which provides only limited benefit to policy makers, scientists or the general public. An example is World Wildlife Fund's DetoX program, where WWF sampled blood from among others 37 Members of the European Parliament, indicating that they had anmedian of 41 different chemicals in their blood, out of a total of 101 chemicals tested (WWF 2004). While this campaign triggers public attention, creates awareness on the state-of-the environment and motivates policy makers to enforce legislations such as the new Registration, Evaluation, and Authorization of Chemicals (REACH), it does not necessarily provide information on the effects of these chemicals, or puts them in a scientifically defendable context. Indeed, one of the comments on the WWF program made by researchers, was that there is an important difference between the exposure to chemicals and the risk of chemicals, and evidence of presence is not evidence of harm.

This example illustrates the sensitive and delicate nature of HBM and underlines the observation that the value of HBM lies mainly in its position as an intermediate measurement between presence in the environment (exposure concentration) and effects on human health (response concentration).

HBM programs are performed to obtain information on the presence and effects of chemicals in human tissues. More specifically, they aim at a number of different goals:

- Find out if there are traces of environmental contaminants detectable in human tissues;
- Establish time trends in contaminant load and evaluate the efficiency of environmental policies;
- Detect whether these traces are uniformly distributed in space, or whether there are differences among different areas;
- Determine which factors may be able to explain the observed variations in chemical concentrations;
- Determine whether there is a relation between the exposure to these pollutants and the occurrence of health effects;
- Evaluate and, if necessary, adjust environment and health policy.

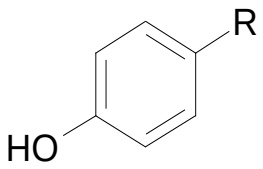
It should be noted that only the first two of these goals depends on HBM as a stand-alone tool (in the sense of the DetoX program, mentioned earlier). All other goals need additional information in order to evaluate, interpret and put into context HBM data. Hence, more than anything, HBM should be seen as only one 'line-of-evidence', that needs to be evaluated in combination with other lines-of-evidence, such as environmental pollutant concentrations, (eco)toxicological data and epidemiological data.

2 ALKYLPHENOLS

☑ General information

Alkylphenols (APs) and alkylphenol ethoxylates (APEOs) are used as additives in plastics and as surface-active ingredients in industrial detergents and emulsifiers. APs are very important environmenally relevant substances and derivatives of phenols. In aquatic systems alkylphenols are formed by degradation of alkylphenol ethoxylates (APEs). The most important group of alkylphenols are the 4-nonylphenols (NPs). Nonylphenols are persistent, toxic, endocrine-disrupting chemicals that are priority hazardous substances of the EU Water Framework Directive (2000/60/EC).

Figure 2.1: p-alkylphenols (AP); R=C8,C9

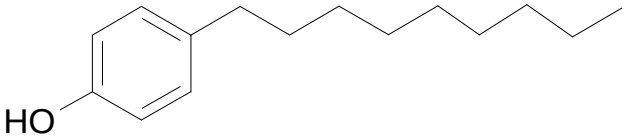


Alkylphenols are widely used as antioxidants in plastics and rubber products. APES and/or other alkyphenol derivatives are also used in pesticides, lube oil, hair dyes and other hair care products, and as nonoxynol-9 in spermicides. The most common APEs are nonylphenol ethoxylates.

Two alkyphenols, nonylphenol and octylphenol, are suspected hormone disruptors; they have been shown to mimic the hormone estrogen. Both nonylphenol and octylphenol show oestrogenic and anti-androgenic activities (Lee et al. 2003, Paris et al. 2002). Alkylphenols have been shown to cause oysters and zebrafish to change sex (Nice et al. 2003). APEs do not biodegrade easily in the water. As a result, nonylphenol has been found in water and sediment downstream from sewage treatment plants, paper pulp mills, and industrial facilities. Some studies have found altered reproduction, feminization, hermaphrodism, and lower survival rates in salmon and other fish living in nonylphenol-contaminated water. These effects have been found in wildlife even at low doses. Nonylphenol has also been detected in a wide range of foods. APEs are identified

in the ingredient list on the labels for personal care products and spermicides. However, they are rarely listed on household products like cleaners, detergents, and pesticides.

Figure 2.2: 4-n-nonoyphenols ; technical nonylphenol is a mixture of more than 22 nonylplhenol isomers



Toxicity testing with various organisms including fish and Daphnia has shown that the breakdown products of alkylphenol ethoxylates are generally about ten times more toxic than the original compounds. Data for nonylphenol show LC50 values of 17-3000 ppb for fish. For invertebrates the LC50 is 20-3000 ppb, and for algae, 27-2500 ppb (Meier et al. 2002). Alkylphenols are rapidly absorbed and accumulate in fish tissues. The bioaccumulation factor (BCF) for long-chain alkylphenols (>C4) is 75-1250 (Servos, 1999). A study using cod found a BCF for heptylphenol just below 600 (Tollefsen et al., 1998). Results of a survey in the United Kingdom showed levels of nonylphenol in the River Aire in Yorkshire had reached levels toxic to wildlife (Blackburn et al. 1995).

Acute toxicity of 4-Heptylphenol as a moderately hydrophobic environmental pollutant was determined in juvenile atlantic cods (Gadus morhua L.) (Tollefsen et al. 1998). The results showed that 4-HP causes mortality in groups of juvenile cod at nominal concentrations down to 2.1 μmol/L. The compound was moderately toxic with a 96-h LC50 of 2.9 μmol/L, and a somewhat lower T-LC50 of 2.7 μmol/L after 168 h of exposure (Tollefsen et al. 1998). The estimated LC50 values were slightly higher than corresponding values for 4-hexyl- and 4-nonylphenol in juvenile Atlantic salmon (Salmo salar)(McLeese et al. 1979).

☑ Matrix

- **Invasive**

  Nonylphenol (NP) and octylphenol (OP) have been analyzed in a study of human blood serum in Netherlands (TNO 2004). Nonylphenol was found in 16 of the 91 samples with a maximum concentration of 16 ng/g serum. Octylphenol was found

only incidentally. A recent study compared alkylphenol concentrations in maternal and cord blood serum (TNO 2005). A study from Japan (Inoue 2000) found NP in serum in concentrations ranging from 14 to 222 ng/g serum while OP was found in only one sample in a concentration of 0.5 ng/g serum.

- **Non-Invasive**

  Breast milk with nonylphenol levels of 0.3 mg/kg (lipid) has been described as a relevant exposure route for human contamination after birth (Guenther et al. 2002). Nonylphenol has also been detected in human umbilical cords (Takada et al. 1999) confirming that NP crosses from placenta from contaminated mother to the growing foetus. A more recent study reaffirmed nonylphenol in human contamination (Greenpeace/WWF 2005). Nonylphenol was detected in 12 of 17 cord blood samples of this study.

- **Comparison**

  For analysis of lipophilic compounds as for example dioxins in breast milk both advantages (toxicological relevance of mother child transfer) and disadvantages (only females can be sampled during a specific time period) have been described. These specifities may be similar for the analysis of alkylphenols in breast milk. Comparisons of alkylphenol levels in maternal blood and breast milk are missing so far. For the analysis of alkylphenols both human blood serum and cord blood serum has been described as a suitable matrix for alkylphenols (TNO 2004 and 2005; Takada et al. 1999).

☑ Kinetics

- **Uptake**
  - *Food*: Despite numerous determinations of NPs in environmental samples few systematical reports exist relating to concentrations of NPs in food. Guenther et al. (2002) analyzed NPs in 60 different foodstuff commercially available in Germany. The results indicated that NPs are ubiquitous in food. The concentrations of NPs on a fresh weight basis varied between 0.1 and 19.4 µg/kg regardless of the fat content of the foodstuff. Based on data on German food consumption rates and these first analyses of NPs in food, the daily intake for an adult was calculated to be 7.5 µg/day NPs. For infants exclusively fed with breast milk or infant formulas daily intakes of 0.2 µg/day and 1.4 µg/day NPs,

respectively, can be estimated (Guenther et al. 2002). Basheer et al. (2004) found up to 530.4 ng g^1 wet weight (w.w.) of total alkylphenols in prawn, crab, blood cockle, white clam, squid, and fish. The highest nonylphenol concentration (197.0 ng g^{-1} w.w.) amongst the seafood samples analyzed was detected in prawns.

- *Drinking water*: Analysis of drinking water in the USA has found a total concentration of alkylphenolic compounds of almost one microgramme per litre. There is no publicly-available data on the levels of alkylphenolic compounds in UK drinking water, though it is believed that certain water companies may have some unpublished data. Water pipe treatments have been described to leach alkylphenols into the drinking water, a cement coating leached 12 mg/m^2 of nonylethoxylate immediately after curing, with continued high leaching for 3 days (ENDS 1999).
- *Air*: Nonylphenol as air pollutant has been determined in the urban athmosphere of New York and New Jersey, USA in concentrations from 2.2 to 70 ng/m^3 (Dachs et al. 1999). NPs occurrence in the athmosphere was apparently due to vapourisation from the Hudson River. NPs occurrence in the athmosphere was suggested to be an important human and ecosystem health issue in urban, industrial, and coastal-impacted areas receiving treated sewage effluents (Dachs et al. 1999). A Japanese study of air pollution by alkylphenols in Tokyo detected 4-tert-butylphenol (4-t-BP), 4-tert-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) in both indoor and outdoor air (Saito et al. 2004). Higher concentrations were found in indoor air with maximum levels of 4-t-BP, 4-t-OP and 4-NP with 387, 45.7 and 680 ng/m^3 (Saito et al. 2004).
- *Other routes* for human exposure may also occur from absorption through skin from shampoos, cosmetics, spermicidal lubricants and domestic and industrial detergents. Further more inhalation and ingestion from pesticide sprays has to be considered.

- **Metabolism**

  Akylphenols are metabolised rapidly, first and foremost by phase 2 enzymes that conjugate intact alkylphenols to their corresponding glucuronides. There is also a certain amount of phase 1 metabolism. The alkylphenols are excreted primarily in the bile, digestive system and liver, but it has also been shown that alkylphenol is

taken up in the brain of fish as salmon (Arukwe et al. 2000), trout (Ferreira-Leach and Hill 2001) and cod (Tollefsen et al. 1998). This is of particular interest with respect to hormone-disrupting effects.

- **Biological half-life**

  The bioconcentration of branched NP in the killifish (Oryzias latipes; medaka) was investigated by Tsuda et al. (2001). The fish (0.16-0.24 g wt) were exposed to measured NP concentrations of 3.6±0.9 micrograms/L NP in a flow-through system for 7 days. BCF values in whole fish (wet weight) reached a plateau after 48 hours of exposure. Exposure of the minnows to 3.6 micrograms/L NP in water for 7 days resulted in a median BCF of 167±23 mL/g-wet weight. Following transfer to clean water the excretion rate constant and biological half-life were determined to be 0.07 h^{-1} and 9.9h, respectively (Tsuda et al. 2001).

  A study of toxicokinetics of 4-heptylphenol as a moderately hydrophobic environmental pollutant in juvenile atlantic cod (Gadus morhua L.) described BCF values for samples collected after 96 and 192h of 555±16 (Tollefsen et al. 1998). Elimination of the radiolabeled compounds followed first-order kinetics with an estimated biological half-life ($t_{1/2}$) of 13 h. Results disclosed that 4-HP accumulates in most tissues of cod to concentrations far higher than the concentration in seawater. Rapid elimination was probably caused by excretion of the parent compound from gills in combination with excretion of 4-HP (and/or metabolites) through the biliary and urinary system. Bile and liver accumulated substantial amounts of labeled material, suggesting the use of these organs for in situ biomonitoring of low-concentration exposures (Tollefsen et al. 1998).

☑ Sampling conditions

- **Seawater and seafood sample collection**

  Surface seawater samples were collected from various locations (Basheer et al. 2004). Samples were obtained at each location during the day in pre-washed glass bottles. Extraction was performed on unfiltered seawater samples. To prevent possible degradation of analytes, samples were collected and processed on the same day. Salinities of the samples varied between 28 and 35‰; seawater temperatures ranged between 28 and 32 °C, and pH between 7.9 and 8.6. Seven varieties of fresh seafood samples were purchased from a local supermarket chain between June 2000

and February 2001. Whole (shucked cockle, clam) or partial (muscle tissue of squid, prawn, crab and fish) samples were cut into small pieces immediately after returning to the laboratory and stored at −80 °C until analysis.

- **Cord blood: Ethics approval and sample collection**

  The Ethics Committee of the University Malaya Medical Centre (UMMC), Malaysia approved the study of contaminants in human umbilical cord blood and collection of the umbilical cord blood upon delivery at labor ward at the UMMC, Malaysia. One hundred and eighty human umbilical cord blood samples were tested for the presence of alkylphenols, bisphenol-A and pesticides (Tan and Mohd 2003). Before delivery, the expectant mother was asked a series of questions about their pregnancy and their personal particulars were recorded. The cord blood was collected in a 6-ml glass lithium heparin Vacutainer (obtained from Becton Dickinson Vacutainer Systems, New Jersey, USA) upon delivery at the UMMC, Malaysia; and centrifuged at 3500 rpm for 10 min. The plasma supernatant was aspirated out and stored in a 14-ml glass vial at −20 °C.

- **Breast Milk: Sample preparation**

  To measure both free and conjugated species, each unknown breast milk sample was prepared in two different ways (Ye et al. 2006): one sample was processed without enzyme treatment, and the other was treated with β-glucuronidase (*H. Pomatia*). Breast milk was thawed and vortex mixed before aliquoting. A 100 μL aliquot of breast milk was mixed with 50 μL of internal standard solution, 10 μL of 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate standard, and 50 μL of enzyme solution in a disposable 1.5 mL CLIKLOK microcentrifuge tube (Simport, Beloeil, Canada). After gentle mixing, the sample was incubated overnight at 37 °C. After incubation, 190 μL of 2-propanol was added to the deconjugated breast milk, the sample was vortex mixed, and then centrifuged at 8000 × *g* for 15 min. Of the milk supernatant, 200 μL was transferred to an autosampler vial, 300 μL of 0.1 M formic acid was added, and the 500 μL sample was vortexed before being placed on the HPLC autosampler for on-line SPE–HPLC–MS/MS analysis. To determine concentrations of the free species, we followed the procedures described above, but added 50 μL of 1 M ammonium acetate buffer instead of the enzyme solution, and skipped the incubation step.

Several studies proposed storage of samples (foodstuffs, breast milk, blood serum) 4°C for immediate analysis (Guenther et al. 2004; TNO 2004). For storage within at most few months freezing at -20°C or lower is the standard for preserving samples.

☑ Analytical aspects

- **Techniques**
  - *Chemical-analytical*: Alkylphenols are commonly determined using gas chromatography and mass spectrometry (GC-MS). Guenther et al. (2002) described a flexible and robust method for both breast milk and foodstuffs using steam distillation/solvent extraction followed by normal phase HPLC clean-up and derivatization. Comprehensive two-dimensional chromatography/time of flight mass spectrometry (GC×GC-TOF-MS) has been applied as a method for isomerspecific technical nonylphenol mixtures (Guenther et al. 2005). 4-Nonylphenol (NP) and Bisphenol A (BPA) was simulteaneously measured in urine samples using isotope-dilution gas chromatography/mass spectrometry (Calafat et al. 2005). Liquid chromatography coupled with mass spectrometry (LC/MS) in the selected ion monitoring mode (SIM) was used for determination of nonylphenol and octylphenol in human blood serum samples (TNO 2004). Reversed-phase HPLC with multi-electrode electrochemical coulometric-array detection was uesd for the determination of NP and OP in human blood plasma and serum samples prepared with a solid-phase extraction method (Inoue et al. 2000).
  - *Bio-analytical*: ELISAs for alkylphenols (AP) and alkylphenol ethoxylates have been developed for assay ranges of 5-500 ppb for AP (Kobayashi et al. 2003). Sensitivity at ppt levels of the ELISA can be achieved by simple solid phase extraction.
  - *Simultaneous measurement of urinary alkylphenols by automated solid-phase extractive derivatization gas chromatography/mass spectrometry. A*lkylphenols (APs) and Bisphenol A (BPA) are widely used industrial chemicals. BPA is used to manufacture polycarbonate plastic and epoxy resins; APs are used to make alkylphenol ethoxylates, common nonionic surfactants. BPA and APs can leach into the environment during industrial production and after degradation of the polycarbonate plastics and nonionic surfactants. Environmental exposure to these

phenolic compounds has been associated with adverse reproductive and developmental effects in wildlife. Kuklenyik et al. (2003) developed a sensitive and robust method for measuring BPA and six APs; 3-tert-butylphenol, 4-tert-butylphenol, 4-n-octylphenol, 4-tert-octylphenol, 4-n-nonylphenol, and technical-grade nonylphenol in urine. The method is based on the use of automated solid-phase extraction (SPE) coupled to isotope dilution-gas chromatography/mass spectrometry (GC/MS). During the automated SPE process, the phenols are both extracted from the urine matrix and derivatized, using pentafluorobenzyl bromide, on commercially available styrene-divinylbenzene copolymer-based SPE cartridges. After elution from the SPE column, the derivatized phenols in the SPE eluate are analyzed by GC/MS. The method, validated on spiked pooled urine samples and on urine samples from exposed persons, has limits of detection of approximately 0.1 ng in 1 mL of urine.

- *Automated on-line column-switching HPLC-MS/MS method with peak focusing for the determination of nine environmental phenols in urine.* Ye et al. (2005) developed a method using isotope dilution on-line solid-phase extraction (SPE) coupled to high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) for the determination in urine of nine environmental phenolic compounds: 4-tert-octylphenol; o-phenylphenol; 2,4-dichlorophenol; 2,5-dichlorophenol; 2,4,5-trichlorophenol; 2,4,6-trichlorophenol; benzophenone-3 (2-hydroxy-4-metoxybenzophenone); triclosan (2,4,4'-trichloro-2'-hydroxyphenyl ether) and Bisphenol A. A unique fully automated column-switching system, constructed using 1 autosampler, 2 HPLC pumps, and a 10-port switching valve, was designed to allow for concurrent SPE-HPLC operation with peak focusing. The phenols present in 100 microL of urine were retained and concentrated on a C18 reversed-phase size-exclusion SPE column. Then, the phenols were "back-eluted" from the SPE column and diluted through a mixing Tee before being separated from other urine matrix components using a pair of monolithic HPLC columns. The phenols were detected by negative ion-atmospheric pressure chemical ionization-MS/MS. The efficient preconcentration of the phenols by the SPE column, analyte peak focusing by the dilution, and minimal ion suppression in the LC/MS interface by the buffer-free

mobile phases resulted in limits of detection as low as 0.1-0.4 ng/mL for most analytes. The method was validated on spiked pooled urine samples and on urine samples from 30 adults with no known occupational exposure to environmental phenols. The method can be used for quick and accurate analysis of large numbers of samples in epidemiologic studies for assessing the prevalence of human exposure to environmental phenols.

- *Determination of alkylphenols in river water by stir bar sorptive extraction with in situ acetylation and thermal desorption-gas chromatography-mass spectrometry.* A method for the determination of seven alkylphenols and bisphenol A by stir bar sorptive extraction (SBSE) with in situ derivatization-thermal desorption (TD)-gas chromatography (GC)-mass spectrometry (MS) is described. SBSE was performed with in situ acetylation and without derivatization for comparison (Nakamura and Daishima 2004). For 4-tert-butylphenol and bisphenol A, in situ acetylation improved the responses in SBSE-TD-GC-MS. The method detection limits ranged from 0.1 to 3.2 ng/l. The recoveries of the analytes from a river water sample spiked with standards at 10 and 100 ng/l were 85.3-105.9% (R.S.D., 3.0-11.0%) and 88.3-105.8% (R.S.D., 1.6-8.3%), respectively.
- *Development and validation of a method for determination of trace levels of alkylphenols in atmospheric samples.* A method has been developed and validated in order to assess the occurrence of the alkylphenols tert-octylphenol and the isomers of technical nonylphenol as well as bisphenol A in gasphase and aerosol samples of a remote area (Berkner et al. 2004). Gasphase samples were adsorbed to XAD2 resin, aerosol samples were taken on glass fiber filters. After ultrasonic extraction, clean-up by column chromatography and silylation of the analytes, ten nonylphenol peaks were quantified separately using a GC-MSD-SIM method. The absolute limits of detection and determination are in the range of a few pg per compound, which is a prerequisite for the quantification of the analytes in relatively unpolluted air. The precision of the whole analytical method is in the range of 1-17% and the recoveries range from 57% to 80%. Problems were encountered during method development due to the tendency of the analytes to sorb to glass surfaces. Silanisation of glassware was crucial to achieve acceptable recoveries. The widespread use of the analytes in plastic

resins resulted in sample contamination. For this reason a careful choice of sampling material was necessary. Measured concentrations in gasphase samples (lower nanogram per m3 range) and aerosol samples (upper picogram per m3 range) are one to three orders of magnitude below already published concentrations.

- **Sensitivity and specivity**

  Guenther et al. (2001) described a detection limit of 15 ng NP absolute in a study of mussels from the German bight. The detection limit was calculated as sum of the procedural blank and three times the standard deviation of the blank.

  Method detection limits in human blood serum using LC/MS were <0.5 ng/g serum for both for nonylphenol and octylphenol. Another study using the LC/MS method described analytical problems with the determination of NP in maternal blood serum deriving from unknown interferences in the chromatograms (TNO 2005). Similar problems were not encountered in the analysis of cord blood from this study (TNO 2005).

  Detection limits of NP and OP were 1.0 and 0.5 ng/ml respectively for the RP-HPLC with multi-electrode electrochemical coulometric-array detection method (Inoue 2000).

- **Units**

  In blood samples (plasma and serum) concentrations of alkylphenols are generally expressed as a weight unit per gram of sample (e.g. ng/g serum). Alkylphenol concentrations in breast milk and several foodstuffs have been identically expressed on a fresh weight basis (Guenther et al. 2002).

☑ Performance characteristics

- **Analytical reproducibility**
- **Intra-laboratory variability**

  Recovery and reproducibility of nonylphenols in foodstuffs and breastmilk measured by GC-MS showed relative standard deviations (RSD) for samples spiked with 25ng of 4-n-NP in methanol of 2.7-4.6% in the procedural blanks but stronger variations (RSDs of 3-25%) for the different food samples (Guenther et al. 2002). For alkylphenols in human blood samples using the LC/MS method RSD of 10% has

been described calculated from 6 analyses (TNO 2004). No information is available about inter-laboratory variability of APs in human blood samples or breast milk.

☑ Validation

Analytical methods for alkylphenols have been validated for different matrices as e.g. human blood (TNO 2004), urine (Kuklenyik et al. 2003), river water (Nakamura and Daishima 2004), athmospheric samples (Berkner et al. 2004) and foodstuffs (Guenther et al. 2001 and 2002).

☑ Confounding factors

Since only very few data about alkylphenols in human samples as blood, breast milk and urine are available no correlations between concentrations and confounding factors like age, socio-economic situation, body mass index (BMI) or gender was described so far.

☑ Concentrations reported in literature

- **Reference values**

  Few data are still available about alkylphenols in human samples. Guenther et al. (2002) found nonylphenol (NP) concentrations of 0.3 mg/kg in breast milk of a 35 year old woman from Germany. Takada et al. (1999) reported NP levels of 2 ng/kg in umbilical cord in a Study of Japan. A study from Japan (Inoue 2000) found of NP in serum in concentrations ranging from 14 to 222 ng/g serum while OP was found in only one sample in a concentration of 0.5 ng/g serum. A survey of alkylphenol in human blood serum of 91 samples from Netherlands showed NP concentrations of 0.58 to 16 ng/g serum (TNO 2004). In a study of Ye et al. (2006) 4-*tert*-octylphenol (4-tOP) OP was detected in some breast milk samples in concentrations below 7.6 ng/ml using automated on-line SPE–HPLC–MS/MS method. Calafat et al. (2005) measured 4-Nonylphenol (NP) in archived urine samples from a reference population of 394 adults in the United States using isotope-dilution gas chromatography/mass spectrometry. NP was detected in 51% of the samples examined > or = 0.1 microg/L. The median and 95th percentile concentrations were < 0.1 microg/L and 1.57 microg/L (1.39 microg/g creatinine), respectively (Calafat et al. 2005). A study of alkylphenols and other environmental phenols in human cord blood of Tan and Mohd (2003) detected nonylphenol and other phenols in 180 cord

blood samples in following concentrations: nonylphenol (non-detectable—15.17 ng ml^{-1}), 4-*n*-octylphenol (non-detectable—4.17 ng ml^{-1}), chlorpyrifos (non-detectable—1.84 ng ml^{-1}), 4-*t*-octylphenol (non-detectable—1.15 ng ml^{-1}), 4-*n*-butylphenol (non-detectable—0.80 ng ml^{-1}) and 4-*n*-hexylphenol (non-detectable—0.54 ng ml^{-1}). Nonylphenol was present in more than 80% of the samples, followed by 4-*n*-octylphenol (53%). The other compounds detected were lower than 20% of the sample size (Tan and Mohd 2003).

- **Critical values**

  Rats were exposed to 200, 650, or 2000 ppm NP in their diet for 3 months. The treatment caused a small decrease in body weight and food consumption in the 2000 ppm dose group. No treatment-related clinical or histopathological changes, including effects on endocrine organs, estrous cycling, or sperm measurements were noted up to *2000* ppm exposure. The NOEL is considered to be 650 ppm NP in the diet (approximately 50 mg/kg body weight) (Cunny et al. 1997). NP was shown to increase uterine weight in the standard uterotrophic assays. The oral NOEL ranged from approximately 50 to 100 mg/kg BW (Cunny et al. 1997).

☑ Dose-response/effect relationship

Objective of the study of Hunag et al. (2004) was to investigate the estrogenic activity of para-nonylphenol in immature SD rats and explore the mechanism and sensitive indicators of its action in uterotrophic assay. The vehicle control (peanut oil), positive control (estradiol benzoate, E2B, 0.4 mg/kg) and p-NP(60 mg/kg, 90 mg/kg and 120 mg/kg) were given orally (by gavage) q.d. on the 21st, 22nd, 23rd postnatal days. Then the rats were sacrificed 24 hours after the last administration. By using ABC immunohistochemistry, the progesterone receptor (PR), estrogen receptor (ER), and proliferating cell nuclear antigen (PCNA) of the rat uterine were analysed. Uterine weight, uterine/body weight significantly increased in E2B 0.4 mg/kg, p-NP 90 mg/kg and 120 mg/kg groups as compared with those of vehicle control group ($P < 0.01$), and a dose-response relationship was observed. The expressions of PR, ER and PCNA in the nuclei of stromal and myometrial cells of uterus were detected in all the p-NP groups, and a dose-effect relationship was noted. Expressions of PR, ER and PCNA as indicators tested by immunohistochemical technique are more sensitive than uterine

weight in uterotrophic assay. Hyperplasia of stromal and myometrial cells of uterus is the reason why the uterine weight of the rat increased.

Fan et al. (2001) observed nonylphenol could decrease the ability of spermatogenesis in rats without morphological changes of spermatogenic cells of the testes and epididymis. They came to this conclusion in their study where pregnant rats were administered with *p*-nonylphenol during gestation and lactation at doses of 0, 50 100 and 200 mg kg^{-1} body weight, respectively. The weights of the testes and prostate of the young male rats of 70 days decreased with the increase doses of exposed nonylphenol. The same trend existed in daily production of sperm in the testes and sperm counts of the epididymis (Fan et al. 2001).

☑ Time trend, geographical variation, susceptible groups

As described alkylphenols (APs) and alkylphenol ethoxylates (APEOs) have a wide range of applications as additives in plastics, surface-active ingredients in industrial detergents and emulsifiers and cosmetic products as hair dyes and other hair care products. There may be groups with frequent exposure to before mentioned products as detergents and cosmetic products which allow dermal uptake of APs. Further more AP uptake from drinking water and athmospheric contamination has to be considered and may show geographic variation with higher AP levels in urban areas. Consequently variations in the levels of APs in humans will be recognized depending on the exposure situation.

3 ALPHA$_1$-MICROGLOBULIN (A_1-M)

☑ General information.

α_1-m, also called protein HC (heterogeneous in charge, human complex forming), is a glycoprotein with a Mr of 27 kD. The protein has a yellow-brown colour due to a strongly attached chromophore. It has a considerable charge heterogeneity at least as extensive as that immunoglobulin (pI = 4.3 – 4.8). α_1-m, like RBP, is a member of the lipocalin superfamily of hydrophobic ligand binding proteins.

☑ Matrix

Untimed or 24-h urine sample.

☑ Kinetics

- **Production**

  The liver is probably the main site of synthesis.

- **Distribution**

  α_1-m occurs free in serum and bound to several high molecular weight proteins such as IgA and albumin. These forms differ considerably in structure and antigenic properties making quantitative immunochemical determination of total α_1-m in serum difficult.

- **Excretion**

  Free α_1-m in serum (normally around 20 mg/l) is eliminated mainly by glomerular filtration followed by reabsorption and catabolism in the proximal tubules. As a corollary serum α_1-m is inversely related to the glomerular filtration rate (GFR).

- **Biological half-life**

  Free α_1-m in plasma has a half-life of 2-3 hours.

☑ Sampling conditions

- **Sampling conditions**

  α_1-m can be determined on a 24-h urine sample or on untimed urine sample. It is advisable, however, to avoid extreme variations in the urinary flow when the results are corrected for the creatinine content of urine. Sampling time is not critical for the assay of urinary α_1-m. A preservative must be added to the urine (e.g. NaN_3 0.1 %).

- **Transport**

  If possible, the sample should be shipped on dry ice. However, shipment at room temperature should not significantly affect the stability of α_1-m provided the urine contains a preservative.

- **Storage conditions**

  α_1-m is stable in urine for several days at room temperature and at least for two weeks at 4 °C. At -20 °C, the protein is stable for several years. In practice, if the analysis cannot be performed within 2 or 3 weeks after collection, it is preferable to store the samples frozen.

☑ Analytical aspects

- **Techniques**

  Radioimmunoassay (RIA), enzyme immunoassay (EIA), latex immunoassay (LIA) or related techniques. Less sensitive techniques such radial immunodiffusion, nephelometry or turbidimetry can also be used.

- **Sensitivity**

  0.001 to 1 mg/l depending on the immunoassay.

- **Units**

  mg/l, mg/24 h or mg/g creatinin

☑ Performance characteristics

- **Analytical reproducibility**

  5-10%

- **Intra-laboratory variability**

  5-10%

- **Inter-laboratory variability**

  Not documented but significant variations can be observed depending on the type of assay and the standards used.

☑ Validation

Published immunoassays are usually validated by comparison with alternative methods. Commercially available calibrators can be used.

☑ Confounding factors

In healthy subjects, age, sex and diuresis are potential confounders for urinary α_1-m. A variety of kidney diseases can also increase the urinary excretion of α_1-m.

☑ Concentrations reported in literature

Reference values. The urinary excretion of α_1-m by healthy subjects aged 20 to 60 years averages 4 to 6 mg/g cr (95^{th} percentile around 20 mg/g cr or 30 mg/24 hours).

☑ Dose –response/effect relationships

Not relevant

☑ Time trend, geographical variation, susceptible groups

No data.

4 ARSENIC

☑ General information

Arsenic (As) is a metalloid which can present the following states of oxidation: -3, 0, 3 and 5. It is largely widespread through the earth's crust; arsenic is the main constituent of more than 200 mineral species, of which about 60% are arsenate, 20% sulfides (auripigment, realgar) and sulfosalts and the remaining 20% include arsenides, arsenites, oxides and elemental arsenic. The most common of the arsenic minerals is arsenopyrite, FeAsS. Concentrations of various types of igneous rocks range from < 1 to 15 mg As/kg, with a mean value of 2 mg As/kg. Similar concentrations (< 1–20 mg As/kg) are found in sandstone and limestone. Significantly higher concentrations of up to 900 mg As/kg are found in argillaceous sedimentary rocks including shales, mudstone and slates. Up to 200 mg As/kg can be present in phosphate rocks (EHC 224, 2001).

So, arsenic occurs naturally in rocks and erosion of these can cause release of arsenic into water. Arsenic is also released into the air from human activities; it comes mainly from the combustion of fossile fuels (particularly of coal), of the production of metal, its agricultural uses and the incineration of waste. Inorganic arsenic (oxide (III)) is produced primarily as a by-product from copper, lead, gold, tin and other metal smelting processes. Arsenic may enter water and soil from some industrial sites or waste disposal plants.

The arsenical products are employed in the trade and industry to form alloys, for the manufacture of electronic components (as gallium arsenide ; transistors, lasers and semiconductors) and in the treatment of glass, the pigments, the textiles, paper, the metal adhesives, ceramics, the preservation agents of wood (Chromated Copper Arsenate, CCA), the ammunition and the explosives. They are also used for the tanning of the skins and, to a certain extent, they enter the manufacture of the pesticides, the additives for food of the cattle and the pharmaceutical products as well as veterinary medicinal products.

Cigarette smoke contains arsenic. If inhalation adds about 1 µg As/day from airborne particulates, approximately 6 µg As /day may be inhaled from 20 cigarettes (EHC 224, 2001).

The metabolism of arsenic and its toxicity are different according to its inorganic or organic form, the inorganic form (mineral; water, environment, industry) being more toxic than the organic one and trivalent arsenic being more toxic than the pentavalent arsenic.

Inorganic arsenic penetrates in water by dissolution of the ores, starting from the industrial effluents and of air deposits. The concentrations of arsenic in unpolluted surface water and groundwater are typically in the range of 1–10 µg/litre. The standard of quality of drinking water in the European countries is 10 µg/L. The limit value in soils in the Netherlands and Austria is 20 mg/Kg. It often happens that natural sources, as the dissolution of a rock base containing of arsenic contributes much to the arsenic content of drinking water and subsoil waters. In well oxygenated surface waters, pentavalent arsenic is generally the most frequent species; in reducing medium, as it is the case in the sediments of the deep lakes or subsoil waters, the trivalent species is more frequent.

Arsenic is present as traces in all the living matter. Dietary arsenic is the major source for most of the population, by far the highest concentrations of total arsenic is found in seafood. Arsenic in foods is a mixture of inorganic species and organoarsenicals including arsenobetaine, arsenocholine. Nevertheless, arsenic in the diet is predominantly organic arsenic which is known to be less toxic than inorganic arsenic. These facts have significant impact on the methodology to monitor and determine potential health outcomes from exposure to inorganic arsenic. The daily intake by food is, on average, of 40-50 µg, but it varies according to the geographical localization (some soils are rich in arsenic) and to the consumption of seafood products.

☑ Matrix

Measurement of inorganic arsenic in the urine is the best way to determine recent exposure, while measuring inorganic arsenic in hair or fingernails may be used to detect chronic exposures.

Urine is the biological sample commonly used to monitor exposure to As. Since the elimination of arsenic takes place mainly via the kidneys, the concentration of arsenic in the urine is a good indication of recent exposure (within the last 1 to 2 days) to inorganic arsenic.

Blood arsenic is a useful biomarker only in the case of acute arsenic poisoning or stable chronic high-level exposure. Usually, arsenic determination in the blood is practically of no importance, because of its short half-life.

Concentrations of arsenic or its metabolites in hair and nails may also be used as biomarkers of arsenic exposure. Arsenic accumulates in keratin-rich tissues such as skin, hair and nails as a consequence of its affinity for sulfhydryl groups. Hair and nails have the advantage of being readily and non-invasively sampled, but a major issue of concern is whether external contamination can be removed. Arsenic levels in hair and nails may be used as an indicator of past arsenic exposure.

☑ Kinetics

- **Absorption**

  Arsenic in inhaled airborne particles that reaches the lungs is well absorbed (deposition of 40 % and an absorption rate of 30 %).

  Nevertheless, the principal way of exposure to arsenic is by ingestion. Both soluble pentavalent and trivalent arsenic compounds are rapidly and extensively absorbed from the gastrointestinal tract (45- 90%, while 90% for organic arsenic compounds); these include arsenious acid and sodium arsenite. Insoluble compounds such as arsenic disulphide are poorly absorbed. Pentavalent arsenic is more water-soluble than trivalent arsenic and is better absorbed.

  Percutaneous absorption rates differ range between <1 % for several inorganic arsenic compounds and a few percent points for several organic arsenic compounds.

- **Distribution**

  Inorganic arsenic is reported to be rapidly cleared from blood where it is strongly linked to plasmatic proteins and to erythrocytes. After 24 h, 1 % of the amount absorbed will be detected in the blood.

  It is widely distributed in the body after either long-term relatively low-level exposure or poisoning: in the liver, the kidneys, the lungs, the muscles, the skin, nails, hair and the bones, where it is fixed durably. Arsenic passes the placental barrier and into breast milk to a minor extent.

  The metabolism and disposition of inorganic arsenic may depend on its valence state, particularly at high doses. The two most common valence states to which humans might be environmentally exposed are the trivalent and pentavalent forms

(interconverted). The metabolism is mainly hepatic. Arsenic metabolism is characterized by two main types of reactions:

- reduction reactions of pentavalent to trivalent arsenic, and
- oxidative methylation reactions in which trivalent forms of arsenic are sequentially methylated to form mono-, di- and trimethylated products (principal metabolic way).

Trimethylated forms are produced in small amounts if humans are administered DMA. Studies suggest that arsenic methylation may be inhibited at high acute exposures. However, in the case of exposure to arsenic via drinking-water, even at very high arsenic concentrations, the methylation of arsenic seems to be relatively unaffected by the dose.

So, dimethylarsenic acid (DMA) seems to be produced by a subsequent methylation of the monomethylarsenic (MMA) precursor. The methylation process is dose dependent and as the dose of arsenic increases, a reduction of the percentage of DMA is observed in urine while retention of arsenic is higher.

- **Excretion**

Inorganic arsenic compounds are mainly excreted via the kidneys but the rate of urinary arsenic excretion depends upon the chemical form of the compound ingested, the route of exposure and the dose level. The sum of arsenic metabolites (inorganic arsenic + MMA + DMA) in urine is a good indicator of the recent exposure to inorganic arsenic.

Nevertheless, adding all arsenic metabolites together can give misleading results unless a careful diet history is taken and/or seafood consumption is prohibited for 2–3 days before urine collection (Buchet et al., 1996). Some seafood, especially bivalves, contain the arsenic metabolites MMA and DMA (particularly DMA) in fairly high amounts and arsenosugars present in seaweeds and some bivalves are extensively metabolized to DMA, which is then excreted in urine.

Other secondary routes of elimination are hair, nails, sweat and faeces. Only a few percent is excreted in faeces (< 10%) probably due to re-absorption by intestines of arsenic eliminated by the bile. Arsenic can be excreted in human milk, although the levels are low. In general, organoarsenicals (MMA, DMA, trimethylarsine (TMA) and trimethylarsine oxide (TMAO), as well as arsenobetaine and arsenocholine) are less extensively metabolized than inorganic arsenic and more rapidly eliminated.

- **Biological half-life**

  Studies in adult human males voluntarily ingesting a known amount of either trivalent or pentavalent arsenic indicate that 45–75% of the dose is excreted in the urine within a few days to a week. So, approximately 70 % of the absorbed inorganic compounds are quickly eliminated in urine, 25 % for MMA and 50 % for DMA when low concentrations, 30 % remaining in unchanged form.

  The half-life of elimination is about 40 to 72 hours. For example, Buchet et al. (1981) reported that an average of 78.3% and 75.1% of a single oral dose (500 µg As) of MMA and DMA, respectively, was eliminated in urine of human volunteers within a 4-day period.

☑ Sampling conditions

Blood and urine (10 mL) should be collected in containers free of any metal contamination. Care must be taken to avoid contamination and prevent speciation changes during sample collection and storage.

Plastic containers should be acid washed and traces of oxidizing and reducing agents avoided to preserve the oxidation state of arsenic compounds. Freezing samples to –80 °C has also been recommended (EHC, 224, 2001).

Sea food products can contain low concentrations of DMA. If the intake of sea food products is high, the quantity of DMA in urine can increase.

It is recommended to avoid consumption of:

- Seafood products at least 72 hours (for five days if possible) before collecting urine samples (i.e. any fish, clams, shrimp, lobster, prawns, calamari, etc);
- Red wine at least 72 hours (for five days if possible) before collecting urine sample; some red wine contain low levels of arsenic;
- Tobacco (one day if possible) prior to the collect.

☑ Analytical aspects

- **Techniques**

  Total As

  Total As has been determined in urine samples, however, it is not very interesting since it includes As from organic origin. First, urine samples are subjected to mineralization by means of oxidizing acids. The arsenic will then be present in the form of inorganic ions. These can be determined by means of the hydride technique as well as by means of ICP-MS.

  The hydride generation atomic absorption spectrometry (hydride AAS) is suitable for the simultaneous detection of inorganic arsenic compounds and MMA, DMA, TMAO and TMA, which, however, must be preceded by adequate reduction. Arsenobetaine and arsenocholine are not detectable by this method.

  The main advantage of ICP-MS is lower detection limits (sub-nanogram to sub-picogram), but it is more susceptible to isobaric interferences arising from the plasma. For example, hydrochloric acid and perchloric acid are not desirable for sample preparation, because the chloride ions generated in the plasma combine with the argon gas to form argon chloride (ArCl). This has the same mass as arsenic (75) which could lead to error if not corrected. Therefore, whenever possible, only nitric acid should be used in sample preparation.

  Speciation

  Speciation of arsenic (i.e. analysis of arsenic compounds or different inorganic species, rather than total arsenic) is considered to be an effective procedure to differentiate between toxic inorganic and less toxic organic arsenic exposure.

  It is usually accomplished by employing separation procedures prior to introduction of the sample material into a detection system. Various type of chromatography or chelation-extraction techniques are most commonly used in combination with AAS, ICP-AES, or ICP-MS detection methods.

  It exists a method for the simultaneous determination of the main arsenic species so far detected in urine: arsenite (As(III)), arsenate (As(V)), methylarsonic acid (MA), dimethylarsinic acid (DMA), and arsenobetaine (AsB). The method is based on anion exchange HPLC coupled on-line to an inductively coupled plasma mass spectrometer (ICP-MS) for element specific detection. Experimental parameters,

such as column type and composition of the mobile phases can be used in order to get best separation, little matrix interferences, lowest detection limits, and short total times of analyses.

Another approach involves selective reduction of arsenate and arsenite (permitting quantification of individual inorganic arsenic species), and selective distillation of methyl arsines to quantify MMA and DMA. It is difficult to measure arsenobetaine with accuracy, because it does not form a hydride.

- **Sensitivity**
  0.1 to 1 µg/L for total As
- **Units**
  µg/l for arsenic in blood and µg/g cr for arsenic in urine

☑ Performance characteristics

- **Analytical reproducibility**
  3-15 % total As
- **Inter- and intra-laboratory variability**
  5-30 %

☑ Validation

Through the participation to intercomparison programmes and the use of certified standards or reference materials.

☑ Confounding factors

Diuresis is a potential confounder of urinary As concentration. Adjustment for variations in diuresis on the basis of urinary creatinine is reliable only when the concentration of creatinine in urine lies between 0.3 and 3 g/l. Other confounding factors are:

- physiological factors: age, sex;
- tobacco
- consumption of sea food products;
- water: tap water consumption in some geologic areas rich in arsenic, consumption of particular bottled water rich in As;
- occupational exposure.

☑ Concentrations reported in literature

The arsenic content in the human body is 3–4 mg and tends to increase with age. Arsenic concentrations in most tissues of the human body are <0.3 to 147 µg/g dry weight, excluding hair, nails, and teeth. Mammals tend to accumulate arsenic in keratin-rich tissues such as hair and nails. The normal concentrations of arsenic range from about 0.08 to 0.25 µg/g in hair and 0.34 µg/g in nails. The normal concentration of arsenic in urine can range from 5 to 40 µg per day (total).

Urinary total arsenic is a poor indicator of exposure because its concentration is very dependent on the organic food contribution.

On the other hand, the result of inorganic As + MMA + DMA is a good indicator of the recent exposure to inhaled and/or absorbed mineral arsenic. It is not useful to measure organic arsenic from food origin (fish, shellfish, shells) whose toxicity is low and who is eliminated for the major part in unchanged form in urine.

In Europe, in the population not occupationally exposed to arsenic and not living in a contaminated area, the concentration of the **sum (Asi + MMA + DMA) is generally lower than 10 µg/g cr** (cf. Cofrac), and most of them are below 15 µg/g cr:

It is what was observed for example in Germany, in the German Environmental Survey.

Tableau 1. Urinary arsenic concentrations ($\mu g.L^{-1}$) in adults (18-69 ans)
German Environmetal Survey 1998 (GerES III) (Backer *et al*., 2003)

Substance	**Number of samples**	**Number of samples <LOQ**	**P 50 $\mu g.L^{-1}$**	**P95 $\mu g.L^{-1}$**
Arsenic (Asi + MMA + DMA)	4741	208	4,1	18,9

In Germany, a reference value of 15 µg/L urine has been fixed based on adults (18 - 69 years) having refrained from fish consumption for 48 hours prior to sampling (GerES).

In Japan where the consumption of sea food products may be high, the sum (Asi + MMA + DMA) can exceed 50 µg/g of creatinine.

☑ Dose –response/effect relationships

Currently risk assessments are based on the uptake of arsenic from water, this is obviously sound when exposure occurs from water. However, the uptake from soil is still under investigation and is likely to be much lower than that from water.

Health effects of chronic exposure

Human carcinogen

Inorganic arsenic is a known human carcinogen classified in group 1 by IARC and acts via a genotoxic mechanism, therefore there is no threshold for such effects. Arsenic is not a chemical mutagen. i.e. it does not attack the DNA directly. However, it leads to chromosomal mutations under both in vitro and in vivo conditions. There is also an inhibition of DNA repair mechanisms. The risk management should ensure that exposures are as low as reasonably practical. There is sufficient evidence that chronic exposure to inorganic arsenic in drinking water causes non-melanoma skin cancers (basal-cell and squamous-cell carcinoma) and an increased risk of bladder and lung cancers in humans.

Reference value for cancer

US EPA in 1998 and OEHHA in 2002 established an oral slope factor of 1.5 $(mg/kg/d)^{-1}$ for a chronic exposure to arsenic by oral route. This value was established from two studies of Tseng (Tseng and al, 1968, Tseng, 1977). The critical effect is skin cancer.

The maximum likelihood estimate (MLE) of skin cancer risk for a 70 kg person drinking 2 L of water per day ranged from 10^{-3} to 2.10^{-3} for an arsenic intake of 1 µg/kg/day.

In 2004, WHO suggested a provisional guideline value of 0.01 mg/L for an excess of risk of skin cancers of 6.10^{-4}.

Non carcinogenic effects

The principal non carcinogenic effects of inorganic arsenic are dermal, neurological, haematologic, cardiovascular, hepatic, endocrine, developmental and reproductive effects. Long-term ingestion of contaminated drinking water may lead to vascular periphery effects: Raynaud's phenomenon, acrocyanosis, progression to endarteritis obliterans and gangrene of the lower extremities ("Black foot disease"). Incidence of

cardiovascular disease is increased. Haematologically, anaemia and leucopenia may occur together with disturbances in haem synthesis. Chronic exposure to inorganic arsenic compounds may lead to peripheral and central neurotoxicity including paresthesiae followed by muscle weakness (electromyographic abnormalities). In the periphery, both motor and sensory neurones are affected. Hyperpigmentation and hyperkeratosis are characteristic dermal lesions observed after chronic oral or inhalation exposure. Other toxic effects associated with chronic exposure to inorganic arsenic include liver injury, cardiovascular disease and diabetes mellitus. Limited data from epidemiology suggest that inorganic arsenic may be a human developmental toxicant; more data are needed. Embryolethality or foetal malformations have been observed in laboratory animals after administration of high doses of inorganic arsenic. Inorganic arsenic may cause irritation of the mucous membranes leading to conjunctivitis and pharyngitis and rhinitis after inhalation. Skin irritation and allergic contact dermatitis may occur after exposure to inorganic arsenic compounds.

Reference values for non carcinogenic effects

US EPA established in 1993 a RfD and ATSDR a MRL in 2005 of 3.10^{-4} mg/kg/j for a chronic exposure by oral way to inorganic arsenic. This value was estimated from the two studies of Tseng (Tseng and al, 1968 and Tseng, 1977). The critical effects retained are hyperpigmentation, keratosis and black foot disease. In the Tseng' studies, people who drank water from wells with arsenic concentrations between 1 and 17µg/L, did not present any symptom. The arithmetic mean of these concentrations (9 µg/L) was used as NOAEL for US EPA. RIVM proposed in 2001 a TDI of 10^{-3} µg/kg/j for a chronic exposure by oral way to inorganic arsenic. JECFA **(**Joint FAO/WHO Expert Committee on Food Additives) proposed a Provisional Tolerable weekly Intake of 0.015 mg/Kg bw.

Health effects of acute exposure

Single doses of inorganic arsenic may be highly toxic by ingestion and inhalation. Oral intake of 0.1 g of arsenic (III) may result in death. Both ingestion and inhalation may cause gastrointestinal effects such as nausea, vomiting, diarrhoea, abdominal pain and gastrointestinal haemorrhage. Multi-organ failure and tissue injuries may occur in severe cases following ingestion. Inorganic arsenic is irritant to the eye and skin.

☑ Time trend, geographical variation, susceptible groups

Some areas of the world have naturally high levels of inorganic arsenic in water from tap water or wells including Argentina, Chile, Mexico, Hungary, Bangladesh, Mongolia, West Bengal in India and Taiwan.

The distribution of contaminated land and water across the EU is an area requiring further research and is of significant Public Health concern.

5 B_2-MICROGLOBULIN (B_2-M)

☑ General information

β_2-m is a small protein (11.8 kD) synthesized by virtually all nucleated cells and is present on their membrane as the light chain of the class I histocompatibility antigens. As a result of the metabolism and degradation of HLA, β_2-m is dissociated from the heavy chain and appears in its free form in extracellular fluids.

☑ Matrix

Untimed or 24-h urine sample.

☑ Kinetics

- **Production**

  In normal subjects, the daily production of β_2-m is between 150-200 mg.

- **Distribution**

  β_2-m circulates as a free unbound protein in all extracellular fluids including plasma.

- **Excretion**

  β_2-m is mainly eliminated via urine. The amount of β_2-m excreted in urine is averages 60-80 µg/g cr in healthy subjects. More than 99.97 % of β_2-m filtering through the glomeruli is reabsorbed and catabolized by the proximal tubular cells.

- **Biological half-life**

  The biological half-life of β_2-m in plasma is estimated at about 2 hours.

☑ Sampling conditions

- **Sampling conditions**

  Because a time- and pH-dependent degradation of β_2-m occurs when the urinary pH falls below 5.6, the β_2-m test must be applied to a spot urine sample (mid-stream ideally) which is immediately neutralized after collection. If possible, the pH of the sample should be measured before adding the buffer. First morning samples should thus be avoided because of the high probability of β_2-m being degraded during the night. Since the urinary excretion rate of β_2-m is to some extent diuresis-dependent, it is advisable also to avoid extreme variations in the urinary flow. The β_2-m can also be degraded by elastase-like proteases in pH-neutral or slightly alkaline urine

infected by bacteria. An anti-bacterial agent must always be added to the urine (e.g. 0.1 % NaN_3).

- **Transport**

  It is always preferable to ship the samples on dry ice. However, if the urine is buffered at a pH > 6 and contains a preservative (NaN_3), a shipment at ambient temperature should not significantly affect the β_2-m levels.

- **Storage conditions**

  In urine with pH > 6 and containing a preservative, β_2-m is stable for several days at room temperature and at least two weeks at 4 °C. At -20 °C, the protein is stable for several years. In practice, if the assay cannot be performed within 2 or 3 weeks after collection, samples should be stored in the freezer. Freezing/thawing cycles, however, should be reduced to a minimum.

☑ Analytical aspects

- **Techniques**

  radioimmunoassay (RIA), enzyme immunoassay (EIA), latex immunoassay (LIA).

- **Sensitivity**

  0.1 to 1 µg/l depending on the immunoassay

- **Units**

  µg/l, µg/24 h and µg/g creatinine

☑ Performance characteristics

- **Analytical reproducibility**

  5 to 10 %

- **Intralaboratory variability**

  5 to 10 %

- **Interlaboratory variability**

  not documented but significant variations can be observed depending on the type of assay and the standards used.

☑ Validation

Published immunoassays are usually validated by comparison with alternative methods. Commercially available calibrators can be used.

☑ Confounding factors

Urinary pH and diuresis are the main confounders. Some lymphoproliferative diseases can increase the urinary excretion of β_2-m regardless the integrity of the tubular function. The assay of urinary β_2-m is a reliable marker of proximal tubule function only when the GFR is normal or only slightly impaired.

☑ Concentrations reported in literature

- **Reference value**

  The mean urinary excretion of β_2-m by healthy subjects ranges from 60 to 80 µg/g cr. Upper limits of normal in healthy subjects aged 20 to 60 years are in the range of 200 to 300 µg/ g cr.

- **Critical value**

  200-300 µg/g cr

☑ Dose –response/effect relationships

See cadmium for examples.

☑ Time trend, geographical variation, susceptible groups

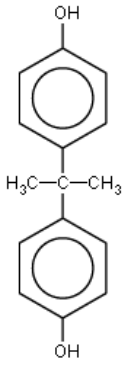
No data

6 BISPHENOL A

☑ General information

Bisphenol-A (BPA) is extensively employed in the production of epoxy resins and polycarbonate plastics used in food and drink packaging industries (Nakazawa et al., 2002; Staples et al., 1998), although the estrogenic activity of this compound was noted nearly seventy years ago (Dodds and Lawson, 1938). Resins containing bisphenol-A are commonly used to coat metal products such as food cans, bottle caps and water supply pipes. Like diethyl stilbestrol (DES), bisphenol-A is capable of binding to DNA after metabolic activation and has estrogenic properties at low concentrations (Hormonally active agents, 2000). Howdeshell et al. (1999) showed that trans-placental exposure of low doses of bisphenol-A in mice could bring on early puberty in females. Much of the concern focuses on unborn babies, because sex hormones play an important role during foetal development (Bolten et al., 1989). Bisphenol-A and alkylphenols primarily enter the marine environment as components of industrial and domestic waste (Fendinger et al., 1995). The consumption of BPA in the European Union is estimated 640,000 tons per year.

Figure 5.1: General chemical structure of Bisphenol A (4,4'-dihydroxy-2,2-diphenylpropane)



BPA and several other environmental phenols are chemicals which exert their effect through mimicking endogenous estrogens (Markey et al. 2001). The release of these chemicals into natural waters and wastewater treatment plants results in exposure of aquatic wildlife and also humans.

BPA is one of the xenoestrogens that are currently produced in large volumes. BPA increases the proliferation of estrogen target cells, induces estrogen-specific genes and reporter genes, increases the wet weight of the uterus, and induces proliferation of the epithelium in the endometrium and vagina (Markey et al. 2001).

BPA is used in the packaging of food and beverages, and in health-related products. This chemical and its derivatives leach from such polycarbonate and epoxy resin products leading to exposure of humans predominantly. Evidence from field studies and laboratory experiments indicate that BPA has the potential to cause ecological problems and affect human health. BPA is able to induce feminization of neonatal amphibia, proliferative activity in the uterus and mammary glands, alterations in the neuroendorine axis, and compromise fertility. In utero exposure to this chemical causes alterations in the onset of sexual maturity in females and changes in the development of male reproductive organs. The most disturbing findings reveal that low doses of BPA, which are physiologically relevant to human exposure, cause the most profound biological effects among a diverse number of chemicals present in the environment. BPA may be detrimental to the development and reproduction of wildlife and humans (Markey et al. 2001).

☑ Matrix

- **Invasive**

  Bisphenol A can be generally analyzed in blood (serum and red blood cells), in cord blood, and in bile (of fish)

- **Non-Invasive**

  Breast milk has been frequently monitored as a toxicologically relevant exposure route for BPA. Also urine and faeces have been described as a matrix for BPA determination

☑ Kinetics

- **Uptake**
  - *Food and Water:* It is generally accepted that food is a main uptake route of bisphenol A. The investigation of bisphenol A (BPA) in powdered milks and infant formulas by gas chromatography–mass spectrometric analysis after trimethylsilylation of Kuo et al. (2004) showed that BPA was detected in all the

samples (n=6) at concentrations from 45 to 113 ng/g (except one infant formula). Del Olmo et al (1997) determined BPA in spring water and seawater in Spain. BPA was also detected in honey samples from different countries (detected levels: ND<2.0–33.3 ng/g) using reversed-phase liquid chromatography (Inoue et al. 2003). Bisphenol-A was also detected in a study of supermarket seafood from Singapore (Basheer et al. 2004). The authors found between 13.3 and 213.1 ng g^{-1} w.w. of bisphenol-A in prawn, crab, blood cockle, white clam, squid, and fish (Basheer et al. 2004). The highest concentrations of bisphenol-A (213.1 ng g^{-1} w.w.) were found in crab samples.

- *Food packaging materials as coatings and films:* Migration of bisphenol A (BPA) from epoxy-coated can surfaces into infant formula concentrates has been reported (Biles et al. 1997). Levels of BPA in the undiluted concentrates surveyed in this study ranged from 0.1 to 13 parts per billion (ppb) as determined by solid phase extraction/high-pressure liquid chromatography with fluorescence detection and confirmation by gas chromatography with mass selective detection. Fourier transform infrared spectroscopy with 30° specular reflectance/transmittance was used to screen formula cans for epoxy coatings.
- European Legislation establishes that the sum of the migration levels of bisphenol A diglycidyl ether (BADGE), its hydrolysis (BADGE·H_2O and BADGE·$2H_2O$) and chlorohydroxy (BADGE·HCl, BADGE·2HCl and BADGE·H_2O·HCl) derivatives shall not exceed the limit of 1 mg/kg in foodstuffs or food simulants. A reversed-phase high-performance liquid chromatographic (RP-HPLC) method combined with mass spectrometry detection using atmospheric pressure chemical ionisation (APCI) was developed from Garcia et al. (2004) for the separation, quantification and identification of the migration of the compounds of interest in different samples.
- Bisphenol A (BPA) is used as an additive in polyvinyl chloride (PVC) products, including stretch films used for food packaging. The BPA contents were investigated of several brands of stretch film bought locally but marketed internationally or throughout Spain and which were presumably produced at different manufacturing plants (Lopez-Cervantes and Paseiro-Losada 2003). Their major components were identified by FTIR (Fourier Transform Infrared Spectrometry) and horizontal attenuated total reflectance, and the migration of

BPA from these materials into the standard European Union food simulants was determined by high-performance liquid chromatography (HPLC) using both fluorescence (FL) and ultraviolet (UV) detection, the identity of the analyte being confirmed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). The two HPLC detection methods had different detection limits (30 microg x l(-1) for UV, 3 microg x l(-1) for FL), but afforded virtually identical BPA determinations for the samples tested. BPA contents ranging from 40 to 100 mg x kg(-1) were found in three of the five PVC-based films analysed, and a content of 500 mg x kg(-1) was found in a fourth; for these determinations, extraction into acetonitrile was used. In standard tests of migration into water, 3% acetic acid and olive oil over 10 days at 40 degrees C, migration from a given film was in all cases greatest into olive oil. Migration from the films with non-zero BPA contents ranged from 3 to 31 microg x dm(-2), values higher than those reported for many other food-contact materials, but lower than the European Union specific migration limit for BPA. PVC stretch film nevertheless may make a significant contribution to contamination of foodstuffs by BPA, and should be taken into account in estimating BPA intake or exposure to this substance.

- *Environment:* Environmental exposure of humans e.g. from river water and atmospheric pollution has to be considered for higher polluted areas.

- **Metabolism**

A number of studies of the metabolism of BPA have been performed in rodents, mainly rats (European Union 2003, Pottenger et al. 2000). The results of these investigations show that BPA is rapidly absorbed from the gastrointestinal tract after ingestion and is then converted to a number of metabolites, mainly BPA glucuronide, in the liver. After 2-3 days, excretion of BPA and its metabolites, mainly in the feces, is mostly complete. A very small fraction, less than 1%, is retained in the tissues. BPA can be transferred to rodent offspring through the placenta and in maternal milk, but quantities found are only a very small fraction of the amount administered to the mother (European Commission 2002). There is no evidence of accumulation of BPA in the fetus.

Until recently, the results from rat metabolism studies were assumed to be directly applicable to humans. However, a recent study performed by Völkel et al. (2002)

with human volunteers indicates that humans metabolize orally administered BPA much more completely and more rapidly than rats. The results showed that BPA glucuronide is very rapidly formed and excreted in urine, and that this process is essentially complete within 24 hours. Practically none of the BPA is retained in the bodies of humans. These differences in metabolism need to be considered when extrapolating the results of toxicology studies from rodents to humans.

- **Biological Half-Life**

  The uptake, metabolism and excretion of the oestrogenic chemical bisphenol A (BPA) were studied in juvenile rainbow trout (*Oncorhynchus mykiss*) from Lindholst et al. (2001). BPA was detectable in plasma, liver and muscle after 2 h of water exposure at 0.44 μM (100 μg BPA/l), and a steady state was reached within 12–24 h. The concentration of the glucuronidated degradation product in the plasma was about twice that of the parent compound. A plasma half life of BPA was calculated as 3.75 h following injection of the compound. The vitellogenin synthesis was measured in response to the BPA treatment, and a lag period of 5 and 7 days between injection of the compound and a significant vitellogenin response was observed for females and males, respectively. At the time of the vitellogenin response no BPA could be detected in the liver tissue from either male or female fish. These results indicate that fish briefly exposed to elevated levels of oestrogenic chemicals might develop a response several days later.

  The kinetics of bisphenol A (BPA) were investigated in zebrafish (*Danio rerio*) exposed to 100 μg BPA/l (Lindholst et al. 2003). BPA uptake was measured during a 7-day period followed by an elimination phase of similar duration. After 2, 6, 12, 24, 48, 72, 120 and 168 h of uptake/elimination, fish were analysed for their content of BPA, bisphenol A glucuronic acid (BPAGA) and bisphenol A sulfate (BPAS). Within the first 24 h steady state levels of BPA, BPAGA and BPAS were reached and the total body concentrations were calculated to be 569, 12 600 and 39.9 ng/g fish, respectively. Elimination rates of the three compounds in zebrafish were estimated by fitting the data to a compartment model. An initial rapid elimination phase was observed for BPA and BPAS with total body half lives ($T_{1/2}$) of <1.1 h and 30 min, followed by a slower second elimination phase with $T_{1/2}$ values of 139 and 71 h, respectively. Excretion of BPAGA occurred from a single compartment with a $T_{1/2}$ of 35 h. The steady state concentration of BPA and its metabolites were

investigated in rainbow trout (*Oncorhynchus mykiss*) exposed to 100 μg BPA/l. The toxicokinetic parameters from zebrafish and rainbow trout were compared; including previously published data on the rainbow trout. The data indicate that the smaller estrogenic sensitivity observed for the zebrafish may be caused by a more rapid metabolism of BPA in the zebrafish liver.

☑ Sampling conditions

- **Seawater and seafood sample collection**

  Surface seawater samples were collected from various locations (Basheer et al. 2004). Samples were obtained at each location during the day in pre-washed glass bottles. Extraction was performed on unfiltered seawater samples. To prevent possible degradation of analytes, samples were collected and processed on the same day. Salinities of the samples varied between 28 and 35‰; seawater temperatures ranged between 28 and 32 °C, and pH between 7.9 and 8.6.

  Seven varieties of fresh seafood samples were purchased from a local supermarket chain between June 2000 and February 2001. Whole (shucked cockle, clam) or partial (muscle tissue of squid, prawn, crab and fish) samples were cut into small pieces immediately after returning to the laboratory and stored at −80 °C until analysis

- **Cord blood: Ethics approval and sample collection**

  The Ethics Committee of the University of Malaya Medical Centre (UMMC), Malaysia approved the study of contaminants in human umbilical cord blood and collection of the umbilical cord blood upon delivery at the labor ward at the UMMC, Malaysia. One hundred and eighty human umbilical cord blood samples were tested for the presence of bisphenol-A, alkylphenols and pesticides (Tan and Mohd 2003). Before delivery, the expectant mother was asked a series of questions about her pregnancy and personal particulars were recorded. The cord blood was collected in a 6-ml glass lithium heparin Vacutainer (obtained from Becton Dickinson Vacutainer Systems, New Jersey, USA) upon delivery at the UMMC, Malaysia; and centrifuged at 3500 rpm for 10 min. The plasma supernatant was aspirated out and stored in a 14-ml glass vial at −20 °C.

- **Breast Milk: Sample preparation**

To measure both free and conjugated species, each unknown breast milk sample was prepared in two different ways (Ye et al. 2006): one sample was processed without enzyme treatment, and the other was treated with β-glucuronidase (*H. Pomatia*). Breast milk was thawed and vortex mixed before aliquoting. A 100 µL aliquot of breast milk was mixed with 50 µL of internal standard solution, 10 µL of 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate standard, and 50 µL of enzyme solution in a disposable 1.5 mL CLIKLOK microcentrifuge tube (Simport, Beloeil, Canada). After gentle mixing, the sample was incubated overnight at 37 °C. After incubation, 190 µL of 2-propanol was added to the deconjugated breast milk, the sample was vortex mixed, and then centrifuged at 8000 × *g* for 15 min. Of the milk supernatant, 200 µL was transferred to an autosampler vial, 300 µL of 0.1 M formic acid was added, and the 500 µL sample was vortexed before being placed on the HPLC autosampler for on-line SPE–HPLC–MS/MS analysis. To determine concentrations of the free species, we followed the procedures described above, but added 50 µL of 1 M ammonium acetate buffer instead of the enzyme solution, and skipped the incubation step.

☑ Analytical Aspects

- **Techniques**
  - *Bisphenol-A in coastal waters and supermarket seafood determined using GC-MS.* Sample handling: The extraction procedure was initially evaluated from Basheer et al. 2004 with artificial seawater (Coral Red Sea Salt® dissolved in deionized water to obtain a final salinity of 33 parts per thousand). This was spiked with a stock solution containing bisphenol-A at a concentration of 50 $\mu g\ l^{-1}$ (per analyte) in 200 ml of sample, which was adjusted to pH 2 using 1 N HCl. Liquid–liquid extraction (LLE) was performed twice with 50 ml of dichloromethane. To remove trace amounts of water, anhydrous sodium sulphate was added to the organic layer. This layer was pre-concentrated in a rotary evaporator to a total solvent volume of approximately 1 ml, and subsequently cleaned-up using an Oasis-HLB (SPE) cartridge. The eluted extract was reduced in volume to less than 1 ml with nitrogen gas bubbling. Finally, 100 µl of bis (trimethylsilyl) trifluoroacetamide (BSTFA) was added, made up to 2 ml with acetone and kept in a 60C water bath for 30 min to complete the derivatization.

From this, 1 µl was injected to a GC-MS for analysis. Biological sample extraction was performed using microwave-assisted solvent extraction (MASE) (Kingston and Haswell, 1997). A small amount (0.2 g) of thawed tissue was placed in a microwave extraction vessel with 10 ml (20% water content) of tetramethylammonium hydroxide (TMOH) solution and 1 ml of n-nonane. The extraction vessel was exposed to microwave radiation at 80% power, and the contents were maintained at 115 °C for 15 min. In MASE, the tissue was completely digested in TMOH. The organic layer was washed and separated with diethyl ether and the extract cleaned-up using an Oasis-HLB (SPE) cartridge. The resulting mixture was derivatized using 100 µl of BSTFA. Finally, 1 µl of extract was injected into the GC-MS for analysis. A six-point calibration with correlation coefficient of >0.997 was made with naphthalene-d_8, pyrene-d_{10} and phenanthrene-d_{10} as internal standards and bisphenol-A-d_{14} as the surrogate standard. Limit of detection; for Bisphenol A 6.30 ng/l (from liquid-liquid extraction) and 1.357 ng/g (for microwave-assisted solvent extraction). A strict quality control procedure was followed that included the analyses of spiked and seawater samples with each set of field samples.

- *Measuring environmental phenols and chlorinated organic chemicals in breast milk using automated on-line column-switching–high performance liquid chromatography–isotope dilution tandem mass spectrometry.* Breast milk is one possible route of exposure to environmental chemicals, including phenols as bisphenol A and chlorinated organic chemicals for breast-fed infants. Ye et al. (2006) developed a highly sensitive method of analyzing breast milk for triclocarban (3,4,4′-trichlorocarbanilide) and eight phenolic compounds: bisphenol A (BPA), 4-*tert*-octylphenol (4-tOP), *ortho*-phenylphenol (OPP), 2,4-dichlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, and 2-hydroxy-4-metoxybenzophenone (BP-3). The method includes adding a solution containing a stable isotope of each chemical, enzymatic hydrolysis of the conjugated chemicals in the milk, and on-line solid-phase extraction coupled with high performance liquid chromatography–tandem mass spectrometry. It can also be used to measure the free (unconjugated) species by omitting the enzymatic deconjugation step. The method, validated using pooled breast milk samples, has inter-day coefficient of variations ranging from 4.8 to 18.9% for

most analytes, and spiked recoveries generally about 100%. Detection limits for most analytes are below 1 ng/mL in 100 μL of breast milk. We tested the usefulness of the method by measuring concentrations of these nine compounds in 20 breast milk samples. BPA, OPP, and BP-3 were detected in more than 60% of the samples tested. The free species of these compounds appear to be most prevalent in milk.

- *Development and validation of a method for determination of trace levels of bisphenol A in atmospheric samples.* A method has been developed and validated in order to assess the occurrence of bisphenol A as well as the alkylphenols tert-octylphenol and the isomers of technical nonylphenol in gasphase and aerosol samples of a remote area (Berkner et al. 2004). Gasphase samples were adsorbed to XAD2 resin, aerosol samples were taken on glass fiber filters. After ultrasonic extraction, clean-up by column chromatography and silylation of the analytes, ten nonylphenol peaks were quantified separately using a GC-MSD-SIM method. The absolute limits of detection and determination are in the range of a few pg per compound, which is a prerequisite for the quantification of the analytes in relatively unpolluted air. The precision of the whole analytical method is in the range of 1-17% and the recoveries range from 57% to 80%. Problems were encountered during method development due to the tendency of the analytes to sorb to glass surfaces. Silanisation of glassware was crucial to achieve acceptable recoveries. The widespread use of the analytes in plastic resins resulted in sample contamination. For this reason a careful choice of sampling material was necessary. Measured concentrations in gasphase samples (lower nanogram per m3 range) and aerosol samples (upper picogram per m3 range) are one to three orders of magnitude below already published concentrations.

- **Sensitivity and specifity**

  The quantitation limits for bisphenol A by gas chromatography–mass spectrometry in a study of powdered milk and infant formulas of Kuo et al. (2004) were 1.0 ng/g for BPA. Limits of detection; for Bisphenol A using GC-MS in an investigation of seawater and seafood of Basheer et al. (2004) was 6.30 ng/l (from liquid-liquid extraction) and 1.357 ng/g (for microwave-assisted solvent extraction). Ye et al. (2006) described detection limits for most analytes are below 1 ng/mL in 100 μL of

breast milk using automated on-line column-switching–high performance liquid chromatography–isotope dilution tandem mass spectrometry. The detection limit was 0.6 ng/ml for BPA in a study of spring water and seawater in Spain using gas chromatography-mass spectrometry (del Olmo et al. 1997).

- **Units**

  For liquid samples as e.g. breast milk, blood or water concentrations of bisphenol A are usually described as weight unit per volume of liquid (usually ng/mL). For solid samples as foodstuffs concentrations are usually given as weight unit per gram of sample (usually ng/g fresh weight).

☑ Performance characteristics

- **Analytical reproducibility**
- **Intra-laboratory variability**

  The method described from Ye et al. (2006) automated on-line column-switching–high performance liquid chromatography–isotope dilution tandem mass spectrometry, validated using pooled breast milk samples, has inter-day coefficient of variations ranging from 4.8 to 18.9% for most analytes, and spiked recoveries generally about 100%.

  Recoveries of bisphenol A and most alkylphenols and pesticides extracted from human cord blood in the GC-MS study of Tan and Mohd (2003) were in the range of 65–120% and the coefficients of variation (CV) of the compounds recovered were below 15%. Intraday and interday extractions showed consistent recoveries; CVs were observed to be below 15%. The intraday and interday recoveries showed that this SPE extraction method has high precision and consistent.

  Recovery efficiencies and reproducibility of extraction methods used for BPA determination seafood and seawater using GC-MS were described from Basheer et al. (2004). The relative standard deviations (RSDs) varied between 4.8% and 18.4% for seawater samples and 3.0% and 8.6% for tissue samples. Recoveries ranged between 73.9% and 96.5% for seawater and between 92.0% and 111.1% for tissue samples respectively.

  The average recoveries of stir bar sorptive extraction with in situ derivatization and thermal desorption-gas chromatography–mass spectrometry in were higher than 95%

with acceptable precision (RSD: <10%, n = 6) for a study of human urine samples spiked with these compounds at 0.5 and 5.0 ng/ml levels (Kawaguchi et al. 2005).

☑ Validation

Analytical methods for bisphenol A have been validated for different matrices as e.g. human cord blood (Tan and Mohd 2003), urine (Kawaguchi et al. 2005), seawater (Basheer et al. 2004), athmospheric samples (Berkner et al. 2004) and foodstuffs (Basheer et al. 2004).

☑ Confounding factors

Bisphenol A patterns may depend on preferences in food consumption.

☑ Concentrations reported in literature

- **Reference values**

  BPA and other environmental phenols were frequently detected in human breast milk samples in a study of Ye et al. (2006) using automated on-line column-switching–high performance liquid chromatography–isotope dilution tandem mass spectrometry. Median concentrations of free BPA (0.4 ng/mL) and total BPA (1.1 ng/mL) of this study were comparable to median levels (0.6 ng/mL) of BPA found in a study of 23 lactating women (Sun et al. 2004). Furthermore, median concentration of BPA in milk in the study of Ye et al. (2006) in a population of 20 women was similar to median urinary concentrations of BPA (1.28 ng/mL) in a group of 394 adult U.S. residents (Calafat et al. 2005). Relatively high frequency of detection of BPA (free, 60%; total, 90%) in this study population suggests a high potential for exposure of breastfed infants to BPA via breast milk. A study of bisphenol A in human cord blood of Tan and Mohd (2003) detected BPA in 88% of the 180 cord blood samples in concentration ranging from non-detectable—4.05 ng ml^{-1}. Hirahara et al. (2002) analysed 1690 maternal blood samples where 1665 samples (98.5%) showed detectable levels of BPA of 0.407±0.007 ng/ml (Meann±SE, n=1665), while, 398 samples obtained from their ubilical cord showed higher concentrations of 1.366±0.115 ng/ml (Meann±SE, n=398).

- **Critical values**

  In 103-week dietary study of the National Toxicology Program (NTP 1982), groups of 50 rats/sex were fed diets containing 0, 1000, or 2000 ppm bisphenol A. All treated groups of rats had reduced body weights, compared with controls, evident from the 5th week of exposure. Food consumption was also reduced, compared with controls, but this effect was not observed until the 12th week of treatment. Reduced body weights in rats, therefore, was considered a direct adverse effect of exposure to bisphenol A.

  In the same study (NTP, 1982), male mice (50/group) were fed diets containing 0, 1000, or 5000 ppm bisphenol A and female mice (50/group) were fed 0, 5000, or 10,000 ppm bisphenol A. Male mice at 5000 ppm and female mice at 5000 and 10,000 had reduced body weights. At 1000 and 5000 ppm, there was an increase in the number of multinucleated giant hepatocytes in male mice. This effect was not considered to be adverse, and this level is a NOAEL in mice. Assuming a food factor for mice of 0.13, this dietary concentration corresponds to a dosage of 130 mg/kg/day. Because the LOAEL of 50 mg/kg/day in rats is less than the NOAEL of 130 mg/kg/day in mice, the NOAEL in mice cannot be chosen as a basis for the RfD. The LOAEL of 50 mg/kg/day in rats, the lowest dosage used in either species in the chronic studies, is chosen as the basis for a chronic oral RfD.

  The Uncertainty Factors (Oral RfD) of 1000 includes 10 for uncertainty in the extrapolation of dose levels for animals to humans, 10 for uncertainty in the threshold for sensitive humans, and 10 for uncertainty in the effects of duration on toxicity when extrapolating for subchronic to chronic exposure.

- **Additional Studies/Comments (Oral RfD)**

  Three subchronic oral toxicity studies of bisphenol A have been considered using dogs, rats and mice (U.S. EPA, 1984a,b,c; NTP, 1982). The only toxic effect seen in beagle dogs fed 1000-9000 ppm bisphenol A in the diet for 90 days was an increase in group mean liver weight in the high-dose group (U.S. EPA, 1984a). The only effect seen in 2-generation bisphenol A feeding studies (100-9000 ppm) conducted with Charles River rats (U.S. EPA, 1984b,c) were decreases in body weight in the F0 generation at 9000 ppm and F1 generation at greater than or equal to 1000 ppm. Rats and mice of both sexes were fed bisphenol A (250 to 4000 ppm rats; 5000to 25,000 ppm mice) in the diet for 90 days (NTP, 1982). Doses >1000 ppm produced

decreased body weight in both sexes of rats with no alteration in food consumption. Male mice receiving >15,000 ppm and all treated females had decreased body weight gain compared with controls. A dose-related increase in severity of multinucleated giant hepatocytes was found in the treated male mice.

In mice, a dosage of 1250 mg/kg/day was associated with fetotoxicity and maternal toxicity, but did not cause a significant increase in the incidence of malformations at any dose level (NTP, 1985a). In rats, dosages of less than or equal to 1280 mg/kg/day were not toxic and did not cause malformations to the fetus (NTP, 1985b).

Confidence in the key study is medium because this study, although well controlled and performed, failed to identify a chronic NOAEL for reduced body weight, the critical effect, in rats, the most sensitive species. Confidence in the database is high, however, because the subchronic studies in rats indicate that the NOAEL for reduced body weight in rats is probably not far below the LOAEL of 1000 ppm of the diet and the uncertainty factor of 10 to estimate a NOAEL from the LOAEL is probably conservative. The developmental toxicity of bisphenol A has been adequately investigated. Confidence in the RfD, therefore, is high.

☑ Dose-response/effect relationship

Vom Saal et al. (1997) reported a series of non-monotonic dose-reponse curves in studying the impact of fetal exposure in mice to subsequent impacts postnatally. Low doses of both bisphenol-A and diethylstilbestrol (DES) caused significant enlargement of the adult prostate weight of mice exposed in the womb. If exposed, however, to higher doses of bisphenol-A or DES, there is no enlargement. In fact, at higher doses, the prostate is smaller than those of mice not exposed in the womb.

This effect has also been found by Gupta (2000). Gupta (2000) demonstrated that when pregnant mice were fed low doses (50 µg/kg/day) of bisphenol A during days 16-18 of gestation the exposure caused a significant increase in prostate size, decreased epididymal weight and a longer anogenital distance. The magnitude of effect on prostate weight (roughly a doubling at day 60 after birth) was greater than that found by vom Saal (1997). The doses used by Gupta (2000) are not quite comparable to vom Saal's (2 and 20 µg/kg/day) but within the same range. Gupta (2000) showed that the chemicals

used (DES and arochlor 1016, a PCB mixture, in addition to BPA) permanently increased androgen receptor (AR) binding activity of the prostate in adulthood.

☑ Time trend, geographical variation, susceptible groups

Consistent data on time trends and geographical variation are not available

7 BROMINATED FLAME RETARDANTS

☑ General information

Brominated flame retardants (BFRs) are comprised of diverse classes of chemical coumounds. There are five major classes of BFRs: brominated bisphenols, diphenyl ethers, cyclododecanes, phenols and phthalic acid derivatives. The first three classes represent the highest production volumes, with the major BFRs tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), and three commercial mixtures of polybrominated diphenyl ethers (PBDEs), namely deca-BDE, octa-BDE and penta-BDE (Letcher and Behnisch 2003, Birnbaum and Staskal 2004). An overview of production volumes of these BFRs is given in table 2:

Table 6.1: Total market demand by region in 2001 in metric tons (MT). (BSEF 2006)

(MT)	Americas	Europe	Asia	Rest of the	Total
TBBPA	18.000	11.600	89.400	600	119.600
HBCD	2.800	9.500	3.900	500	16.700
Deca-BDE	24.500	7.600	23.000	1.050	56.150
Octa-BDE	1.500	610	1.500	180	3.790
Penta-BDE	7.100	150	150	100	7.500
Total	53.900	29460	117.950	2.430	203.740

There are important differences in BFR use in different regions, with Europe having banned the use of penta- and octa-BDE in electrical and electronic equipment by 2006. Hence, Deca-BDE is the main PBDE of consern in Europe, and consists of a mixture of 97-98% BDE-209 and up to 3 % NonaBDEs (Table 2).

Table 6.2: The general composition of PBDE-based frame retardant commercial products given in percent of BDE congeners present (WHO/ICPS, 1994)

Technical	Congener %						
Product	TetraBDE	PentaBDE	HexaBDE	HeptaBDE	OctaBDE	NonaBDE	DecaBDE
PeBDE	24-38	50-60	4-8				
OcBDE			10-12	44	31-35	10-11	<1
DeBDE						<3	97-98

Overall, TBBPA, HBCD and Deca-BDE as the main BFRs in Europe, and the focus in the following will be on these three compounds.

☑ Matrix

- **Invasive**

  Invasive determination of BFR concentrations are performed in adipose tissue and blood.

- **Non-invasive**

  Because of the relatively high fat content of breast milk, BFRs are transferred to the milk. Concentrations in human breast milk are strongly correlated with the fat content of the milk and reflect the accumulated levels in adipose tissue. Accordingly, milk is an excellent matrix to monitor the long-term exposure to liphophilic, bioaccumulating substances such as BFRs (Koren and Meironyte 2000). Hakk and Letcher (2003) reported good correlations of PBBs in human serum to adipose tissue, ranging from 1:140 to 1:260, and did not change in pregnant women or maile chemical workers

- **Comparison**

  Because of its specificities, breast milk has both disadvantages (sampling restrictions) and advantages (toxicological relevance of mother-child transfer of BFRs).

☑ Kinetics

- **Uptake**

  In order to fully appreciate the biotic fate of BFRs, a distinction needs to be made between reactive and additive flame retardants. Most BFRs are additive flame retardants, meaning that they do not form a chemical bond with the materials they are added to. Reactive flame retardants on the other hand are mixed with the plastic before polymerization to form covalent bonds and thus become part of the polymer matrix. This difference makes that additive BFRs are much more likely to leach out of goods and products during their lifetime (Sjödin et al 2003). PBDEs and HBCD are examples of additive BFRs, while TBBPA can be used as either a reactive (90% of total use) or an additive flame retardant (10% of total use). If BFRs are persistent enough to bioaccumulate, the major route of exposure will be through food. This means that fatty fish and mother's milk are two major sources. Sjödin et al (2000) found significant correlations between fish consumption and BDE-47 in Swedish

and Latvian males. Nonpersistent BFRs, such as TBBPA (see further) are not thought to biomagnify and are mainly ingested due to direct exposure via inhalation (Sjödin et al 2003). Schauer et al (2006) suggested that of TBBPA was absorbed from the gastrointestinal tract and rapidly metabolized by conjugation resulting in a low systemic bioavailability of TBBPA. An overview of the estimated daily PBDE intake is presented in the following table:

Tan et al (2007) regarded house dust as the most important source of PBDE exposure in Singaporese children, although there are important differences in the percentage congener distribution. BDE 209 is more firmly bound to dust particles, while others, such as BDE 47, 99 are more likely to remain in the gas phase. Also others reported that exposure to PBDEs is to a large extend due to uptake through dust (Wilford et al 2005; Stapleton et al 2005).

- **Metabolism**

  Experiments, mainly on rats and other mammals, have shown that TBBPA and DeBDE have a very low uptake rate, with between 90% and 99% of the administered dose eliminated in the feces and gut (WHO/ICPS 1995, de Wit 2002, Hakk and Letcher 2003). Urine excretion of TBBPA in rats is generally < 1%, and although there is some metabolisation in rats, most of the TBBPA is excreted unmetabolized (Hakk and Letcher 2003). Although DeBPE (mainly BPE-209) is rapidly excreted in rats (see further), experimental evidence indicates that BDE-209 can be readily metabolized via oxidative debromination if absorption is achieved (Hakk and Letcher 2003). Little is known about the metabolism of HBCD. Under laboratory conditions, TBPPA can be microbially metabolized due to anaerobic reductive debromination to bisphenol-A, with subsequent aerobic mineralization by gram-negative aerobic bacteria (Ronen and Abeliovich 2000). TBBPA is also photolytically decomposed when exposed to UV light, with the main breakdown product 2,4,6-tribromophenol. Also DeBDE is rapidly debrominated in both laboratory and field experiments by UV light and sunlight to lower brominated PBDEs (de Wit 2002). DeBDE braks down to Nona- to Hexa-BDEs with a half-life of <15 min in toluene and approximately 15, 100, and 200 hr in sand, sediment, and soil, respectively (Birnbaum and Staskal 2004).

Table 6.3: Estimates of PBDE intake from food for different countries (Source: Washington State Department of Ecology 2004)

Daily PBDE intake (µg/kg bodyweight)	Country	Age	Source of exposure	PBDE congeners
0.0007	Sweden	adult	food	47, 99, 100, 153, 154
0.01	Sweden	Infant (0-6 mo.)	Breast milk	47, 99, 100, 153, 154
0.00062 (0.044 µg/day)	Canada	adult	Food	28, 47, 99, 100, 153, 154
0.00019-0.003	The Netherlands	Adult	Food	28, 47, 99, 100, 153, 154
0.0014-0.0011 (0.097-0.082 µg/day)	Spain	Adult	Food	Sum of tetra-to octa-BDEs
0.00059 (0.041 µg/day)	Sweden	Adult	Food	47, 99, 100, 153, 154
0.00013 (0.091 µg/day)	U.K.	Adult	Diet, air, occupational	47, 99, 100, 153, 154
0.00073	Canada	Infant	Breast milk	Sum of tri- to hepta-BDEs
0.00043	Canada	Adult	Diet, air, occupational	Sum of tri- to hepta-BDEs
0.2-2.6	Canada	0-6 mo, 0.5-4, 5-11, 12-19, 20-59, 60+ yrs.	Air, water, food, breast milk, and dust	Sum of tetra- to deca-BDEs
0.36	USA	Nursing infants	Breast milk	17, 28, 47, 66, 77, 85, 99, 100, 138, 153, 154, 183, 209
0.011	Germany	Nursing infants	Breast milk	17, 28, 47, 66, 77, 85, 99, 100, 138, 153, 154, 183, 209
0.004	North America	Children	Food (fish, meat, fowl)	Sum of mono- to deca-BDEs
0.003	North America	Adults	Food (fish, meat, fowl)	Sum of mono- to deca-BDEs
0.04-0.9	USA	<1, 1-2, 3-5 yrs	Multiple pathways	Penta-BDEs
0.014-0.04 (0.86-2.4 µg/day)	USA	Adult women	Back-calculated from tissue levels	Total, mostly 47, 99, 100, 153, 154

- **Biological half-life**

  In a recent review on the metabolism, toxicokinetics and fate of BFRs, Hakk and Letcher (2003) argued that PBDEs are the most important class of BFRs due to their environmental occurence and persistence in abiotic compartments. Thuresson et al (2006) recently calculated apparent half-life values of hepta- to decaBDPE for occupationally exposed electronics dismantlers and workers manufacturing flame-retrded rubber compounds. Half-life values are presented in the following table:

  Table 6.4: Apparent half-life values for selected BDEs.

BDE	$t_{1/2}$ (in days)		
congener	Estimated	Standard Error	95% confidence interval
BDE-209 (deca-BDE)	15	1.7	11-18
BDE-208 (Nona-BDE)	28	5.5	17-39
BDE-207 (Nona-BDE)	39	17	4-73
BDE 206 (Nona-BDE)	18	2.5	15-20
Octa-1	72	39	0-150
Octa-2	85	28	29-140
BDE-203 (Octa-BDE)	37	11	16-59
Octa-3	91	95	0-280
BDE-183 (Hepta-BDE)	94	13	68-120
BDE-47	2 years		
BDE-153	26 years		
TBBPA	2		

  These values for PBDEs indicate that the apparent half-life increases with decreasing number of bromine substituents, which was already postulated by other authors (Sjödin et al 1999). There are up till now no other data on PBDEs based on observational human data, but estimates based on animal experiments indicate long half-lives for BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154, less brominated PBDEs (Geyer et al 2004, Thuresson et al 2006). For TBBPA, very little direct human data is available. Hakk and Letcher (2003) and Sjödin et al (2003) quote an estimated half-life of 2.2 days, from a study by Hagmar et al (2000). No other data on humans were available, but this rapid elimination is consistent with half-lives estimated in rats or fish. Little is known about the metabolism of HBCD, but studies

by manufacturers show half-lives in rats of approximately 2 hours (Hakk and Letcher 2003).

☑ Sampling conditions

Covaci et al (2003) in detail describe sample pretreatment issues for the determination of BFRs. Although methods for BFR determination are very sensitive, the omnipresence of BFRs makes the risk for contamination an obvious risk. Furthermore, the light- and UV sensitivity of several BFRs makes prolonged sample storage precarious. Generally, samples are stored frozen at -20°C until analyzed.

☑ Analytical aspects

- **Techniques**

  Because of the global presence of BFRs in human and environmental samples, analytical methods for the determination of BFRs has shown a rapid development, with methods usually based on established methods for chlorinated pollutants, such as PCBs (Covaci et al 2003; Brede and Bjergaard 2004). Methods generally are analysed using GC-MS, with a wide variety of pretreatment, extraction and clean-up procedures. A detailed overview of methods, with recommendations, is provided by Covaci et al 2003. However, the first two international intercalibration excercises have shown that there still is a large interlaboratory variability, and methods need further improvement and refinement (de Boer and Cofino 2002). Further interlaboratory testing is currently occuring within the QUASIMEME Laboratory Performance Studies (QUASIMEME 2005), and are scheduled for the future (QUASIMEME rounds 46 and 48 for respectively 2006 and 2007). Recently, Cariou et al (2005) proposed a new method for the multi-residue analysis of HCBD, TBBPA and PBDEs from human biological matrices. Analytical reference materials and standards for brominated flame retardants are commercially available in indoor dust and environmental materials such as cod-liver oil, fish or mussel tissue, or sediments (Stapleton et al 2006, Stapleton et al 2007)

- **Sensitivity and specificity**

  Because of the availablility of a wide variaty of internal and syringe standards for BFR analysis, including radioactive standards, sensitivity and specificity of analytical techniques is high. Thomsen et al (2002) reported levels of detection

(LOD) in human milk of 0.3-0.7 pg/g milk for different PBDEs, Cariou et al (2005) reported levels of quantification of < 10 pg/g serum fat or breast milk for TBBPA and tri-to heptaBDEs, and 30-1500 pg/g for HBCD and decaBDE using a multiresidue analytical method.

- **Units**

  Since BFRs generally are lipophylic, the concentrations of individual conceners are generally expressed as a weight unit per gram of fat (usually pg/g fat). When taking into account this standardisation towards fat content, concentrations of BFRs in different matrices (blood, human milk, adipose tissue) are comparable.

☑ Performance characteristics

- **Analytical reproducibility**

  For the determination of BFRs, the addition of internal standards, evaporation steps, extract handling and standard solution stability are critical steps. The imprecision level is highly dependent on the compound analyzed, and ranges between 10-20% for tri-to hepta-BDEs and around 25% for BDE 209 and HBCF (Covaci et al 2003). Thomsen et al (2002) reported repeatability of BFR determination in the range of 4.7-8.4% and 0.6-10% relative standard deviation for GC/LRMS and GC/HRMS respectively.The recent establisment of certified reference materials should continue to increase the reproducibility and precision of BFR determinations (Van Leeuwen et al 2006).

- **Inter- and intralaboratory variability**

  Following four earlier comparable studies, an international laboratory performance study among 21 laboratories on the analysis of BFRs in environmental samples was organised by QUASIMEME (QUASIMEME 2005). The study concluded that most laboratories were able to determine most BDEs without major problems. Relative coefficients of variance (CV%) generally were below 30% for BDE 28, 47, 99, 100, 153, 154 and 183. However, adequately quantifying BDE 209 (decaBDE) remains a problem. Reported CV% for two sediment samples were 63% and 53%, although possible sources of errors have been identiefied previously (QUASIMEME 2005). Also, there are only few laboaratories that can adequately measure HBCD or TBBPA.

☑ Validation

Very recently, the German Federal Institute for Materials Research and Testing (BAM) developed standard methods for penta- and octaBDEs in products (Kemmlein et al 2005). However, there are no uniform, internationally accepted guidelines describing the measurement of BFRs in biological tissues.

☑ Confounding factors

Contradictory results have been obtained in different studies with regard to the effect of sex and age on BFR concentrations (Smeds and Saukko 2003). Occupational exposure is important through computer work, evidenced by the elevated serum levels of BDE-153, BDE-183 and BDE-209 found in computer technicians, which may be due to uptake through dust particles (Jakobsson et al 2002). Exposure through diet is possibly a main route of exposure to PBDEs (Domingo 2004), though data on the occurence of these compounds in food items is scarce. In a recent spanish study, the highest PBDE levels (in ng/kg wet weight) were found in fats and oil (587.8), fish and shellfish (339.2) and meat (109.2) (Domingo 2004). Also Sjödin et al (2000) reported a strong association between fish consumption and serum PBDE concentrations, which was confirmed for nursing Japanese women (Ohta et al 2002).

☑ Concentrations reported in literature

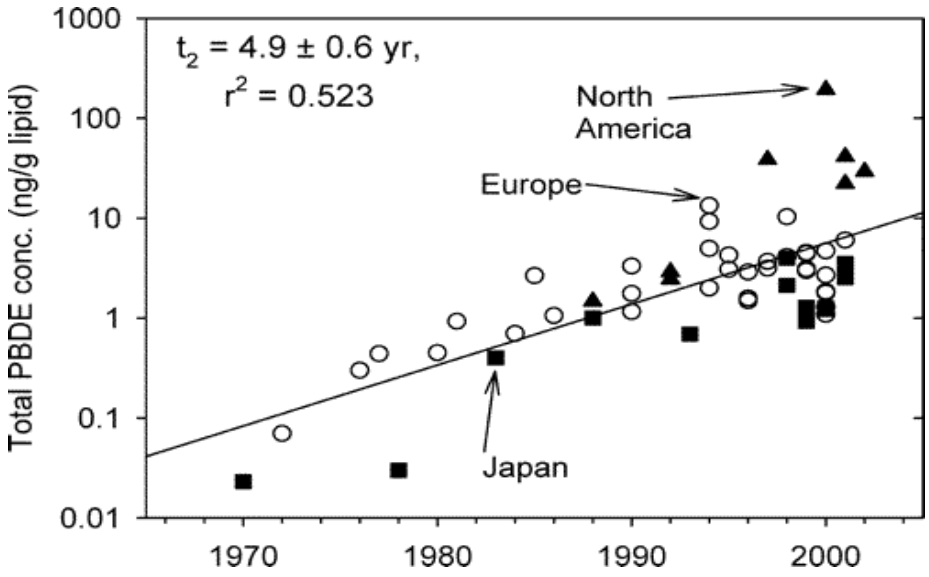
- **Reference values**

  Since there are no natural sources of BFRs in the environment, reference values should be taken below detection limit. However, the global use and bioaccumulative properties of certain BFRs have resulted in a global presence of these compounds. Figure 6.1 provides an overview of global ambient PBDE concentrations in human tissues over time.

- **Critical values**

  Because of the lack of adequate toxicological data on the effects of BFRs, there are no clearly defined critical values above which there is reason for concern. However, the main concern regarding BFRs is that their presence in the environment is increasing at an exponential rate, which in itself is enough reason for concern and close monitoring.

Figure 6.1: overview of global ambient PBDE concentrations in human tissues over time.



☑ Dose –response/effect relationships

In a review by Darnerud (2003), it was concluded that there is a general lack on high-quality toxicity data. However, studies have established a number of observations on the effects of different BFRs. Generally, acute toxicity is low, and no mutagenic potency is observed for BFRs. Lowest observed adverse effect levels (LOAELs) generally range from 0.6-0.8 mg/kg body wt for PentaBDE (neurobehavioral effects in neonatal mice), to 2 mg/kg body wt for OctaBDE (early developmental effects) and 80 mg/kg body wt for DecaBDE (thyroid hyperplasia, liver enlargement and hyaline degeneration in kidney). Very little data is available for humans, and four epidemiologic studies did not show adverse effects of occupational exposure in the working place. Also for TBBPA and HBCD, acute toxicity is in the grams per kg body wt range for rat, mouse, or rabbit, yet more data would be desirable.

Additionally, there is concern that polybrominated dibenzo-p-dioxins (PBDDs) and dibenzofurans (PBDFs) may be formed due to the combustion of BFRs and as low-level byproducts in some commercial mixtures. The limited studies with PBDD/Fs have shown that these compounds demonstrate all the clasical disease effects that are

observed for dioxins (PCDD/Fs), and that PBDFs also have tendency to bind with the Ah receptor. Keeping this in mind, it is assumable that PBDD/Fs will contribute to the overall dioxin body burden, which is already near the maximum TDI level at which dioxin-associated health effects may occur (Birnbaum et al 2003).

☑ Time trend, geographical variation, susceptible groups

- **Time trend**

  Since the first measurements of BFRs in human tissue in 1970, there has been an exponential increase in BFR concentrations with a doubling time of about 5 years. This implies that PBDE concentrations in people have increased by a factor of 100 during the last 30 years (see table x.x). However, in Sweden, a recent study by Lind et al (2003) shows that there may be a reduction in PBDE in breast milk from 1998 onwards. Also Thomsen et al (2003) reported similar trends for Norway. This trend is also reflected in e.g. guillemot eggs in from Sweden, mussels in the Seine estuary, or pike from Lake Bolmen in Sweden (de Winter-Sorkina et al 2006).

- **Geographical variation**

  From a meta-analysis of PBDE concentrations in human tissues, Hites (2004) concluded that people in the USA are exposed to higher levels of PBDEs than Europeans and Japanese. Median concentrations in US of blood (fetal and maternal) or breast milk samples are about 25-40 ng/g lipid, while concentrations in Sweden, Belgium, Germany and many other European countries in the same matrices are much lower at about 2-6 ng/g lipid.

- **Susceptible groups**

  Apart from occupationally exposed groups (e.g. through computer work) and babies (through breast feeding), no obvious susceptible groups could be identified.

8 CADMIUM (Cd)

☑ General information

Cd, a by-product of zinc production, is a cumulative poison that can cause kidney and bone damage following prolonged exposure in the environment and the industry.

☑ Matrix

Blood and urine (untimed urine sample) are the biological samples commonly used to monitor exposure to Cd.

☑ Kinetics

- **Absorption**

  In the industry and for the smoker, inhalation represents the main route of Cd exposure. The absorption rate of inhaled cadmium varies with particle size and solubility of Cd. For cadmium oxide, absorption has been estimated between 25 to 50 %. A part of the Cd deposited in the respiratory tract is cleared and once swallowed is absorbed in the gastro-intestinal tract. For non smoker with no occupational exposure, Cd from foods contributes to more than 95% to the total Cd intake. The oral absorption of Cd is relatively low (2-7%) but can reach up to 20% in case of iron deficiency. Absorption of Cd across the skin can be considered as negligible.

- **Distribution**

  Absorbed Cd is mainly stored in the liver and the kidneys, two organs that contain about 50 % of the total Cd body burden. Transfer of Cd across the placental and the brain-blood barriers is very limited. Cd is mainly excreted via urine.

- **Excretion**

  Cd is mainly eliminated via urine.

- **Biological half-life**

  The biological half-life of Cd is estimated at about 100 days in blood and at more than 10 years in urine.

☑ Sampling conditions

Blood and urine should be collected in containers free of any metal contamination. Samples can be stored at 4°C or frozen.

☑ Analytical aspects

- **Techniques**
  atomic absorption spectrometry (AAS) or ICP-MS.
- **Sensitivity**
  0.01 to 0.1 µg/l
- **Units**
  µg/l for Cd in blood and µg/g cr for Cd in urine

☑ Performance characteristics

- **Analytical reproducibility**
  1-2 %
- **Inter- and intralaboratory variability**
  5-10 %

☑ Validation

Through the participation to intercomparison programmes and the use of certified standards or reference materials.

☑ Confounding factors

Diuresis is a potential confounder of urinary Cd concentration. Adjustment for variations in diuresis on the basis of urinary creatinine is reliable only when the concentration of creatinine in urine lies between 0.2 and 2 g/l. Smoking is a confounder of both blood and urinary Cd.

☑ Concentrations reported in literature

- **Reference values** (upper limit of normal for non occupationally exposed populations)
  - *Cd in blood*
    - Adult non smokers <2 µg/l
    - Adults smokers <5 µg/l

- Children <0.5 µg/l

- *Cd in urine*
  - Adults <2 µg/g cr
  - Children <0.5 µg/g cr

- **Critical values**
  - *Cd in urine*: 5 µg/g cr
  - *Cd in blood*: 5 µg/l

☑ Dose –response/effect relationships

Renal-dysfunction induced by Cd develops in a dose-dependent manner according to the internal dose of Cd as assessed on the basis of Cd levels in kidney (renal cortex), urine or blood. Depending on the sensitivity of the renal biomarker and the susceptibility of the population (workers or general population), the thresholds of urinary Cd associated with an increased risk of renal effects vary from 2 to 10 µg/g creatinine. The thresholds associated with the development of tubular proteinuria, the critical effect predictive of a decline of the renal function, is estimated between 5 and 10 µg/g creatinine (see Table). Recent studies in populations of children or adults with low environmental exposure to Cd in Europe suggest that Cd can cause subtle tubular effects at much lower concentrations (below 2 µg/g creatinine). The significance of these early effects is however unknown.

Table 7.1: Risks of tubular proteinuria according to Cd levels in urine or blood

Cd in blood (µg/l) or in urine (µg/g cr)	
< 2	Normal
2-5	Tubular proteinuria unlikely
5-10	Risk of tubular proteinuria in susceptible individuals
> 10	Dose-dependent increase in the risk of tubular proteinuria

Tubular proteinuria defined as a urinary excretion of ß2-microglobulin and retinol-binding protein (RBP) > 300 µg/g cr.

☑ Time trend, geographical variation, susceptible groups

Few comparative data exist about the geographical and temporal variations of Cd body burden in Europe. Although a decrease of the Cd body burden has been reported in the

general population of some countries, human exposure shows little change compared to the drastic decrease of lead exposure.

9 CHIRAL POPS/EDCS

☑ General information

When racemic chiral POPs/EDCs (C-POPs/EDCs) enter the environment, enantioselective biodegradation might occur in water, soil (sediment) (Falconer et al., 1995; Pakdeesusuk et al., 2003; Robson and Harrad 2004) and their enantiomeric ratios (ERs), which have been widely used as tracers of air-soil transportation process (Bidleman et al., 1998; Hühnerfuss H 2000; Robson et al., 2004), could tell the processes information.

- **Enantioselective Residuals**

  The enantioselective biotransformations of C-POPs/EDCs have been well documented in aquatic biota. Early studies showed the ER and the correlation between the ERs and the concentrations of α-HCH and γ-HCH, respectively, could characterize the different microbiological degradation pathways in the North Sea (Faller et al., 1991). Different enzymatic characters were also revealed in different marine animals from the liver of Common eider ducks, the liver of flounders, blue mussels, and even the North Sea water in German Bight by ERs (Pfaffenberger et al., 1992). Additionally, enzyme activity might be affected by health status (Mössner et al., 1992). The other C-POPs/EDCs, such as OXC, c-HE in Baltic herring, Baltic salmon, Baltic seal even in human adipose tissue (Buser et al., 1992), c, t-CHL, o,p'-DDT, o,p'-DDD in cod liver oils and fish oils (Koske et al., 1999) had been observed. Except 2 brain samples, all organs showed that (-)-α-HCH was enantioenriched in pork (Covaci et al., 2004). In human samples (Chu et al., 2003), the racemic α-HCH was found in three liver samples, while chiral PCB95, 149, and 132 showed racemic or nearly racemic in muscle, kidney, and brain and the higher ERs for the three chiral PCBs were found in liver samples. Recent researches have paid more attention on the metabolites of C-POPs/EDCs, including some chiral metabolites, which formed from chiral of prochiral parental compounds. For example, the methyl sulfonyl ($MeSO_2$-) substituted of PCBs and DDE, they are also persistent and bioaccumulate like their parents and might be more toxic. The preferential formation one atropisomer of certain chiral $MeSO_2$-PCB have been observed in animals, such as arctic ringed seal and polar bear (Wiberg et al., 1998), harbor porpoises (Chu et al., 2003) and grey seal (Larsson et al., 2004). In grey seals,

all of the investigated metabolites found in significantly higher concentrations in the liver than in lung and blubber and the equal enantiomeric specificity for chiral metabolites in all tissues (Larsson et al., 2004) might suggested their formation in liver and redistribution among the other tissues.

- **ER as tracer of biotransformation in biota**

  The investigations in the polar bear food chain (*arctic cod-ringed seal-polar bear*) (Wiberg et al., 2000) and Lake Superior aquatic food web (Wong et al., 2004) suggested ERs might be magnified through predator-prey relationships with the increasing enzyme activity along the trophic levels. In the Lake Superior aquatic food web, chiral PCBs (91, 95, 136, 149, 174, 176, and 183) showed no biotransformation potential in phytoplankton and zooplankton (low trophic level organisms); macrozooplankton (diporeia and mysids) might stereoselectively metabolize them or, alternatively, obtained their nonracemic residues from feeding on organic-rich suspended particles and sediments; forage fish (lake herring, rainbow smelt, and slimy sculpin) and lake trout suggested a combination of both *in vivo* biotransformation and uptake of nonracemic residues from prey because of the widely nonracemic PCB residues (Wong et al., 2004). In polar bear food chain, cod showed near-racemic mixtures for α-HCH and CHL related compounds and, in contrast, ringed seal and polar bear were frequently nonracemic. Along the food chain, (+)-α-HCH became more abundant relative to (-)-α-HCH (Wiberg et al., 2000). The first order kinetic depuration rate models have been used to calculate the relative half-life time (Walter et al., 2001) and minimum biotransformation rates (Wong et al., 2002; 2004). The metabolic elimination rates calculation suggested that at least 58% of the t-CHL and the entire PCB-136 depuration rate could be attributed to metabolism in Rainbow Trout (Wong et al., 2002) and minimum biotransformation rates (calculated from enantiomer mass balances between predators and prey) suggested that significant biotransformation might occur for PCB congeners over the lifespan of trout and sculpins (Wong et al., 2004). Chiral biomagnification factor (relative to CB-153) analysis in polar bear food chain indicated that OXC might be formed by ringed seal and metabolized by polar bears and the linear relationships of ERs of some highly recalcitrant CHLs in polar bear adipose with the bears' age (Wiberg et al., 2000) might suggested a continuous feeding and enantioselective depuration mode. Relationships between ER of PCB95

with 1/ER of PCB132 and ER of PCB149 with of 1/ER 132 suggest that the bioselective metabolism of chiral PCBs has the same trend in human, although the ratio is different (Chu et al., 2003). Additionally, multivariate statistical methods revealed the ERs were good indexes of sample groupings in response of the enzyme activates (Wiberg et al., 2000).

☑ Matrix

- **Invasive**

  The common materials used to investigate the (prenatal) exposure are adipose tissue, maternal blood, cord blood, placenta as partly invasive, and even amniotic fluid (Foster et al., 2001; 2002).

- **Non-invasive**

  One of the widely used matrix for evaluation of human body burden to C-POPs/EDCs is breast milk since it's easy collecting, enriching of lipophilic compounds and exposure to suckling infant (Smith, 1999).

  Hair has high potential – especially for children - and is strongly recommended to be applied (Schramm 1992, 1993, 1995, 1997)

☑ Kinetics

Two processes, bioaccumulation in adipose tissue and excretion to breast milk, are the result of PBT levels in milk (Czaja et al. 1997). However, many factors like age of mother, parity, length of previous lactation and sampling method might affect the investigated levels (Harris et al. 2001). For example, the predictors of plasma concentrations of DDE and PCBs investigated in a group of 240 USA women showed the most reliable predictors of DDE were age and serum cholesterol, and the most important predictors of PCBs were age, serum cholesterol, and residence in the Midwest or Northeast (Laden et al., 1999). For general people, diet should be one of the major factors that influence the exposure levels of C-POPs/EDCs, with patterns in fish consumption playing a particularly significant role (Solomon et al., 2002). Despite of some negative results (Rauhamaa-Mussalo et al., 1988), mothers with contaminated fish consumption exhibited a higher level of C-POPs/EDCs (Kostyniak et al., 1999; Stewart et al., 1999; Laden et al., 1999; Harris et al., 2001). For example, Mohawk mothers (Fitzgerald et al., 2001), with the greatest estimated local fish consumption, had a

significantly higher geometric mean of *p, p'*-DDE level in milk than that of the control women, but no differences in mirex or HCB concentrations. Following the recommendation against the consumption of local fish by pregnant and nursing Mohawk women, the reduction of *p, p'*-DDE levels from 1986 to 1990 in Mohawk women breast milk was found. Great Lakes fish consumption was also associated with increased blood plasma PCB levels in men and mirex levels in both men and women. Waterfowl consumption was associated with higher plasma PCB (men and women), DDE (men only), and mirex levels (men and Cornwall women) (Kearney et al., 1999). The age of the mothers positively correlated with the C-POPs/EDCs levels (Covaci et al., 2002; Rauhamaa-Mussalo et al., 1988; Brunetto et al., 1996; Czaja et al., 1997) because of the longer exposure time and the possible higher daily uptake for the older mothers. Some reports showed that PCBs and DDE were significantly negatively related to both parity (Nair et al., 1996) and duration of lactation (Nasir et al., 1998; Kostyniak et al., 1999) and some showed unrelated (Czaja et al., 1997). It might be due to the correlation studies attributed to the simultaneous operation of many factors, such as age, number of deliveries, body weight, dietary habits, and sample collection time (Czaja et al., 1997; Harris et al., 2001). For example, *p, p'*-DDE in maternal adipose tissue was positively correlated with *p, p'*-DDE in cord blood ($P = 0.0001$) and breast milk ($P<0.0001$) and marginally correlated with change in BMI ($r = -0.03088$; $P = 0.06$) (Dorea et al., 2001). Historical and current local use patterns might be another reason for of the differences.

The very high fasting triglyceride levels (Haggarty, 2002) suggest that maternal body fat has higher turnover during pregnancy than in the non-pregnant state, with the total lipid increasing in maternal blood from 6.17 to 9.0 g/l (DeKoning et al., 2000). Except the instant nutrition supply to fetus, the relatively high ratio of milk energy preparation also demands a cycle of energy storage during pregnancy. In situations of low-energy supply, the stored lipid can be critical for the establishment of lactation and maintenance of maternal health (McNamara, 1999). All these may lead to the release of lipophilic contaminants from the adipose tissues into maternal blood in similar situation with their release into plasma in response to subcutaneous adipocyte basal lipolysis (Chevrier et al., 2000; Imbeault et al., 2001; Charlier et al., 2002). The release of β-HCH in response lipolysis of fasting can be in quantities sufficient to stimulate estrogen target uteri of ovariectomized mice (Bigsby, 1997). Although placenta is a predominantly non-fatty tissue, the difference of total lipids between cord blood (3.47 g/l serum) and maternal

blood (9.0 g/l serum) (DeKoning et al., 2000) forms a gradient between the two sides of the placenta membrane resulting in PBT diffusion. For the production of milk, the human body employs approximately 75% of endogenous fats during pregnancy (Waliszewski et al., 1999), which might also result in the release of C-POPs/EDCs from the adipose tissue stores.

Although not quite clear for the mechanism, all researches confirmed the transplacental transfer of C-POPs/EDCs. It was suggested these xenobiotics travel with the lipid components of blood, such as very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL), and chylomicron remnants, and likely gains access the many tissue compartments of the human body by utilizing the lipoprotein lipase mechanism (Dekoning et al., 2000). For examples, Aroclor 1254 increased hepatic lipid synthesis, but decreased hepatic production of albumin and apolipoproteins in pigeon (Borlakoglu et al., 1990a).

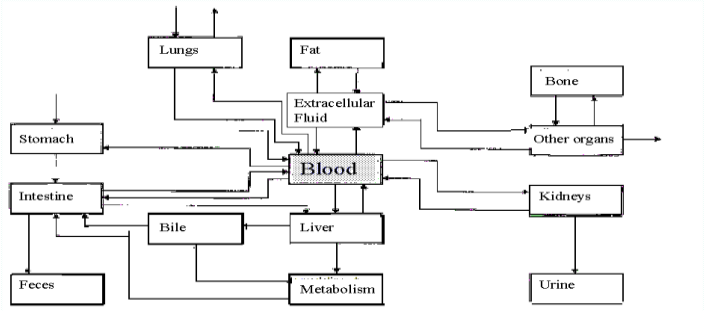
It used to be thought that the placenta created a perfect bulwark against toxic exposure and thus came the term 'placental barrier'. In fact, the placenta sorts out chemicals primarily on the basis of molecular weight, electrical charge, and lipid solubility. Small, neutrally charged molecules that readily dissolve in fat are afforded free passage regardless of their capacity for harm. Although the early suggestion that placenta restricts the transformation of C-POPs/EDCs to some extent (Eckenhausen et al., 1981), the followed researches (Saxena et al., 1981; Rosival et al., 1983; Cariati et al., 1983; Ando et al., 1985) all suggested the transfer of C-POPs/EDCs to fetus through placenta. Most of the results come from the comparison of PBT levels between maternal substance and placenta or cord blood, some data involved fetal tissues (Bosse et al., 1996; Waliszewski et al., 2000).

Paired sample analysis, especially using maternal blood *vs.* cord blood, is in principal a direct method to test potential transfer of persistent pollutants through the placenta. Many investigations found the significant correlation of C-POPs/EDCs, such as PCBs, PBBs, HeCB, HCHs and DDT with related compounds, between paired blood samples (Jacobson et al., 1984; Waliszewski et al., 2000; Covaci et al., 2002; Dallaire et al., 2004). For example, *p, p'*-DDE concentrations of the mean venous blood (7.12 μg /g) and cord blood (6.39 μg /g) were not significantly different, but were significantly correlated (Dorea et al., 2001). A more recent work showed not only lipophilic PCBs, but also their more polar metabolites OH-PCBs, can be transfered across the placenta to

the fetus (Soechitram et al., 2004). A more broad list of compounds, from β-HCH, *p, p'*-DDE, *p, p'*-DDT, HeCB, mirex, OXC, trans-nonachlor, PCB 99, 118, 138, 153, 156, 170, 180, 187 and Aroclor 1260 to Parlar 26, 50 and total toxaphene, had been shown to correlate between maternal blood *vs.* cord blood samples (Walker et al., 2003). Generally, maternal serum levels were higher than cord serum levels for C-POPs/EDCs mightily because of higher lipid content (DeKoning et al., 2000) in maternal than in cord serum.

Apart from maternal blood *vs.* cord blood, a broad type of maternal-fetus paired samples, such as placenta *vs.* serum (Schlebusch et al., 1994), placenta *vs.* cord blood (Ando et al., 1985) and cord blood *vs.* milk (Jacobson et al., 1984; Stewart et al., 1999) had been used to test the C-POPs/EDCs incorporation to fetus. For example, both cord serum and maternal milk levels of PCBs and PBBs were examined in relation to maternal serum levels (Jacobson et al., 1984). A significant linear correlation had been found between the HeCB concentration in placenta and that in cord blood (Ando et al., 1985).

Figure 8.1: Distribution of C-POPs/EDCs among body through blood circuit



However, the linear correlations did not always exist for all investigated compounds. Cord blood *vs.* milk analysis show that blood PCB homologues of light (Cl 1-3) or moderate (Cl 4-6) chlorination did not correlate with their breast milk homologues, the most persistent and heavily chlorinated PCB homologues (Cl 7-9) were significantly and positively correlated with breast milk levels (Stewart et al., 1999). Although no significant correlation between maternal serum and placental concentrations was observed, the placenta / serum ratios of HCH isomers, HeCB, total DDT, total PCB with 0.48, 0.99, 0.45, 0.32 show the incorporation of these C-POPs/EDCs into fetus

(Schlebusch et al., 1994). All these findings suggest the essence of a dynamic equilibrium of PBT among human tissues through blood circuit (Figure).

Despite of the dynamic equilibrium potential, there might be many factors that affect the correlation of C-POPs/EDCs between the paired samples. Firstly, PBT distribution might be lipid type related. Comparing the lean charr (the most tissues and organs had a substantially lower triacylglycerols but only a slightly lower phospholipids and cholesterol) with the fat charr (Jorgensen, et al., 1997), the tissue concentration of OCS was positively correlated with the concentration of triacylglycerols and negatively correlated with phospholipids and cholesterol. The proportion of the total body burden of OCS deposited in extra-adipose tissues was higher in the lean charr (28%) than in fat charr (4 times higher in brain of the lean charr than in fat charr). Also the partitioning of PBT pesticides between adipose tissues and serum might relate to the variation of lipid content in serum due to the association of C-POPs/EDCs with the lipid (lipoproteins) (Waliszewski et al. 1999). The congener specific distribution of PCBs in plasma fractions is more complex than can be explained solely by their solubility in the lipid components of plasma fractions (LDL, HDL and the lipoprotein-poor (predominantly albumin) fractions), and may suggest a complex association with apolipoproteins and plasma proteins that are important in transporting PCB to tissues (Borlakoglu et al. 1990b). Also DDT and HCH showed a positive correlation between paired breast milk and maternal serum while no correlation could be established either between breast milk and cord serum or maternal serum and cord serum (Nair et al., 1996). The fasting effect suggested there was different mobility for β-HCH and *o, p'*-DDT from fat depots by lipolysis in animals (Bigsby et al., 1997). An altered hormonal status, a different degree of metabolic activity and an increased deposition of fat in the breast during pregnancy perhaps favored degradation and selective partitioning of a few metabolites from the blood serum to the breast. It might explain that the light (Cl 1-3) or moderate (Cl 4-6) chlorinated PCBs in cord blood did not correlate with their breast milk homologues, the most persistent and heavily chlorinated PCB homologues (Cl 7-9) were significantly and positively correlated with breast milk levels (Stewart et al., 1999). The detected α-HCH in the similar level with p, p'-DDE but could not detected β-HCH in the non-fatty amniotic fluid (Foster et al., 2001; 2002) suggested the sample status responded to the variation of PBT patterns. The variation of reliability in the measurement of compound

levels **(**Jacobson et al., 1984**)** and some other unpredictable factors might also cause the deviation from the dynamic equilibrium hypothesis.

The children serum levels of PBB, PCB and DDE increasing with months of breast-feeding (Anderson et al. 2000) indicated breast milk as the main source of these pollutants after the delivery. The paired analyses of adipose tissue *vs.* mature milk (colostrums *vs.* mature milk) indicate a high degree of coherence, principally of total DDT; suggest lactation as a more effective decontamination means than through placenta (Waliszewski et al., 2001), especially, for primigravidae mothers (Nair et al., 1996). Other study also reported that breast milk was the main source of pesticide contamination to the newborn because the levels of maternal serum and cord serum are nearly equal but much lower than breast milk (Nair et al., 1996). Factually, the lipid content of the fetal tissue was 0.65% (8-14 week) much less than the amount of usually present in adults, which is generally from 15 to 30% of the body weight. That means the fetus has low potential to store C-POPs/EDCs. The estimated body burden of mother (from milk), placenta (lipid content 0.85%) to fetal tissue are 16.7, 10.1 to 5.3 TEQ (ng/kg lipid) for each (Schecter et al., 1996). Although a lower burden, transplacental exposure might be more relevant with regard to physical development and cognitive functioning of the child than postnatal exposure via breast milk (Przyrembel, et al., 2000).

☑ Sampling conditions

Midwives collected placentas at birth and froze them in LD-polythene bags in –20 ^{o}C. When defrosted, every placenta is mechanically homogenized, shared in small glass bottles (*Neolab scintillation tubes 20 ml, code: 9-0621*) and stored in –20 ^{o}C until analyses. A whole deep frozen placenta (laboratory code 0202027) is frosted in a refrigerator and homogenized using automatic mill. The homogenized sub-samples are packed in sealed glass bottles and stored under –190 ^{o}C.

The protocol of milk preparation is the following: 1. Open the cork/cap/stopper of the frozen bottle. 2. Place the bottle in a "minigrip" pack or suchlike (other glass) in order to avoid possible loss of sample if bottle will break during defrosting. 3. Defrost the bottle in a fridge. 4. Temperate the bottle to room temperature after thawing. 5. Shake/mix the sample at 38-40 ^{o}C for 1 hour 6. Close the cork/cap/stopper of the bottle and shake

vigorously. 7. Aliquot the sample 8. Freeze the aliquots as soon as possible and send them to other laboratories on dry ice.

☑ Analytical aspects

- **Cold extraction**

  The extract glass column (25 × 2 cm) is packed with 10 g placenta, which is homogenized with about 30 g anhydrous sodium sulfate and 15 g sea sand. The packed column is spiked on its top with 10 µl internal standard ^{13}C-standards. The extracting solvent is a mixture acetone and n-hexane (2:1 v/v) of 250 ml. The elute flow was controlled in mediate drops but not in flow (~2 drops/min) and collected in a carefully weighted (using analysis-grade balance) 250 mL round bottom flask.

- **Lipid determination**

  Evaporate the solvent by rotary vacuum evaporator with a water-bath temperature up to 45ºC and a mediate rotary rate; the vacuum is controlled around 600 mbar. When the extract is condensed to about 0.5 ml, removed the round bottom flask from the evaporator and then evaporated it to hemi-dry with very mediate stream of nitrogen. After that, the flask is placed for 6 hours into a desiccator until stable weight is achieved (i.e. error of two separate weight measurements over at least two hours ±0.0005g). The lipid content is calculated on the base of wet weight sample. Like the organic solvent, dry lipids also can keep the chlorinated organic compounds. So the lipid residue can be used to for further compound analysis and the results show that the recovery is sound. The use of lipid residue after lipid determination can save the extraction step for analysis of the chlorinated organic compounds. After recording of the lipid data, the lipid residue is resolved using 2 mL toluene and sealed and kept at –28 ^{o}C for cleanup.

- **Cleanup procedure**

  The first cleanup step is done on a GPC column. It is a 1.27 × 95 cm column packed with *Bio-Beads S-8*, spherical porous styrene-divinylbenzene copolymer with 8% crosslinkage, a constant-volume pump, an autosampler and an automatic fraction collector (*Gilson*) worked together with the column to perform the automatic chromatographic processes. The collected fraction is from around 32 min to 42 min for toluene eluent at a flow rate of 2ml/min (the result was checked and adjusted in the whole procedure of the two cohorts sample preparation).

Most part of lipids can be removed after GPC. However, the additional cleanup is needed to reach the analysis condition of HRGC-HRMS. Further cleanup is performed on a sandwich glass cartridge (75×12 mm, *Merck*), which is packed with alumina B 0.8g, anhydrous sodium sulfate 0.3g, florisil 0.5g, silica gel 1g, and anhydrous sodium sulfate 0.5g from bottom to top. Here 25 ml toluene is used to elute the pesticides residues from the cartridge.

Condense the cleaned elute by *Turbo Vap 500* (with the operation parameters: 5 min after sensor detection, temperature of water-bath 45 °C and fan speed level C). The eluent is evaporated to about 200 µl, and then, the sample is transferred to a 200µl micro-vial and is condensed by a gentle stream of nitrogen to a final volume about 10 µl in a fume hood. Add 10ul 1,2,3,4-TCDD standard as the recovery standard to the final volume. Finally, the about 20 µl samples is sealed and stored at -28 °C for analysis.

- **HRGC-HRMS analysis and data collection**

  HRGC device equipped with a capillary column *DB-XLB* (length 60m, internal diameter 0.25mm and thin film 0.25 µm). A capillary chiral column BGB-172 (two columns with 30m length and 0.25 and 0.18µm film thickness) is used for the enantiomers separation. (+) and (-)-isomers for o,p'-DDT are determined according literature (Falconer et al., 1997). Because the stationary phases are bleeding slowly in the processes, the GC programs are changed to suit the enantiomeric separation. Carrier gas is helium. HRMS engine using electron impact ion source mode is emplyed at multiple ion detection modes, combined with retention control and is used to identify the investigated compounds.

  Limit of detection (LOD) is set as signal/noise (S/N) = 3. The deviation of the ion ratios of the calibration mass to the reference mass for the ^{12}C-investigation compounds from the counterpart ratios of ^{13}C-internal standard compounds are controlled in range ±15%; the retention time differences are controlled ±3s. To some compounds without the labeled standard, the native standards are run at the same chromatographic condition of the real samples, and the mass information and retention time are used to screen these compounds in the samples. Only the data supported the upper conditions are considered for collection. Two blank samples and one reference (placenta or milk) sample are applied in each 30 real samples treating

procedure. The data higher that 3 time mean blank values are used as quantified data and, on the contrary, the lower ones are taken as unquantifiable data.

☑ Performance characteristics

The relative deviation (R-SDT: STD / Mean ×100%) of all abounding components like β-HCH, HeCB, OXC, c-HE, p,p'-DDE, END-1 and dieldrin can be controlled under 20-25% in the whole sample analysis procedure. Additionally, because the STD changed with the level of compound approximately by formula STD = 0.2~0.3 $Mean^{0.7\sim0.8}$, it is difficult to control the relative deviation at low concentrations. The uncertainty is larger for the low level compounds, especially, when concentrations become less than 1 ng/g. The final factor of error induction is the blank. It is not important for the higher concentrated compounds, but it can be a problem for compounds like α- HCH and γ-HCH because in some situation, their contents are around 1~2 ng/g lipid. The lipids detection R-STD is about 6 % for lipid reference samples and about 10 to 12 % for milk reference samples.

The robustness of the method for lipid determination has been checked intra-laboratory and inter-laboratory. The fat contents of paired placenta sub-samples (N = 2 × 30) were applied for intra-laboratory evaluation. Suggesting that the paired sub-samples have been homogenized well, theoretically, their lipid contents should exhibit the same values (Y = X). Factually, both homogenization procedures for sample aliquot and the lipid determination at each time could introduce some uncertainties. When using the ideal model to fit the data, the inter-laboratory residuals are 0.11 (±0.05) with the average of the relative errors (Y-residual / Y-prediction) are reported to be about 12%, and the intra-laboratory residuals were 0.03 (±0.03) with the average of the relative errors 5.11% (STD 4.06%). This suggested that, generally the detection results could be predicated between laboratories.

☑ Validation

No reference material or interlaboratory exercises for C-POPs/EDCs available

☑ Confounding factors

Human health and human disease result from three interactive elements: environmental factors, individual susceptibility, and age (Perera, 1997). Children's responses to

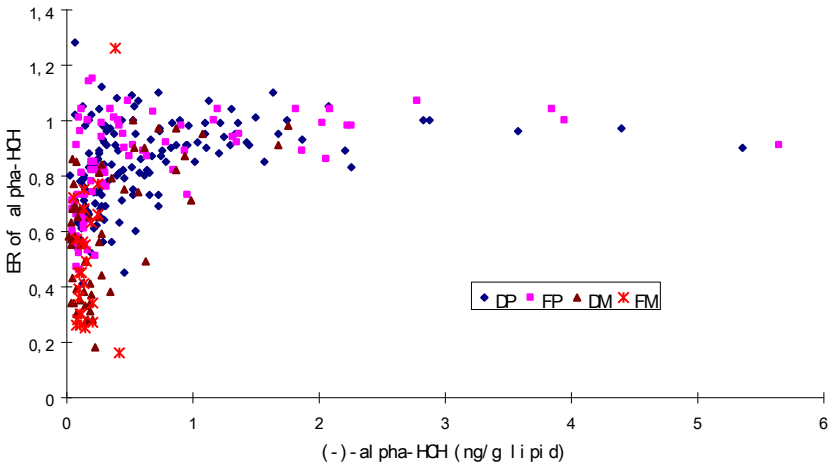
environmental toxicants will be affected by toxicokinetic factors (such as their systems absorb, distribute, metabolize, and excrete chemicals) and toxicodynamic factors (inferred toxic mechanisms and mode of action). These vary during development, in utero where maternal and placental processes play a major role, to the neonate in which emerging metabolism and clearance pathways are key determinants (Ginsberg et al., 2003; Daston et al., 2004).

Scientifically sound documents support that environmental chemicals are capable of acting as endocrine disrupters in lab. Clinical data linking between the environmental exposure levels and the present human adverse reproductive outcomes, such as early pregnancy loss, birth defects, reduced birth weight, and altered functional development is limited (Feldman, 1997; Johnson et al., 2000). The scientific community awaits further epidemiological assessment. However, one of the major limitations in environmental studies is the use of crude exposure matrices. Surrogate exposure measures, such as time living in a contaminated area (Dolk et al., 1998), do not adequately reflect the true nature and / or degree of real exposure to the chemical substance(s) of interest. Also, as they are not quantitative measures of absorbed dose, the data cannot give numerical measures of a dose response relationship, limiting their use in quantitative risk assessment (Sim, 2002). On the other hand, people exposed many pollutants, which have been documented to be EDCs, and surrogated measure might not reflect the real xenoentrogenic exposure personally. Additional testing systems are therefore required to screen for estrogenicity and to identify appropriate biomarkers of human exposure (Rivas et al., 2001). The time of sample collection is also important because of the might transient chemical exposure and the sensitive period of reproduction organ development (Kelce et al., 1997; Sultan et al., 2001). For example, by evaluating the incidence rates of acute infections with perinatal exposure to PCBs and DDE, the researchers suggested that there is a possible association between prenatal exposure, not postnatal exposure, to these C-POPs/EDCs and the acute infections in early life in the investigated Inuit population (Dallaire et al., 2004). The association between C-POPs/EDCs and the higher levels of total IgE in newborns (with higher allergic sensitization) supported the higher incidence rate of atopic eczema casesin the industrial region (Reichrtová et al., 1999).

☑ Concentrations reported in literature

The physical processes (leaching, volatilization, and atmospheric deposition) and abiotic reactions (hydrolysis and photolysis) for chiral compounds are unaffected (Bidleman et al. 1999). ERs show only the information of biotransformation or biodegradation of persistent pollutants. It is helpful because we usually not need consider these factors when we focus on biotic processes of these compounds. However, ER values were usually difficult to determine at high precision because of the lower concentrations. Although tissue specific ERs for certain chiral C-POPs/EDCs had been well document, these differences could be the results of different enzyme activities or blood-brain-barrier in different tissues, especially for tissue liver, kidney and brain (Kallenborn et al., 2000). Assumed equilibrium of C-POPs/EDCs between breast tissue and placenta, the ERs of the paired samples for certain chiral compounds should be similar for the less enzyme activity tissues such as placenta, milk and adipose tissue, the ER results for these tissues depended on the enzyme activity tissue, with emphasizing the liver. However, only some data could be used to conclude the assumption from the present data. For α-HCH, c-HE (might include o,p'-DDD), ER versus each enantiomer concentration plots suggested the concentration tendency of the Ers (Figure). Because of the conituing exposure mode and the possible variation in ER sources, the ER-concentration patterns could be more complex than the well-documented single dose model (Walter et al., 2001; Wong et al., 2002; 2004). No structural characters had been found for chiral OXC. The possible reasons moght be that OXC is consisting of metabolites of both cis-CHL and trans-CHL, they have been reported the apparent different OXC enantioselective in mammalians (Buser et al, 1992). According the *in vitro* results, the estrogenic activity of o,p'-DDT residuals might be overestimated because the enantioselective biodegradation of the more (-)-o,p'-DDT, which was the active estrogen mimic whereas the hER activity of (+)-o,p'-DDT was negligible (Hoekstra et al., 2001).

Figure 8.2: (-)-α-HCH is usually the isomer enantioselective enriched in samples of human females (P- Placenta, M – breastmilk D-Denmark, F – Finland).



☑ Dose –response/effect relationships

- **Ambient endocrine disrupters**

  The endocrine disrupting action mode of POPs/EDCs has been suspected to associate with the wide range of human health effects (Kavlock et al., 1996). Factually, EDCs range from natural plant oestrogens (e.g. genistein, coumesterol) and mycoestrogens (e.g. aflatoxins, zearalenone) to growth promoting pharmaceuticals (e.g. trenbolone acetate, melengastrol acetate) to chemicals spread in water, sewage sludge or the atmosphere such as detergents and surfactants (e.g. octylphenol, nonylphenol), plastics (e.g. bisphenol-A, phthalates), pesticides (e.g. methoxychlor, dieldrin, DDT) and industrial chemicals (e.g. PCB, TCDD) (Torres, 2002). The various environmental chemicals are capable of acting as endocrine disrupters as either hormone agonists or antagonists, which can potentially alter the hormonal balance in animals and people. However, controversy of the adverse effects on human health still remains (Feldman, 1997).

- **Low-dose effects**

  Beyond the traditional threshold model (assumes the threshold dose as no observed adverse effect level) and linear non-threshold model (extrapolates risks to very low doses of adverse effect), especially the later, as the dose below the standard threshold, the response becomes more likely to exceed the control value, so called hormetic-like. Hormesis (Calabrese et al., 2003ab) has been defined as a dose-response relationship in which there is a stimulatory response at low doses, but an inhibitory response at high doses, resulting in a U- or inverted U-shaped dose response, which is also called low-dose effect (Kaiser, 2000). Sex differentiation period is sensitive to low-dose chemical effects and the exposure might be transient, the effects are irreversible and not easy discovered until after puberty (Kelce et al., 1997). For examples, Utero exposure of mice shown the low-dose stimulating responses to diethylstilbestrol and estradiol **(**vom Saal et al., 1997), and the effects are organ and strain specific, transient and not sustained into adulthood for natural estrogens (Putz et al., 2001). *In vitro* research shown that EDCs, like coumestrol, endosulfan, dieldrin, and DDE, p-nonylphenol but bisphenol A, produced rapid (3–30 min after application), concentration (10^{-14}–10^{-8} M)-dependent ERK-1/2 phosphorylation but with distinctly different activation patterns. These actions may help to explain the distinct abilities of EDCs to disrupt reproductive functions at low concentrations via multiple membrane-initiated signaling pathways (Bulayeva et al., 2004). Although the low-dose effects was argued to appear unlikely during human pregnancy because of the species differences (particularly higher estrogen levels attained in human pregnancy compared to the mouse) (Witorsch, 2002), low levels of POPs/EDCs exposure have raised concerns for future generations. For example, levels of PCBs now shown to affect human brain development are nearly one million-fold lower than levels previously believed safe (Solomon et al., 2002).

- **Combined effects**

  As Daughton C (EPA in Las Vegas) said: no organism is ever exposed to a single chemical in isolation; the individual compounds are just part of the story and the biggest unknown right now is interactions. It is necessary to consider the impact of combined effects, which are commonly assessed in terms of synergism, additivity, or antagonism by comparing of the observed response with the usually expected one (Payne, 2001). Many reports have focussed on the mixture EDC activities. For

examples, pig immature cumulus-oocyte complex exposed to EDC mixture, which mimic contaminants of the Arctic marine food chain and the highly exposed populations of women, supported the concerns that such pollutants harm reproductive health in human and other mammalian species (Campagna et al., 2001). The extracts of air, subsurface soil, and superficial dust from a landfill, was used to examine multiple biological responses by a 2-day prepubertal female rat bioassay, where soil, dust, and air extracts effectively reduced serum total thyroxine (T4) with similar dose-response relationships, despite the significantly different TCDD toxic equivalent (TEQ) values of these three extracts (Li et al., 1996).

- **Additivity**

  Many reports support that mixtures of EDCs at doses that are individually inactive can give active response. For example, the mixtures has been reported at doses that are individually inactive in the immature rat uterotrophic assay, can give an uterotrophic response (Tinwell et al., 2004). The additive effect of o,p'-DDT, p,p'-DDE, β-HCH, and p,p'-DDT acted together on MCF-7 cells could be predicted on the basis of data about single agent concentration–response relationships (Payne et al., 2001). Similar results have been reported on hydroxylated PCBs, benzophenones, parabenes, bisphenol A, and genistein mixture using a recombinant yeast estrogen screen (Silva et al., 2002) and on estradiol-17β and ethynylestradiol-17α mixture (equi-potent fixed-ratio) using vitellogenin induction in a 14-day *in vivo* juvenile rainbow trout screening assay (Thorpe et al., 2003). Based on the pharmacologically well-founded models of concentration addition and independent action, the contribution of bisphenol A (BPA) or o,p'-DDT to the overall mixture effect combined with 17ß-estradiol (E2) was tested using yeast estrogen screen (***Rajapakse et al., 2001***). At molar mixture ratios proportional to the levels normally found in human tissues (i.e., below 1:5000 of E2:BPA or o,p'-DDT), the effects of individual xenoestrogens are too weak to create an impact on the actions of steroidal hormones; however, at mixture ratios more in favor of the xenoestrogens (1:20,000 and 1:100,000 for E2: BPA or o,p'-DDT), a significant contribution to the overall mixture effect was predicted and the predictions were tested experimentally. The researchers suggest that the assumption that weak xenoestrogens are generally unable to create an impact upon the already strong effects of endogenous steroidal estrogens (Safe, 1995) is not supported.

- **Synergism**

  When screened in a simple yeast estrogen system (YES) containing human estrogen receptor (hER), combinations of two weak environmental estrogens, such as dieldrin, endosulfan, or toxaphene, were 1000 times as potent in hER-mediated transactivation as any chemical alone. Hydroxylated PCBs shown previously to synergistically alter sexual development in turtles also synergized in the YES (Arnold et al., 1996). After this report, at least five teams using the same chemicals in 10 standard endocrine test systems to look for the synergy and only additive in every case (Kaiser, 1997), for instances, estrogenic activity of a dieldrin / toxaphene mixture in the mouse uterus, MCF-7 human breast cancer cells, and yeast-based estrogen receptor assays (Ramamoorthy et al., 1997). Other kinds of EDCs, from the representative alkylphenols and phthalates, the pesticides dieldrin and toxaphene, to the mycoestrogen zearalenone and the phytoestrogen genistein, interacted with three major teleost steroid-binding sites (estradiol receptor (ER), testosterone receptor (TR) and cortisol receptor (CR)) was evaluated. These compounds are exclusively estrogenic in rainbow trout, albeit weakly so, and do not display any synergistic effects (Knudsen et al., 1999). However, curcumin and genistein, which inhibit the growth of estrogen-positive human breast MCF-7 cells induced individually or by a mixture of the pesticides endosulfane, DDT and chlorease or 17-beta estradiol when present at micromolar concentrations, can synergistically inhibit the induction was noted (Verma et al., 1997). Although most of the negative results in endocrine disruption toxicity, synergism can be found in other toxic tests or exposure reports, such as induced EROD activities (Borlakoglu et al., 1992; Basu et al., 2001). There are also report might refer the synergistic effects by testing manufactured gas plant-PAHs mixture *in vitro* test (Chaloupka et al., 1993) and by epidemiological investigating of combined exposure of high environmental tobacco smoke (plasma cotinine) and PAHs (using BaP-DNA adducts as a molecular dosimeter) (Perera et al., 2004).

- **Antagonism**

  A recent report (Rajapakse et al., 2004) on combined effect of 17 β-estradiol, 17α-ethinylestradiol, genistein, bisphenol A, 4-nonylphenol, and 4- *tert*-octylphenol using MCF-7 human breast cancer cells by E-SCREEN fell short of the additivity expectations because of the weak antagonism when the presence of 4-nonylphenol

and 4- tertoctylphenol in mixture. It implies that, in sometimes, some interactions might compromise the predictability of estrogenic combination effects. Due to the inappropriate of the simple addition activities, methodology, it is suggested that isobole analysis is only suitable for 2- or 3-component mixtures, and concentration addition requires access to dose response data and EC50 values for the individual components of the mixture (Tinwell et al. 2004).

☑ Time trend, geographical variation, susceptible groups

The first evolution of human milk for C-POPs/EDCs contamination occur in 1951 when 32 non-occupationally exposed black women were surveyed in Washington, DC, 30 of whom had detectable levels of DDT with a mean value of 0.13 ppm (Laug et al., 1951). After more than a decade, many reports have been published on the levels of C-POPs/EDCs in breast milk. DDT concentrations in human milk have declined in most area of the world, from 5000-10 000 μg DDT/kg milk fat to around 1000 in 1999 in many areas. Nevertheless, levels can be high in areas still using DDT, ever higher than the WHO's recommended limit for infants (Smith, 1999). Also there is a declined trend for other C-POPs/EDCs such as HCH, dieldrin, CHL and relative compounds, and PCBs because of the ban or regulating throughout many global areas (Smith, 1999; Waliszewski et al., 1998; Noren et al., 2000; Solomon et al., 2002). Comparing the change of *p, p'*-DDE by reduced consumption of local fish and elevations of mirex from 1986-1992, HeCB showed no difference at any point due to the ban in Mohawk women milk (Fitzgerald et al. 2001), which might confirm its continuous release as industry by-products (van Birgelen, 1998). Although a decline of the time trend of C-POPs/EDCs in human samples, the exposure levels still can be detected easily because of their persistence.

The level of total DDT had decrease much from 1570 (N = 49) or 2320 (no sample size) in 1974 (Smith 1999), 699 (N = 50, recalculated using lipid data 4.5% g/g wet weight) in 1982 (Nasir et al., 1998), 570 (N = 165) in 1985 (Mussalo-Rauhamaa et al, 1988), and to the present data 84.06 ng/g lipid (N =65). In Denmark milk samples (N = 57), total DDT was 1150 total HCH 80, and dieldrin 40 ng/g lipid in 1982 (Nasir et al., 1998) (Table 3-2-1 (2)). The reported β-HCH, HeCB, dieldrin, p,p'-DDT, p,p'-DDE and total DDT were 116.43, 282.76, 33.10, 1123.69, 143.02, 1373.78, 1526.30 ng/g (recalculated the mean values of two time measurements) in serum of samples from 1976-1978 (Hoyer et

al. 2000). Considering the possible sample specific difference (Table 3-2-1 (3)), the body burden of the DDT of Finland people could be lower than Denmark people at the beginning of the ban and the following clearance periods. Apparently, all the other investigated PBTs/EDCs, could also decrease with the year because of the ban. The general level these OCs have decreased in first order rate in human samples (Smith, 1999; Noren et al., 2000; Solomon et al., 2002) from the ban.

It is suggested that maternal exposure is more crucial to children health. But the recent report referred paternal exposures as potential factor of cryptorchidism (Pierik et al., 2004). Also based on self-reported parental pesticide application, an increased risk of cancer was supported among children whose fathers did not use chemically resistant gloves compared with children whose fathers used gloves (Flower et al., 2004).

Many paediatric diseases have been suspected with the environmental factors. Children exposed transplacentally to PCBs' levels considered to be background have hypotonia and hyporeflexia at birth, delay in psychomotor development at 6 and 12 months, and poorer visual recognition memory at 7 months in the USA (Tilson et al., 1990). The general PCB exposure, measured by both contaminated fish consumption and cord serum PCB levels examined during the immediate postpartum period, had been shown can predicate lower birth weight and smaller head circumference (Fein et al., 1984). However, the other report (Gladen et al., 2004) showed the exposure to p,p'-DDE correlated with the increase of height and weight in adolescent boys. The recent epidemiological risks based on biological measures (chemical analysis combined with bioassay) showed that the moderate exposure of polychlorinated aromatic hydrocarbons (PCB 138, 153, and 180 level in serum and dioxin-like compounds by chemically activated luciferase expression assay) might interfere with sexual maturation and in the long run adversely affect human reproduction (Den Hond et al., 2002). Case-control investigation of 117 male schoolchildren (10–19 years of age) lived in a more than 20 years endosulfan aerially sprayed region found that sexual maturity rating (scoring for development of pubic hair, testes, penis, and serum testosterone level) was negatively related to aerial exposure to endosulfan (using serum endosulfan levels) and serum LH levels were significantly positively related to serum endosulfan levels after controlling for age. Further they suggested that the prevalence of congenital abnormalities related to testicular descent among study and controls subjects was 5.1% and 1.1%, respectively, might (not significantly) correlate the long-term endosulfan exposure

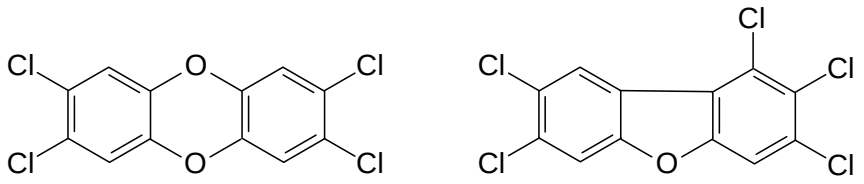
(Saiyed et al., 2003). Although only the concentration on lipid basis of cis-nonachlordane was significantly increased in testicular cancer cases, the *case mothers* showed significantly increased concentrations of the sum of 38 PCB congeners, HeCB, trans- and cis-nonachlordane, and the sum of CHLs (61 cases 58 age-matched controls) (Hardell et al., 2003). Recent case-control study based on the interview information (pregnancy aspects and personal characteristics, lifestyle, occupation, and dietary phyto-oestrogen intake of both parents) showed, apart from small-for-gestational age for hypospadias, and preterm birth for cryptorchidism, paternal pesticide exposure was significantly associated with cryptorchidism and paternal smoking was associated with hypospadias in male offspring (Pierik et al., 2004). However, to reach the dose-outcome relation of EDCs exposure with adverse effects like in lab (Ulrich et al., 2000) might need more detail information include multiple routes of exposure; the timing, frequency, and duration of exposure; need for qualitative and quantitative data; sample collection and storage protocols; and the selection and documentation of analytic methods (Rice et al., 2003).

10 DIOXINS

☑ General information

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two groups of tricyclic, chlorine-substituted, organic compounds that are generally identified by the name ”Dioxins”. For both groups of compounds, the 8 hydrogen atoms on positions 1-4 and 6-9 can be replaced by chlor-atoms, which results in a theoretical maximum of 75 PCDDs and 135 PCDFs. The chemical structure formula of two examples of congeners are shown in Figure 9.1:

Figure 9.1: Two examples of dioxin/furan congeners



2,3,7,8-TCDD 1,2,3,7,8-PCDF

Because dioxins are normally present only as complex mixtures of congeners, the concept of toxic equivalents (TEQs) has been introduced, where relative toxicities of the different congeners are expressed in relation to the toxicity of 2,3,7,8-TCDD (i.e. toxic equivalency factors, TEFs). The analytical data on concentrations of different congeners within a given sample can thus be converted to provide a TCDD toxic equivalency. Hence, 2,3,7,8-TCDD has a TEF value of 1, and the toxicity of all other dioxins and furans is expressed relative to this value. There are a number of TEFs available, with the WHO and International systems (resp. WHO-TEF and I-TEF) predominantly used. There is a general move in the EU towards using the WHO-TEFs, though research on air emissions still uses I_TEQ. In June 2005 a WHO-IPCS expert meeting was held in Geneva during which the toxic equivalency factors (TEFs) for dioxin like compounds, including some polychlorinated biphenyls (PCBs), were re-evaluated. For this re-evaluation process the refined TEF database recently published by Haws and coworkers (2006) was used as a starting point. Decisions about a TEF value were made based on a combination of unweighted relative effect potency (REP) distributions from this database, expert judgement and point estimates.

Previous TEFs were assigned in increments of 0.01, 0.05, 0.1, etc., but for this re-evaluation it was decided to use half order of magnitude increments on a logarithmic scale of 0.03, 0.1, 0.3 etc. Changes were decided by the expert panel for 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (TEF=0.3), 1,2,3,7,8-pentachlorodibenzofuran (PeCDF) (TEF=0.03), octachlorodibenzo-p-dioxin (OCDD) and octachlorodibenzofuran (OCDF) (TEFs=0.0003), 3,4,4',5-tetrachlorbiphenyl (PCB 81) (TEF=0.0003), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) (TEF=0.03) and a single TEF value (0.00003) for all relevant mono-*ortho* substituted PCBs.

Additivity, an important prerequisite of the TEF concept was again confirmed by results from recent *in vivo* mixture studies. Some experimental evidence shows that nondioxin-like aryl hydrocarbon receptor (AhR) agonists/antagonists are able to impact the overall toxic potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds and this needs to be investigated further.

Certain individual and groups of compounds were identified for possible future inclusion in the TEF concept, including 3,4,4'-TCB (PCB 37), polybrominated dibenzo-*p*-dioxins (PBDDs) and dibenzofurans (PBDFs), mixed polyhalogenated dibenzo-*p*-dioxins and dibenzofurans, polyhalogenated naphthalenes and polybrominated biphenyls (PBBs). Concern was expressed about direct application of the TEF/TEQ approach to abiotic matrices such as soil, sediment etc., for direct application in human risk assessment. This is problematic, as the present TEF scheme and TEQ methodology is primarily intended for estimating exposure and risks via oral ingestion (e.g., by dietary intake). A number of future approaches to determine alternative or additional TEFs were also identified. These included the use of a probabilistic methodology to determine TEFs that better describe the associated levels of uncertainty and 'systemic' TEFs for blood and adipose tissue and total toxic equivalency (TEQ) for body burden.

The criteria to include a compound in this TEF scheme are (WHO 2000):

- shows a structural relationship to PCDD/Fs;
- binds to the aryl hydrocarbon (Ah) receptor;
- elicits dioxin-specific biochemical and toxic responses;
- is persistent and accumulates in the food chain.

WHO-TEFs also include coplanar PCBs, the so-called dioxin-like PCBs. Yet these compounds are not taken into account in this report.

Table 9.1: Toxic equivalency factors (TEFs) for PCDDs and PCDFs

	Congener	***I-TEF***	***WHO-TEF***	***New TEF***
PCDD	2,3,7,8-tétraCDD	1	1	
	1,2,3,7,8-pentaCDD	0,5	1	
	1,2,3,4,7,8-hexaCDD	0,1	0,1	
	1,2,3,6,7,8-hexaCDD	0,1	0,1	
	1,2,3,7,8,9-hexaCDD	0,1	0,1	
	1,2,3,4,6,7,8-heptaCDD	0,01	0,01	
	OCDD	0,001	0,001	0.0003
PCDF	2,3,7,8-TCDF	0,1	0,1	
	1,2,3,7,8-pentaCDF	0,05	0,05	0.03
	2,3,4,7,8-pentaCDF	0,5	0,5	0.3
	1,2,3,4,7,8-hexaCDF	0,1	0,1	
	1,2,3,6,7,8-hexaCDF	0,1	0,1	
	1,2,3,7,8,9-hexaCDF	0,1	0,1	
	2,3,4,6,7,8-hexaCDF	0,1	0,1	
	1,2,3,4,6,7,8-heptaCDF	0,01	0,01	
	1,2,3,4,7,8,9-heptaCDF	0,01	0,01	
	OCDF	0,001	0,0001	0.0003
PCB	3,3',4',5-TCB (81)	-	0,0001	0.0003
non ortho	3,3',4,4'-TCB (77)	-	0,0001	
	3,3',4,4',5-PeCB (126)	-	0,1	
	3,3',4,4',5,5'-HxCB (169)	-	0,01	0.03
PCB	2,3,3',4,4'-PeCB (105)	-	0,0001	0.00003
mono-ortho	2,3,4,4',5-PeCB (114)	-	0,0005	"
	2,3',4,4',5-PeCB (118)	-	0,0001	"
	2',3,4,4',5-PeCB (123)	-	0,0001	"
	2,3,3',4,4',5-HxCB (156)	-	0,0005	"
	2,3,3',4,4',5'-HxCB (157)	-	0,0005	"
	2,3',4,4',5,5'-HxCB (167)	-	0,00001	"
	2,3,3',4,4',5,5'-HpCB (189)	-	0,0001	"

☑ Matrix

- **Invasive**

  Dioxins are generally analyzed in adipose tissue or blood (both whole blood and serum). Levels of dioxins are hghest in adipos tissue because of the high lipofilicity of dioxins and the slow turn-over rate of lipid-concentrations.

- **Non-invasive**

  Breast milk has been frequently monitored as an important and toxicologically relevant exposure route (Uehara et al 2006; Wang et al 2004). Also cord blood and placental tissue have been used, especially to highlight the possible correlations between pre- and postnatal exposure in mother-child cohorts (Schecter et al 1998; Wang et al 2004).

- **Comparison**

  When concentrations are expressed on a lipid basis, there are generally good correlations reported between whole blood, breast milk and adipose tissue levels, (Päpke 1998, Schuhmacher 1999, Wang et al 2004, Nakano et al 2005, OSPAR 2005). Good correspondence is observed when expressed in TEQ-values, lower correspondence when data are gathered at a congener-specific level. Because of its specificities, breast milk has both disadvantages (only females can be sampled during a specific time period, usually no recent occupational exposure) and advantages (toxicological relevance of mother-child transfer of dioxins). Placenta, breast milk and venous serum all offer excellent opportunities for biomonitoring mother-child transfer of PCDD/Fs. Cord blood has the negative aspect that dioxin concentrations are generally low due to the low fat concentrations, and has a precarious sampling procedure, but features the advantage that a relatively large amount is present without invasive sampling of babies or mothers (Wang et al 2004).

☑ Kinetics

- **Uptake**
  - *Food:* It is generally agreed that food is the main uptake route of dioxins. Päpke (1998) estimated that for the normal population, more than 90% of the total daily intake derives from food. Since PCDDs and PCDFs are lipophilic and accumulate in the food chain, food of animal origin is the most important source. Several studies on dietary intake indicated a daily TEQ intake for an average

adult population of 0.8-1.2 pg I-TEQ/kg body weight (bw) per day in Germany (Fürst et al 1990, Grün et al 1995), 1.1-2.8 pg TEQ/kg bw/day in the Netherlands (Baars et al, 2004), 1.5 pg TEQ/kg bw/day[1] in Finland (Kiviranta et al 2004), 0.86 pg TEQ/kg bw/day[1] for Belgium (Focant et al 2002), 0.62-1.26 pg TEQ/kg bw/day[1] for Catalonia, Spain (Bascompta et al 2002; Llobet et al 2003), or 2.05 pg I-TEQ/Kg bw/day in France (Arfi et al 2001). These values are generally well within the TDI range of 1-4 pg TEQs/kg bw/day that was proposed by the WHO (Van Leeuwen et al 2000) or the total weekly intake values of 14 pg TEQ/kg bw and a provisional tolerable monthly intake of 70 pg TEQ/kg bw proposed by respectively the EU Scientific Committee on food and the FAO/WHO Expert Committee on food additives respectively (European Commission 2001; WHO 2002). Based on these data, there is genuine concern within the European commission that the dietary exposure to dioxins exceeds the TDI for at least a considerable part of the European population (EC, 2001). Since food is the determining factor in dioxin accumulation for non-occupationally exposed humans, it should not be surprising that diet can have a profound effect on dioxin levels. Svensson et al (1991) reported consistent correlations between the amount of fish eaten and plasma levels of several PCDD/Fs. Also high consumption of meat and dairy products increases the level of dioxin intake (Kiviranta et al 2004). However, Welge et al (1993) did not observe lower mean PCDD/F concentrations in vegetarians compared to non-vegetarians, which highlights the complexity of dioxin accumulation through dietary exposure.

- *Mother's milk:* Because breast milk is rich in fat and dioxins are highly lipophilic, uptake through mother's milk in nursed infants is of high importance. Päpke (1998) estimates that the median daily intake of a nursed baby is 77 pg I-TEQ/kg bw for a 5 kg baby, which about 50 times higher than the average daily PCDD/F intake for an adult, and also Wang et al (2004) estimated that babies had a mean daily intake that was 10-38 times higher than the WHO tolerable daily intake. It is also documented that breast milk samples of secundiparas contain only 61% of PCDD/Fs of those taken from primiparas (Uehara et al 2006).

[1] Assuming an average body weight of 76 kg

- *Drinking water:* In Japan, average PCDD and PCDF levels of respectively 0.002 and 0.016 pg WHO-TEQ/l were reported in drinking water. Most congeners were efficiently removed by water treatment, but levels of TeCDFs were shown to increase slightly as a result of clorination (Kim et al 2002). Maystrenko et al (1997) reported PCDD/F concentrations in drinking water in the Republic of Bashkortostan below 5 ppq, with most concentrations for different congeners below 1 ppq. Concentrations increase with chlorination. WHO concluded from the sparse data available in literature that intake of PCDD/Fs through drinking water is negligible (WHO 1992).
- *Air:* Dioxins and furans are mainly available in air through the high enrichment on respirable dustparticles which have a very high relative pulmonary bioavailability (Nessel et al 1992). Measurements of PCDD/Fs in the UK (Clayton et al 1993), Germany (Liebl et al 1993) or Slovakia (Stenhouse et al 1998) reported values that were generally below 0.15 pg I-TEQ/m³. Also OSPAR reported background levels in rural and unpolluted areas range from 0.01-0.05 pg/I-TEQ/m³, while concentrations of 0.3 pg I-TEQ/m³ or higher are indicative for local emission sources (OSPAR 2005). On the basis of ambient air levels from Germany, WHO concluded that the intake of PCDD/Fs via air is low, amounting to 4 pg TEQs/person/day. It was noted that exposure could be substantially higher in indoor air in situations where e.g. wood had been preserved using pentachlorophenol or PCB-treated coatings, up to 1-3 pg TEQ/m³ (WHO/EURO 2000, OSPAR 2005).

- **Metabolism**

  Dioxins, especially those with four and more chlorine atoms in the lateral (2, 3, 7 and 8) positions are generally very resistant to chemical and biological degradation. However, partitioning of PCDD/Fs in the body is not passive in lipid, and one cannot correctly extrapolate from blood or adipose tissue to body burden (Schlecter et al 1998). Based on the reports by WHO (1998,), the PCDD/F body burden at steady state can be calculated as follows:

$$\text{Body burden at steady state} = f * \text{intake} * \text{half life} / \ln 2$$

With: Body burden: in ng/kg bw
Intake: in ng/kg bw/day
Half life: ± 7.5 years or 2737.5 days (for TCDD)
F: absorbed fraction (50% for TCDD)

A physiologically-based pharmacokinetic model (PBPK) for the calculation of human toxicity thresholds of 2,3,7,8-TCDD has been developed by Zeilmaker and Van Eijckeren (1998), which was further extended and validated by Zeilmaker et al (1999) . Also other modeling approaches have been developed to describe dioxin pharmacokinetics and are still being refined ((Buckley 1995, Maruyama et al 2004, Emond et al 2005). PBPK-models have extensively been validated and may at least partially be used to explain differences in tissue concentrations among individuals.

- **Biological half-life**

Due to the slow metabolism of PCDD/Fs, it is difficult to gather detailed information on the biological half-life of different congeners. Compared to both monkeys and rats (which both have half-lives measured in days), humans have reported half-lives in years (Table x.x):

Table 9.2: Half-lives ($t_{1/2}$) of PCDDs in rats (in days) and adult humans (in years) (data taken from Geyer et al 2002), PCDFs (Masuda 2001) or both through occupational exposure (Flesch-Janys et al 1997).

Congener	Rat $t_{1/2}$ (days)	Human $t_{1/2}$ (years)	Occupationally exposed (years)
TCDD	18.7	5.8-9.7	7.2
PeCDD	30.9	12.6-15.7	15.7
HxCDD	110	8.4-45	4.9-13.1
HpCDD	251	25-102	3.7
OCDD	322	6.7-132	6.7
TCDF			7.2
PeCDF		6.1	15.7-19.6
HxCDF		3.9	5.8-6.2
HpCDF		3.5	3-3.2
OCDF			6.7

An important uncertainty to estimate human kinetics of PCDD/Fs is the time- and concentration dependency of the biological half-life. Masuda reported increasing half-lives with time-to-exposure for PCDFs (see table x.x), and toxicokinetic elimination modelling illustrated that elimination is concentration dependent, with half-lives for TCDD of less than 3 years at 100.000 ppt to over 10 years at serum lipid levels below 50 ppt (Aylward et al 2005).

One toxicologically relevant pathway for PCDD/F elimination from the body is through mother milk in breast feeding females. It has been observed that pre-delivery maternal blood levels and TEQs are higher than postpartum blood levels (Schlecter et al 1998).

☑ Sampling conditions

- **Operational aspects**
  - Adipose tissue: samples should be stored at -20°C or lower in the dark, should be extracted within 30 days and completely analyzed within 45 days of collection.
  - Blood: after clotting, blood samples are centrifuged at 3000 G for 15 minutes and the serum is stored frozen at -20°C or lower. Although the standard for preserving samples is freezing, Schecter et al (2004) have described a potentially more convenient, practical and cost-effective method using potassium dichromate.
  - Breast milk: samples are gathered using standard, commercially available breast pumps. Samples are stored at -20°C until analysed.

☑ Analytical aspects

- **Techniques**
  - Chemical-analytical: Internationally accepted methods generally use high resolution gas chromatography and high resolution mass spectrometry (HRCG/HSMS). Step-by-step guidance on extraction, purification, cleanup and measurement of dioxins in adipose tissue is presented in detail by Method 8290A, chapter 7 (USEPA, 1998).

    Recently, the DIAC and DIFFERENCE projects aimed at developing new and improved methods using GCxGC with Electron Capture Detection (ECD) as an alternative for the GC-HRMS method (DIAC project), while alternative

biochemical and analytical detection methods (e.g. Calux and LRMS) were studied in the DIFFERENCE project (Van Loco et al 2004).

- Bio-analytical: In the last decade, biotechnological advances have promoted the development of a battery of in vitro bioassays and ligand binding assays for dioxins (See Behnisch et al 2001 for a full review on pro's and cons). Although it is unlikely that these bio-analytical screening methods will fully replace chemical-analytical methods, they can be extremely useful as a rapid, sensitive and relevant addition to the chemical measurements (WHO 2003).

- **Sensitivity and specificity**

  The sensitivity of the methods depend upon the level of interference within the matrix, but for a 10 g human adipose tissue sample USEPA (1998) reports a calibration reach of 1 to 200 parts per trillion (ppt) for TCDF/PeCDD/PeCDF, 2.5 to 500 ppt for HxCDD/HxCDF/HPCDD/HpCDF and 5 to 1000 ppt for OCDD/OCDF. Van De Weghe and Vanermen (2006) indicate that for milk and serum, levels of detection for PCDD/Fs are 0.05 pg/g lipid for each congener.

- **Units**

  Since dioxins are extremely lipophilic, the concentrations of individual congeners are generally expressed as a weight unit per gram of fat (usually pg/g fat). Because dioxins are usually present as complex mixtures and are also reported as such, measurement data on individual congeners are generally combined using the TEQ values (see higher), so data are expressed as pg TEQ/g fat.

☑ Performance characteristics

- **Analytical reproducibility**
- **Inter- and intra-laboratory variability**

  To assure the reliability of analytical data and to assess inter-laboratory variability of dioxin measurements, WHO/EURO has been coordinating inter-laboratory quality assessment studies on levels of PCDDs and PCDFs in breast milk and blood.:

Time	Matrix	# labs participating	Reference
1988-1989	Human milk and blood	19	WHO (1991)
1991-1992	Human milk and blood, cow's milk and fish	30	WHO (1995)
1996-1997	Human milk and blood plasma	24	WHO (2000)

Also the Norwegian Institute of Public Health performs annual inter-laboratory comparison exercises on the determination of PCDD/Fs. They concluded that there is generally a good inter-laboratory comparability for TEQ-values at higher contaminant levels, with a relative standard deviation of 13 % in breast milk, analyzed by 52 laboratories from 24 countries worldwide. However, variability for individual congeners was considerably higher (Becher et al 2001). The EU has established that a trueness of 20 % is required for the analytical procedures between half and two times the maximum level (Becher et al 2004)

☑ Validation

Analytical methods have been validated by different agencies: no specific guidelines for human tissues are available, however there are validated and internationally accepted methods for measuring PCDD/Fs in food, animal tissues and fodder.

Authority	Method
U.S. Environmental Protection Agency (1994)	Method 1613: Tetra- through Octa-chlorinated dioxins and furans by isotope dilution HRBC/HRMS.
U.S. Environmental Protection Agency (1998)	Method 8290A: Polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by high-resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS)
European Commission	Guidelines for dioxin sampling in food and fodder (Guidelines 2002/69/EC and 2002/70/EC, PB L 209, 6.8.2002, p 5-21)

☑ Confounding factors

- **Age**

  The influence of age on the PCDD/F body burden has been reported to increase between 0.4 and 0.8 pg I-TEQ/year of age (Päpke 1998;). In a large-scale study on the dioxin content of human milk in Japan, Uehara et al (2006) reported a positive correlation between primiparas age and dioxin content of breast milk, but no significant correlation for secundiparas.

- **Socio-economic situation**

  Nadal et al (2004) showed that contrary to their initial hypotheses, subjects with a higher socioeconomic level are more exposed to PCDD/Fs due to higher uptake through food.

- **Body mass index (BMI)**

  Although the effect of BMI as a confounding factor is under discussion, many papers adjust for this. Tsukino et al (2006) found no significant correlation (p = 0.22) between mean BMI and PCDD/Fs among Japanese women, nor did Falk et al (1999) for frequent consumers of Great Lakes sport fish. However, many models include BMI to account for body fatness (Salvan et al 2001, Kamagai et al 2000). Potentially more important than BMI alone can be the change in weight. More specifically, periods of significant weight loss have been documented to lead to a strong increase in the serum concentrations of different congeners;

- **Gender**

  Several authors presented data showing that men generally have lower PCDD/Fs than women. Chen et al (2005) discussed that this might be due to differences in food pattern, where Taiwanese women eat more diary products, fish and fruit and vegetables than men, and thus have a higher intake, in combination with a greater percentage of body fat. Also Schuhmacher et al (1999) and Landi et al (1998) found higher concentrations in respectively Spanish and Italian women. Arisawa et al (2005) on the other hand did not find gender-related effects in Japan, while Falk et al (1999) found significantly higher levels in males. The complexity of gender as a confounder is illustrated by Fierens et al (2005), who found slightly higher PCDD/F levels in females, though these last authors noted a gender dependent effect of dioxins in smokers, where male smokers show increased dioxin levels, while female smokers have significantly decreased dioxin levels compared to non-smokers.

☑ Concentrations reported in literature

- **Reference values**

Table 9.3: data on dioxin-levels in different matrices in Europe (data from ELICC 2004)

Country	Area	Tissue	Year	N° of samples	Median PCDD/Fs (TEQ pg/g fat)
Austria	Vienna (urban)	Human milk	1996	13	10.7
	Tulln (rural)	Human milk	1996	21	10.9
	Brixlegg (industrial)	Human milk	1996	13	14.0
Belgium	Brabant Walon	Human milk	1996	8	20.8
	Liege	Human milk	1996	20	27.1

Country	Area	Tissue	Year	N° of samples	Median PCDD/Fs (TEQ pg/g fat)
	Brussels	Human milk	1996	6	26.6
	Flanders	Blood plasma	1998	341	22.8
	Flanders (rural)	Blood serum	1999	22	47.9
	Flanders (urban)	Blood serum	1999	25	49.2
Czech Republic	Kladno	Human milk	1996	11	12.1
	Uherske Hradiste	Human milk	1996	11	18.4
	-	Adipose tissue	1996-99	61	98.0
	-	Adipose tissue	2001	29	21.8
	Ostrava	Adipose tissue	1997	12	32.3 (mean)
Denmark	Urban	Human milk	1996	48	15.2
Estonia	-	Human milk	1991	-	13.5 - 21.4 [a]
	Urban	Human milk	1993	-	14.4
	Rural	Human milk	1993	-	12.4
Finland	Helsinki	Human milk	1996	10	21.5
	Kuopio	Human milk	1996	24	12
	-	Human milk	1987	-	16.0 - 17.9 [a]
	-	Blood	2000		33.0
France	National	Milk	1988	244	18.8
Germany	Berlin (urban)	Human milk	1996	10	16.6
	-	Adipose tissue	1996		16.5
	-	Blood	1996		15.6
	-	Blood	1992	85	49
	-	Adipose tissue	1992	4	79
Hungary	Budapest	Human milk	1996	20	8.5
	Scentes	Human milk	1996	10	7.8
Lithuania	Palaanga (coastal)	Human milk	1996	12	16.6
	Anykshchiai (rural)	Human milk	1996	12	14.4
	Vilnius (urban)	Human milk	1996	12	13.3
Norway	-	Human milk	1987		14.9 - 20.4 [a]
Slovak Republic	Michalovce	Human milk	1996	10	15.2
	Nitra	Human milk	1996	10	12.6
	Occupational exposure	Blood	1995-97	7	24.5 – 61.3

Country	Area	Tissue	Year	N° of samples	Median PCDD/Fs (TEQ pg/g fat)
Spain	Bizkaia	Human milk	1996	19	19.4
	Gipuzkoa	Human milk	1996	10	25.5
		Adipose tissue	1996		31.0
	Occupational exposure	Blood	1999		27
	-	Adipose tissue	1993	17	46
Sweden	-	Human milk	1987	-	20.8-23.8 [a]
		Adipose tissue	1994		18.6
The Netherlands	-	Human milk	1996	17	22.5
United Kingdom	Birmingham	Human milk	1996	20	17.9
	Glasgow	Human milk	1996	23	15.2

[a] concentrations in Nordic TEQ values

Table 9.4: Dioxin levels in human milk reported in the Third round of the WHO-coordinated exposure study (Van Leeuwen and Malisch 2002)

Country	Number of pools	PCDD/Fs (WHO-TEQ pg/g fat, median)	
		Median	range
Australia	2	5.57	5.39 – 5.75
Belgium	2	16.92	14.78 – 19.07
Brazil	11	3.92	2.73 – 5.34
Bulgaria	3	6.14	5.08 – 7.11
Croatia	2	6.4	5.99 – 6.80
Czech Republic	3	7.78	7.44 – 10.73
Egypt	9	22.3	14.90 – 51.50
Fiji	2	3.34	3.17 – 3.51
Finland	2	9.44	9.35 – 9.52
Germany	4	12.53	11.14 – 12.72
Hong Kong SAR	11	8.69	5.80 – 10.09
Hungary	3	6.79	5.26 – 7.46
Ireland	4	7.72	6.19 – 8.82
Italy	4	12.66	9.40 – 14.83
Luxembourg	2	14.97	13.68 – 16.25

Country	Number of pools	PCDD/Fs (WHO-TEQ pg/g fat, median)	
		Median	range
New Zealand	3	6.86	6.08 – 7.00
Norway	2	7.30	7.16 – 7.43
Philippines	2	3.94	3.64 – 4.24
Romania	3	8.86	8.37 – 12.00
Russia	7	9.36	7.16 – 12.93
Slovak Republic	4	9.07	7.84 – 9.87
Spain	6	11.56	10.24 – 18.68
Sweden	1	9.58	
The Netherlands	3	18.27	17.09 – 21.29
Ukraine	3	10.04	8.38 – 10.16
USA	2	7.18	6.22 – 8.14

- **Critical values**

  Critical values for TCDD/Fs have been established based on TDIs. WHO proposed a TDI of 1-4 pg TEQs/kg bw/day (Van Leeuwen et al 2000) while weekly values of 14 pg TEQ/kg bw and provisional monthly values of 70 pg TEQ/kg bw were proposed by resp the Scientific Commission on Food of the EU and the Joint FAO/WHO Expert Committee on food additives (European Commission 2001; WHO 2002).

- **Normal range in populations (including power calculations)**

☑ Dose –response/effect relationships

There is a vast amount of evidence that TCDD, the most potent dioxin congener, is a human carcinogen. TCDD has been classified as such by the International Agency for Research on Cancer (IARC, 1997), and recent evidence strengthens this classification (Steenland et al 2004). Moreover, there is strong evidence that this classification is valid for both acute, short term exposure like the Seveso Incident (Warner et al 2002, Steenland et al 2004) and long term, chronic exposure such as occupational exposure (Crump et al 2003, Steenland and Deddens 2003). Crump et at (2003) concluded that the available dose-response assessments for dioxin and cancer indicate that dioxin TEQ exposures within roughly 3-fold of current background levels may be carcinogenic. There appears to be a general increase in all types of cancer, which lead Steenland and Deddens (2003) to postulate that TCDD might be the first true all-site human

carcinogen. There appears to be some controversy on the fact whether there is evidence of a dioxin cancer treshold. While Kirman et al (2000) and Hayes et al (2001) reported a threshold of about 60 ng/kg bw (which is well above the estimated average background exposure of 3-5 ng/kg), Mackie et al (2003) concluded there is no such limit.

Following-up of a number of birth cohorts in the US, The Netherlands, Japan and Taiwan have highlighted that (neuro)developmental delays (Huisman et al 1995), chloracne (Baccarelli et al 2005), alteration of thyroid hormone status (TT4, TSH) (Pluim et al 1993), decreased lung function (ten Tusscher and Koppe 2004), and other effects could be related to in utero exposure to total TEQs (WHO 1998). Finally in the Seveso studies, a change in sex ratio towards females was observed in children born to parents highly exposed to TDCC (Mocarelli 2001).

Moreover, advances in the understanding of mechanisms of action of dioxins concerning the AhR, strengthen the evidence that molecular mechanisms occurring downstream of AHR are associated with cancer development such as changes in cytosolic signaling proteins, calcium mobilization, tumor suppressor proteins, oncogenes,... (Steenland et al 2004).

☑ Time trend, geographical variation, susceptible groups

- **Time trend**

  Exposure assessments within the past 20 years show that PCDD/F levels in breast milk are showing a continual decline, in some countries up to 50 % (Brouwer et al 1998; ELICC 2004). This is confirmed by the investigation of sediment cores, that have shown that dioxin deposition dramatically increased until the 1970ties, and halved again by 2000 (Kjeller et al 1991, Baker and Hites 2000).

- **Geographical variation**

  Globally, there is considerable geographical variation in measured PCDD/F concentrations in human tissue. The most wide-spread monitoring program (the WHO human milk program) indicates that concentrations are lowest in the Southern hemisphere, with lowest values in Fiji and the Philippines (respectively 3.34 and 3.94 pg TEQ/g fat). Values in Europe, especially in Western Europe, are among the highest in the World (< 10 pg TEQ/g fat), with Eastern European countries somewhat in between these two extremes.

- **Susceptible groups**

  Babies and people depending on fish for a large portion of their diet.

11 DISINFECTION BY-PRODUCTS (TRIHALOMETHANES & TRICHLOROACETIC ACID)

☑ Biomarker description

Trihalomethanes (THMs) and trichloroacetic acid (TCAA) may be used to measure human exposure to drinking water disinfection by-products.

☑ General information

The treatment of both drinking water and water for recreational use such as swimming represents a major advancement in the control of water-borne infectious diseases (Arbuckle et al. 2002).

Drinking water disinfection by-products (DBPs) are formed through reactions between residual chlorine (and other halogen compounds) and natural organic materials such as fulvic and humic acids in the water. In the case of chemical disinfection using chlorine, a number of DBPs may be formed including trihalomethanes (THMs) and non-volatile haloacetic acids (HAAs), which are the two most abundant (Kuklenyik et al. 2002, Bader et al. 2004). THMs include the four primary species chloroform ($CHCl_3$), bromodichloromethane (BDCM – $CHBrCl_2$), dibromochloromethane (DBCM – $CHBr_2Cl$), and bromoform ($CHBr_3$) (Nuckols et al. 2005). The five most common HAAs include monochloroacetic acid (MCAA – $CH_2ClCOOH$), dichloroacetic acid (DCAA – $CHCl_2COOH$), trichloroacetic acid (TCAA – CCl_3COOH), monobromoacetic acid (MBAA – $CH_2BrCOOH$) and dibromoacetic acid (DBAA – $CHBr_2COOH$).

Since the discovery of these disinfection by-products in the 1970s, studies in the fields of epidemiology and toxicology have produced evidence to suggest that exposure to the chemicals may be linked to cancers and other disease outcomes. One of the criticisms made of such studies is that quantification of human exposure to DBPs is inadequate.

Human exposure has often been estimated through the use of indices such as type of water source; type of disinfection method; total concentrations of THMs or HAAs or their component species in water (Arbuckle et al. 2002). However, exposure to DBPs occurs through multiple routes and varies considerably across any population due, in part, to differences in the ways in which water is used and its quantity. Common water uses associated with DBP exposure include consumption of food, drinks and drinking water (ingestion exposure); washing, bathing, and showering (dermal and inhalation

exposures); swimming (dermal, inhalation and – particularly in children - ingestion exposures); dishwashers, washing machines and humidifiers (inhalation exposure) (Weisel et al. 1999; Nieuwenhuijsen et al. 2000; Wallace 1997; Gordon et al. 2005; Aggazzotti 1995).

Owing to the problems associated with assessing external exposure, measuring internal exposure using biomarkers has drawn considerable attention in recent years. According to Arbuckle et al. (2002), ideal biomarkers should be:

- sensitive and specific to the exposure of interest;
- readily accessible;
- inexpensive to measure;
- technically feasible to measure;
- have an elimination half-life appropriate to the exposure window of interest;
- be indicative of duration, intensity, and pattern of exposure; and
- be consistently and quantitatively related to exposure.

THMs behave as volatile chemicals, whereas HAAs (including TCAA) are classified as non-volatile. This difference in volatility has implications not only for their behaviour in exposure or the choice of matrix in which they can be sampled, but also on their biological half-lives.

☑ Matrix

According to Froese et al. (2002), the only DBPs evaluated for use as biomarkers of exposure in humans have been THMs and HAAs. To date most work has concentrated on developing biomarkers for THMs in blood and exhaled breath and HAAs in urine (Arbuckle et al. 2002).

As mentioned above, the different volatilities, biological half-lives and biochemical properties of these compounds result in different behaviour in each matrix in the human body. This has implications for the potential for use of the biomarkers in each of these matrices. There has also been a general preference for using THM in exhaled alveolar air or blood as biomarkers of DBP exposure (Nieuwenhuijsen et al. 2000). Increasingly levels of HAAs in urine are measured for this purpose.

For example, most determinations of uptake of THMs by dermal absorption, inhalation, or ingestion resulting from showering, bathing, or drinking water have been based on

measurements of exhaled breath. Rather fewer studies have focused on measurement of THMs in venous blood (Gordon et al. 2005).

- **Invasive**

  Blood: Whole blood sampling is the most common invasive method of testing for THMs. THMs in blood have been demonstrated as a feasible means of evaluating background THM exposure (Froese et al. 2002). The drawback of this method is the degree of complication that arises with the use of invasive methods in terms of cost, practicality etc.

  When sampling for THM concentrations in the blood of subjects exposed to THMs under controlled conditions (including ingestion, showering and bathing), Backer et al. (2000) showed that levels of three measurable THMs increased significantly as a result of showering of bathing, but only very slightly upon drinking tap water. The relatively rapid hepatic metabolism of ingested THMs is responsible for lowering the blood concentrations of THMs absorbed in the gut. Miles et al. (2002) studied correlations between the levels of THMs in tap water and in blood. In general, the correlation was found to be weak.' (Polkowska et al. 2003)

- **Non-invasive**

  - *Urine:* The potential of TCAA in urine to act as a biomarker of DBP exposure from ingestion has been proven by studies carried out, although it has been found necessary to adjust for volume of water consumed, the temperature of the water and the use of home filters (Froese et al. 2002).

    The short biological half-life of THMs makes urine an unsuitable matrix in which to measure them. The longer half-life of TCAA makes it a more suitable candidate biomarker, particularly of chronic ingestion exposure to HAAs from chlorinated drinking water (Calafat et al. 2002). Cammann and Huebner (1995) examined THM levels in blood and urine of swimmers and employees of an indoor swimming pool. They concluded that at least 3–7% of the THMs were secreted via the urine. (Polkowska et al. 2003) In addition, urinary trichloroacetic acid excretion rates were found to correlate well with ingestion exposure. (Polkowska et al. 2003) TCAA in urine was sufficiently stable to allow monitoring in this study with rapid turnaround of analyses. Urinary sampling for TCAA is relatively noninvasive, particularly when FMU samples are used. Although this study has established that monitoring urinary TCAA is technically

feasible, analytical costs and analytical resources would likely be a serious constraint for a larger scale study. Despite several qualifiers, TCAA remains the most promising prospect for a biomarker of ingestion exposure to DBPs in drinking water. (Froese 2002)

- *Exhaled alveolar air:* The non-invasive nature, rapidity of collection and potential for continuous real-time monitoring makes exhaled alveolar air a particularly advantageous means of gathering samples of volatile organic compound (VOC) biomarkers (Gordon et al. 1995; Linstrom et al. 2002). The concentration of VOCs such as THMs in the blood also exist in dynamic equilibrium with concentrations in exhaled breath (Wallace et al. 1996), which allows the estimation of body burden and changes of body burden with time (Gordon et al. 1995).

  While exhaled alveolar air can indicate exposure to THMs following exposure to high concentrations during swimming or showering, various studies have been unable to detect background levels attributable to chronic exposure from drinking water (Froese et al. 2002). One of the problems in using exhaled breath samples to measure exposure to DBPs is that THMs do not persist for longer than the minutes-hours timeframe. Another drawback is that THMs in breath only represent those THMs absorbed through the skin or inhalation routes, ingested THMs being metabolised in the liver.

  The lack of volatility of TCAA means that blood concentrations do not reach dynamic equilibrium with concentrations in alveolar air. As a result, measuring TCAA concentrations in exhaled air is not a good means of estimating TCAA exposure. Weisel et al. (1999) found strong correlations between the THM concentrations in breath after a shower and THM water concentrations. (Polkowska et al. 2003)

- **If different methods are available, compare these**

  The use of different methods depends on the source of exposure and the timescales of concern i.e. an alveolar air method is best suited to exposure to THMs from showering due to their high volatility and short half-lives, whereas TCAA in urine can give information on exposure to DBPs in drinking water.

☑ Kinetics

- **Uptake (by different routes)**

  There are a number of different routes of uptake of THMs and TCAA. These include ingestion, inhalation and dermal absorption. Studies have shown that ingestion of drinking water represents the highest source of exposure for two THMs (chloroform and bromodichloromethane), followed by the other two routes depending on the behaviour of individuals.

  A study conducted by the Total Exposure Assessment Methodology group (Wallace 1997) demonstrated that indoor residential air contributed 25-30% of the total air-tap-water daily intake of $CHCl_3$ and BDCM.

  Chloroform levels in breath have been correlated with concentrations in shower water and air and with time spent showering and water temperature (Jo et al. 1990; Weisel et al. 1999; Gordon et al. 1998; Gordon et al. 2005).

  - *Drinking water:* TCAA exposure is thought to be largely accounted for by ingestion of chlorinated drinking water, since its dermal and inhalation uptake is low (Calafat et al. 2002).
  - *Swimming pool water:* Routes of THM uptake from swimming pool water include inhalation of volatilised THMs into indoor air, ingestion of THMs and TCAA from the water (particularly important with children, who have been shown to ingest much larger quantities than adult swimmers), and dermal absorption from contact with the water. 'Chloroform is readily absorbed into the body mainly through the lungs and intestinal mucosa. However, when subjects take a bath or swim in chlorinated water, skin absorption may also be significant. In fact, the skin, the largest organ of the body, acts as a lipid sink for lipid-soluble contaminants.' (Aggazzotti et al. 1995). Aggazzotti et al. (1990, 1993) showed that chloroform values in plasma and alveolar air samples collected from swimmers after 90 min training are significantly related to the levels in water and in ambient air, the number of swimmers inside the pool, the intensity of swimming and the age of the swimmers, but not to the number of training hours during the week. L´evesque et al. (1994) studied dermal and inhalational chloroform exposure in swimmers using alveolar breath analysis. In agreement with Aggazzotti et al. (1993), they found that chloroform concentration in the surrounding air is correlated to the level in water and to the intensity of the sport

activity. Weisel and Shepard (1994), studying exhaled breath measurement to evaluate chloroform exposure in swimmers, suggested that inhalation is more important than dermal absorption in exposure deriving from recreational swimming. However, more recently, Lindstrom et al.(1997) estimated that the dermal exposure route may account for 80% of the blood chloroform concentration in swimmers during a typical 2-h training period. Cammann and Hubner (1995) confirmed that high levels of physical activity are correlated with high concentrations of THMs in blood and urine. They also found that the secretion of THMs in urine is less than 10%.' (Fantuzzi 2000)

- **Metabolism**
  - *THMs:* The pharmokinetics of absorbtion and excretion of THMs associated with low-rate exposure from ambient air and drinking water are not well understood. Existing studies demonstrate that absorption in the gut is a rapid and complete first-order passive process. Pulmonary uptake and subsequent elimination also occur by way of passive diffusion processes. THMs are eliminated from the body mainly through pulmonary elimination of unchanged chloroform as well as metabolism in the liver, kidneys and other tissues; this metabolic process is dependent on dose (lower dosages are metabolised more quickly) and saturable (Aggazzotti et al. 1995). Chloroform in the liver is largely metabolised by oxidative processes.

    Some studies have indicated that internal doses of THMs increase to a much greater extent from inhalation or dermal exposure compared with dose levels related to ingestion. Studies in the rat have shown that blood kinetics of trihalomethanes differ when THMs are administered as a single chemical or as a combination. Da Silva et al. (2000) found that binary mixtures of THMs caused significantly raised THM concentrations in blood and may indicate metabolic inhibition effects of mixtures as well as mutual metabolic interaction between individual THMs.
  - *TCAA*: TCAA is absorbed in the gastrointestinal tract following ingestion exposure in humans (Kim and Weisel, 1998; Schultz et al., 1999). The blood concentration of rats following oral ingestion showed a maximum concentration at approximately 2 hour post-dosing (Schultz et al., 1999).

No evidence of significant dermal absorption of TCAA has been observed in humans in vivo (Kim and Weisel, 1998).

Some TCAA is metabolized in the liver. Larson and Bull (1992) reported that the metabolic process involved was reductive dehalogenation to dichloroacetic acid. Following oral and intravenous administration in rats, TCA binds significantly to plasma proteins and is also distributed to the liver (Schultz et al., 1999). Because of the significant binding to plasma, only the free TCA is available to tissues for uptake and elimination (Yu et al., 2000).

A pharmacokinetic model developed by Allen and Fisher (1993) estimated that, in humans, 93% of total TCAA eliminated was excreted unchanged in the urine, while the remainder may be metabolized or eliminated by other routes, thus supporting the consensus that TCAA could serve as a good biomarker of DBP exposure in humans.

The high degree of urinary excretion, hepatic metabolism of remaining TCAA, and low degree of volatility of TCAA all indicate that, in contrast to THMs, it would make a poor exhaled breath biomarker of DBP exposure.

- **Biological half-life**

THMs have a very short biological half-life, only measured in minutes following exposure. They are detectable primarily in alveolar air and primarily indicate exposure through inhalation e.g. showering or swimming in an organohalogen-treated pool.

Aggazzotti et al. (2005) found that chloroform absorbed through swimming was cleared very rapidly after ending the activity, the process complete after about 10 hours.

TCAA has a longer biological half-life than THMs and may be measured in both urine and blood. The persistence of TCAA in blood and urine makes it a useful biomarker for drinking water exposure to TCAA. The urinary half-life is sufficiently long to integrate drinking water exposure over several days (Bader et al., 2004). In order to obtain a closer estimate of half-life it is necessary to adjust for creatinine Bader et al. (2004) found urinary elimination half-lives for TCAA of between 2.1 and 6.3 days in a study of five participants.

Froese (2002) notes the lack of research on the pharmcokinetics of TCAA excretion. Since TCAA may also be produced in the metabolism of chloral hydrate (CH) or

trichloroethane (TCE), research has tended to focus on occupational exposures of humans to these chemicals, and half-life estimations based on this data may differ greatly when compared to exposure to DBPs in drinking water. Quoting from several studies Froese et al. (2002) present a range of half-lives for TCAA (both as a metabolite of TCE and as a biomarker of exposure to TCAA) of 2.1 to 4.2 days, noting that the doses of exposure were up to four orders of magnitude greater than that experienced in drinking water exposure.

The greater half-life of urinary TCAA as compared to urinary THM biomarkers would suggeset that it can provide a much better estimate of longer-term exposure to DBPs. However, the experiments carried out by Froese et al. (2002) into the urinary excretion of TCAA raises doubts about its specificity as a marker of drinking water ingestion.

The biological half-lives (t l / 2) of chloroform are very short and range from 20 to 27 min; our data are not sufficient to link this variable to specific factors. (Aggazzotti et al. 1995).

☑ Sampling conditions

- **Blood**

  Blood sampling for DBP-related studies generally infers the sampling of venous blood by either venipuncture with hypodermic needle (for single samples) or venous catheter (for time-series samples or multisampling) carried out according to standard phlebotomic procedures:

  - Blood samples were collected in 18 sampling sessions. Questionnaire also used at session to establish: activity practised in the swimming pool; frequency of attendance and length of time spent at the swimming pool in the course of a week. Duration of the swimming session and the time elapsed between end of session and blood sampling noted. Data gathered regarding the possibility of exposure outside the swimming pool (e.g. occupational exposure or handling of solvents at home). (Aggazzotti et al., 1995)
  - Blood samples were collected into vacutainers with potassium-ethylenediaminetetraacetic acid (EDTA) as anticoagulant and then centrifuged. Plasmatic aliquot was transferred into borosilicate glass vials with hole caps sealed with Teflon-faced silicone-rubber septa. (Aggazzotti et al.,1998);

- Blood samples collected from each participant into vacutainers were prepared by heating, restoration of vacuum, and resterilization in order to eliminate background contamination from the blood collection device. We collected samples approximately 5 min before and after each activity, using a multisample adapter (venous catheter). Additional blood samples were collected 30 min after the shower and bath activities. The catheter remained in the participant for the duration of each day of the study, approximately 12 hr. We collected blood samples from each participant over the course of the 2-day study. After collection, each blood sample was refrigerated and packed into coolers with ice packs, and at the end of each day shipped by overnight express courier to be analysed. (Nuckols et al. 2005);
- Obtained two blood samples, just before and just after the participant's shower. THM levels in blood have a short half-life, so blood samples taken as early in the morning as possible (before water-use activities were initiated) to provide estimate of participant's baseline or steady-state blood levels (assumes no exposure while sleeping). Similarly, because inhalation of THMs while showering is one of the most important and significant routes of exposure, the samples that were taken as soon after showering as possible were considered an estimate of the peak blood THM level. (Lynberg et al. 2001);
- Each participant provided several blood samples over the course of study. Samples were taken approximately 5 min before and after each activity (and 30 min after the end of the shower and bath activities), using a venous catheter that remained in the subject's arm throughout each study day. Blood collection (Vacutainer) tubes were specially treated before use to remove background contamination. After collection, samples were refrigerated and packed into coolers with ice packs for shipping by overnight express courier. (Gordon et al. 2005)

o **Urine**

Urine samples tend to be collected by a similar method. The listing below gives some examples of methods used:

- Entire urine voids were collected in high-density polyethylene bottles. All samples were extracted within 24 hours of collection. (Bader et al. 2004)

- Urine samples were collected at different times throughout the day; not necessarily first-morning voids. Following shipment, samples were stored securely at –70°C and were not subjected to inadvertent thawing ensuring integrity of the samples. (Calafat et al. 2002)
- Participants brought entire FMU voids to the testing centre. If the participant anticipated a delay of more than 4 hours before the sample could be delivered to the lab, he or she stored the sample in a cooler with ice or in a refrigerator. Samples were extracted for analyses within 6 hours of collection. (Froese et al. 2002)
- Urine samples were collected from volunteers living in various parts of the TriCity. The part of the TriCity where the subjects lived during the study was noted (which, in turn, determined the quality of the drinking water they consumed at home), and the major sources of drinking water they consumed during the day (home, work, or both) were identified. Samples were collected in Erlenmeyer flasks equipped with ground glass stoppers. In most cases, samples were analyzed immediately after collection. In cases when samples were not analyzed within 5 min of collection, they were kept in closed flasks at 4°C. (Polkowska et al. 2003)
- Entire first morning urine voids were collected in polycarbonate or linear high-density polyethylene containers. Samples were stored in a refrigerator (4°C) until extraction, which was carried out within 48 hr of collection. (Weisel et al. 1999)

o **Exhaled alveolar air**

There are a variety of methods available for the collection of exhaled alveolar air. These are summarised below:

- Alveolar air samples were collected in one-way glass tubes with two valves. Subjects were asked to breathe normally into the tube with open valves. At the end of expiration, the valves were closed. In order to avoid problems with risk of contamination, septa and screw caps were washed in water, acetone, n-pentane, and then dried. Vials and all pieces of glassware were washed in water, acetone, n-pentane and then placed in a muffle furnace to purge any volatile contaminants. For analysis the tubes were heated to 37°C to recreate the conditions at the time of sampling. (Aggazzotti et al. 1998)

- Samples collected using a self-administered procedure in which subjects exhaled alveolar air directly into an evacuated single breath canister (SBC methodology developed by Pleil and Lindstrom 1995). Samples were made into 1L stainless steel canisters fitted with a short Teflon tube disposable mouthpiece. Subject began sample collection near the end of a normal resting tidal breath to provide mostly alveolar breath. Samples were shipped at the end of each day by overnight express courier for analysis. (Gordon et al. 2005)
- Alveolar air samples were taken according to the SBC method using a specially passivated, evacuated 1L stainless steel canister (with electropolished interior) fitted with a small Teflon tubing stub. At the end of a normal exhalation, with the dead-space (i.e. non-alveolar) portion of a breath eliminated, the subject placed the end of the Teflon collection tube in his or her mouth and opened the canister valve to fill the volume with 1 litre of the lungs' expiratory reserve. Because the canister is initially evacuated, the sample is collected until it comes to atmospheric pressure. The subject closes the sample valve and may resume normal activities. After collection, the samples were shipped back to the laboratory where they are inventoried and the absolute pressure of each sample is measured. Samples were then pressurized using laboratory-grade nitrogen gas and a dilution factor is calculated based on initial and final pressures. CO_2 levels were measured to ensure that initial sample was indeed alveolar air. (Lindstrom et al. 2002)
- Exhaled breath samples previously carried out by inhaling pure humidifed and charcoal-scrubbed air from a 20-liter Tedlar bag that has previously been filled from a pure air cylinder, then exhales into a second 20-liter Tedlar bag. The second bag is emptied by pumping through a glass cartridge containing 1.5 g Tenax. The entire system is mounted in a van to allow house calls to the participants to be made. The time required to collect a breath sample is about 5 min. Vans may be equipped with a helium positive-pressure system to bathe the bags while in storage on the van. An improvement to this method employs a charcoal face mask to allow breathing clean air without the need for a cylinder of clean air and a separate inhalation bag. Several (2-4) breaths are taken through the charcoal filter to flush the alveoli and bronchial tubes of ambient air before collecting the breath sample. The exhalation bag is replaced by an evacuated

electropolished 1.8-liter stainless-steel cylinder with a critical orifice (although other suitable collecting devices such as Tenax or other sorbents could also be used). The subject exhales through a 1-m long perfluoroethylene tube, which retains the latter part of the breath (the alveolar portion) for a few seconds during the resting and inhalation parts of the respiration cycle, during which time the alveolar air in the tube is pulled into the cylinder through the critical orifice. The canister collects approximately 98% alveolar air. (Wallace et al. 1996)

- Post-shower exhaled breath samples. Post-shower whole-breath samples were collected by having the subject blow into a Tedlar air sampling bag at the completion of a shower. To quantify the breath levels, 1-2 liters of the breath were transferred onto a Carboxen 569 (Supelco, Inc., Bellefonte, PA) adsorbent trap using a personal sampling pump at a flow rate of 1 l/min as soon as the bag was returned to the laboratory. Storage tests demonstrated that the THMs were stable in the sampling bag for up to 48 hr. (Weisel et al. 1999)
- SBC procedure used to collect breath samples from each of the five subjects. Breath samples collected 5 min after completion of the activity (unless in room adjacent to shower). Background samples were obtained once each morning before any water-use activities began. Samples were shipped at the end of the day by overnight express courier for THM analysis. (Gordon et al. 2005)

☑ Analytical aspects

- **Techniques**
  - *Blood*

    Analysis performed on plasma aliquots for chloroform. Samples analyzed by a head-space GC technique. Calibration performed by the external standard method. The identity of the chloroform was confirmed by GC-MS. Both standard samples of chloroform in n-pentane and standard samples of chloroform-fortified human plasma examined at increasing concentrations after extraction with n-pentane. The identification of chloroform is based both on retention times measured on a total ion-current chromatogram and on mass chromatograms of the molecular ion and the most significant fragments of chloroform. (Aggazzotti et al. 1995)

Analysis of THMs in blood samples using a variation of method reported by Ashley et al. (1992). This method includes spiking blood samples with isotopically labelled standards, extracting with solid-phase microextraction, and analysis by GC followed by high-resolution magnetic-sector MS. We quantified blood THM concentrations using calibration curves generated from dilutions of pure samples of each THM species. Blanks and quality control materials were analyzed with each analytical run. Detection limits were in the parts per quadrillion range, allowing the quantification of most samples even at background levels. (Nuckols et al. 2005)

Plasma samples were analysed by a head-space GC technique for individual THMs. Quantitative analysis was performed by Chromato-Integrator Merck Hitachi D2000. Calibration was performed by the external standard method and the limit of detection for all THMs was 0.1 mgl^{-1}. Repeatability was evaluated using a standard solution containing 1 mg of each THM and was calculated from five duplicate determinations on five different days. For plasma, the repeatability expressed as coefficient of variation CV was 2.8% for $CHCl_3$. The detection limit was calculated during 30 different series of THM determinations of blank value. Based on 2.5 times the standard deviation of the mean blank concentration, the detection limit for each THM, both in plasma and water samples, was set at 0.1 mgl^{-1}. (Aggazzotti et al. 1998)

Analysis of blood samples for THMs. Volatile compounds (e.g. bromoform) are present in commercial vacutainer rubber stoppers and these can interfere with the accurate measurement of THMs in blood; these compounds must therefore be removed by heating the stoppers under vacuum prior to use. Current detection limits for THMs in blood are below 1 pg/mL, which is sufficient to provide quantitative levels of THMs in the blood of most users of chlorine- or chloramine-treated water. It is possible to store blood samples under refrigeration for up to 10 weeks before analysis. Less progress has been made in measuring HAAs in blood. Currently, HAAs have a considerably higher detection limit in blood (at least 1,000 times greater) and improved detection methods are needed. (Arbuckle et al. 2002)

Whole blood samples analysed for THM species. Individual THM species levels (parts per trillion) in whole blood were determined by a modification of the

previous heated purge and trap gas chromatography isotope dilution mass spectroscopy procedure. The use of selected ion monitoring and a mass spectrometer lowered detection limits into the parts-per-quadrillion range. (Lynberg et al. 2001)

The whole blood samples were analyzed for THMs using solid-phase microextraction GC/isotope dilution MS, with the mass spectrometer operating in the selected ion monitoring mode. Stable isotopically labelled analogues of the compounds of interest were added to the samples as internal standards, and quantification was accomplished by measuring specific ion responses relative to those of the corresponding labelled analogues. (Gordon et al. 2005)

TCAA and THMs analyzed in whole blood samples. The TCAA analytical methodology is performed by liquid-liquid microextraction (LLME), head-space solid phase microextraction (SPME), gas chromatography (GC) and electron capture detector (ECD). Liquid-liquid extraction (LLE), GC and ECD methods are used for the analysis of THMs (trihalomethane). (Chen et al. 2003)

- *Urine*

  TCAA concentrations in the urine samples were measured according to a modified version of US EPA Method 552.28 employing the use of solid phase microextraction (SPME). (Bader et al. 2004)

  Before analysis, urine samples used for this study (stored at –70°C) were left to thaw overnight at 5°C. No chemical degradation of TCAA was detected in the quality control samples and standards used for this study under these conditions. TCAA was measured using a method described previously (Kuklenyik et al. 2002), which involved the use of solid-phase extraction followed by the analysis of TCAA by isotope-dilution high performance liquid chromatography–tandem mass spectrometry; with this method, specific fragment ions of TCAA and its 13C-labeled analog were monitored for quantification and confirmation. The limit of detection (LOD) for TCAA in a 1mL urine sample was 0.5 μgL^{-1}. Because the log-transformed TCAA concentrations were less skewed than were the nontransformed values, log-transformed values were used in the analyses. Results are reported both as micrograms TCAA per liter of urine (μg/L) and micrograms TCAA per gram of urinary creatinine (μg/g creatinine). Creatinine adjustment was used to correct for urine dilution; only those urine samples with

creatinine values ≥300 mgL^{-1} were included in the analysis because those with values < 300 mg/L are too dilute to provide results that can be interpreted correctly. (Calafat et al. 2002)

Sample preparation and analysis of TCAA in urine performed according to a modified version of U.S. Environmental Protection Agency (EPA) method 552.2 and the method used by Kim and Weisel. Samples were analysed with gas chromatography. These methods provided excellent performance [relative standard deviation (RSD) of 4.8% on 11 triplicate analyses of tap water and 8.5% on 17 triplicate analyses of urine] and allowed sensitive detection of TCAA in urine. Our method detection limit (MDL) for these analyses was approximately 0.3 µg/L for urine, based on three times the SD for triplicate sample analyses, averaged for triplicate sets with TCAA ≤ 2 µg/L. (Froese et al. 2002)

A variant of thin layer headspace (TLHS) analysis with autogenous generation of the liquid sorbent was applied for the determination of volatile organohalogen compounds in urine. (Polkowska et al. 1999)

Volatile organohalogen compounds were determined in urine using continuous flow TLHS analysis with autogenous generation of the liquid sorbent. The extract obtained by this technique was analyzed by direct aqueous injection of the sample into a capillary column of a gas chromatograph equipped with electron capture detector. The details of the procedure, developed at the Department of Analytical Chemistry, Technical University of Gdansk, have been described elsewhere. The concentration factors of the volatile analytes, determined in earlier experiments, ranged from 7 to 70 (30 for chloroform) (Polkowska et al. 2003)

The volume of each urine sample was measured in the laboratory. TCAA concentrations were determined by extraction with ethyl ether and derivatisation with 10% sulphuric acid in methanol, followed by GC/electron capture detector (ECD). To correct for variations in the volume of urine excreted, creatinine was also analyzed in the urine using a calorimetric method. The total amount (nanograms) of TCAA excreted in a urine void was calculated by multiplying their concentrations in the urine by the volume of the urine void. An excretion rate was then calculated by dividing the total amount of TCAA in the urine void

by the time interval between the first morning urine void and the previous urination. (Weisel et al. 1999)

- *Exhaled alveolar air*

  Alveolar air samples were analysed for THMs by direct injection of samples into a GC using a gas-tight syringe. Calibration was performed as before and the limit of detection was 0.1 μgm^{-3}. Repeatability was evaluated using a standard solution containing 15 mgm^{-3} of each THM and was calculated from five duplicate determinations on 5 different days. The repeatability CV was 5.3% for CHCl3, 2.7% for CHBrCl2, 3.7% for CHBr2Cl and 4.1% for CHBr3. DL was calculated during 30 different series of THM determinations of blank values. Based on 2.5 times the standard deviation of the mean blank concentrations, the detection limit for each THM, both in environmental and alveolar air samples, was set at 0.1 μgm^{-3}. The identity of THMs was confirmed by GC-MS. (Aggazzotti et al. 1998)

  The breath samples were analyzed by thermal desorption coupled to gas chromatography/mass spectrometry (GC/MS) (Weisel et al. 1999)

  Exhaled alveolar air samples were analyzed for THMs by automated gas chromatography/mass spectrometry (GC/MS) using a modified version of U.S. Environmental Protection Agency (EPA) Method TO-14 (Winberry et al. 1990). The mass spectrometer was operated in the full scan mode. Target analytes were identified by matching the mass spectra acquired from the sample to a mass spectral library. During each analysis period, a single-point calibration was run; precision for each THM was typically < 20% relative SD. Detection limits were 0.5 $\mu g/m^3$ for $CHCl_3$, 0.7 $\mu g/m^3$ for $CHBrCl_2$, 0.8 $\mu g/m^3$ for $CHBr_2Cl$, and 1.0 $\mu g/m^3$ for $CHBr_3$. (Gordon et al. 2005)

- ***Sensitivity and specificity***

  The range of detection limits varies according to the matrix in question and the analytical method used. The details of detection limits for both THMs and TCAA for selected analytical methodologies are provided in tables 1-6. Table 7 gives an indication of the range of detection limits expected for each matrix, for both THMs and TCAA (where appropriate).

Table 10.1: Range of detection limits for various matrices for biomarkers of DBPs

Matrix		THMs	TCAA
Invasive	Blood	≤1 pgL^{-1} – 0.1mgL^{-1}	>1 ngL^{-1}
Non-invasive	Urine	N/A	0.3 μgL^{-1} – 0.5 μgL^{-1}
	Exhaled alveolar air	0.1μgm^{-3} – 0.5μgm^{-3}*	NA

*depending on method used and species measured (e.g. Gordon et al. 2005 reported detection limits of 0.5 $\mu g/m^3$ for $CHCl_3$, 0.7 $\mu g/m^3$ for $CHBrCl_2$, 0.8 $\mu g/m^3$ for $CHBr_2Cl$, and 1.0 $\mu g/m^3$ for $CHBr_3$).

- ***Units***

Table 10.2: Units used in expressing concentrations of biomarkers of DBPs in various matrices

Matrix		THMs	TCAA
Invasive	Blood	Concentrations expressed as μgL^{-1} of whole blood or plasma (or pgL^{-1} and ngL^{-1} in the case of the most sensitive analytical methods)	
Non-invasive	Urine	Concentrations of THMs and TCAA are given as µgL-1 of urine or, in order to adjust for the dilution of the urine, in µgg-1of urinary creatinine. Often both sets of units are presented in results.	
	Exhaled alveolar air	Concentrations of THMs are given in mgm^{-3} of exhaled alveolar air.	

☑ Performance characteristics

- ***Analytical reproducibility***

The figures for analytical reproducibility given in table 9 are derived from the studies listed in tables 4-6.

Table 10.3: Analytical reproducibility (precision) in analysis of concentrations of biomarkers of DBPs in various matrices

	Matrix		THMs	TCAA
express	Invasive	Blood	2.8% for $CHCl_3$*	-
	Non-invasive	Urine	-	8.5% of relative standard deviation
		Exhaled alveolar air	5.3% for $CHCl_3$, 2.7% for $CHBrCl_2$, 3.7% for $CHBr_2Cl$ and 4.1% for $CHBr_3$	-

expressed as a coefficient of variation (CV)

- ***Inter- and intralaboratory variability***

  'Another key difference though between available studies is the accuracy of the sampling and analysis method. While the Miran infrared analyser utilized by Giardino et al. is useful for providing near-real-time readings to examine relative concentration changes during a shower event, the accuracy for this instrument is limited, especially in humid environments and at relatively low analyte concentrations (e.g. below the detection limit range of 1,000 to 5,000 $\mu g/m^3$). Moreover the results of Giardino and colleagues are consistently higher on average than those studies using gas chromatography (GC) quantitation, and the influence of humidity or the presence of other chemicals tends to cause an artificially higher instrument response on a Miran analyzer. In this case, interferences would cause an artificially higher reading for aribone concentration of TCM or TCE using the Miran analyzer, which could not occur using GC techniques. Accordingly, it seems likely that the discrepancies between the Giardino et al. results and those of Jo et al, Tancrede et al and Keating et al. could be substantially due to analytical quantitation techniques.' (Kerger et al. 2000).

☑ Validation

- **OECD:** N/A
- **EPA:**
  - *THM:* US EPA Method 524.2: Measurement of purgeable organic compounds in water by capillary column gas chromatography/mass spectrometry.
  - *THM:* US EPA Method 551: Determination of trihalomethanes, total by liquid-liquid extraction and gas chromatography with electron capture detection (LLE & GC/ECD), EPA report number.
  - *TCAA:* US EPA Method 552.28: Determination of haloacetic acids by liquid-liquid extraction and gas chromatography with electron capture detection (LLE & GC/ECD), EPA report number 600/4-90-020.

☑ Confounding factors

- **Age**

  A number of studies have found an inverse association between THMs in alveolar air and age (Aggazzotti et al. 1993; Fantuzzi et al. 2000). The mechanism upon which this association is based is yet to be established.

- **Sex**

  A study by Calafat et al. (2002) found that the relation of TCAA concentration and place of residence (urban or rural) varied also according to sex. The authors of this study postulated that without good demographic data such a variation is hard to account for. They did, however, propose that such factors as personal habits (wearing dry-cleaned clothing, use of chlorinated household products, diet etc.), occupational exposure and differences in metabolism could play a role in the effect of sex on TCAA concentration and place of residence.

- **Occupational exposures**

  Since TCAA is also produced in human metabolism of TRI, TCE and PERC (common industrial chemicals) and chloral hydrate (a drug), there is the possibility for the specificity of urinary and plasma TCAA biomarkers of exposure to DBPs to be compromised (Calafat et al. 2002).

☑ Concentrations reported in literature

- **Reference values**

  Table 10.4: Reported chloroform (trichloromethane) levels in different media in various studies (from Nieuwenhuijsen et al. 2000b)

Study	Medium	Chloroform levels	Comment
Drinking water			
Krasner et al., 1989	drinking water	9.6–15 μg/l	quarterly means
Kuo et al., 1997	drinking water	18, 19, 69 μg/l	means 3 areas
Chen and Weisel, 1998	drinking water	14 and 33 μg/l	cold and warm, season respectively
Keegan, 1998	drinking water	50 μg/l	mean 1 company
Shin et al., 1999	drinking water	20 μg/l	estimated mean
Showering and bathing			
Jo et al., 1990a	shower water	27 μg/l	while showering
	shower air	186 μg/m^3	while showering
	exhaled breath	13 μg/m^3	normal shower
	exhaled breath	7 μg/m^3	inhalation only shower
Weisel and Jo, 1996	exhaled breath	0.01 m^3/min per	mean amount
	showering	1 mg/l $CHCL_3$ in H_2O	expired $CHCL_3$
Gordon et al., 1998	bath water	86 μg/l	mean level
	exhaled breath	45 μg/m^3	mean of max observed levels
Weisel et al., 1999	shower water	31 μg/l	mean level
	shower air	0.4 μg/m^3	overall median
	exhaled breath	4.0 and 54 μg/l	collection after 5 min for low and high water concentration
Swimming			
Aggazotti et al., 1990	pool water	274.4 nmol/l	estimated AM
	pool air	1789.2 nmol/m^3	estimated AM
	plasma	9.26 nmol/l	estimated AM
Aggazotti et al., 1993	pool water	312.9 nmol/l	estimated AM
	pool air	1152.6 nmol/m^3	estimated AM
	alveolar air	758.9 nmol/l	estimated AM
Aggazotti et al., 1995	pool water	32.7 μg/l	AM (n=18)
	pool air	213.6 μg/m^3	AM (n=18)
	plasma	1.06 μg/l	AM (n=127)
	pool water	36.0 μg/l	AM (n=6)
	pool air	140.3 μg/m^3	AM (n=6)
	alveolar air	94.1 μg/m^3	AM (n=163)
Aggazotti et al., 1998	pool water	33.7 μg/l	AM
	pool air	91.7 μg/m^3	AM, before swim
	pool air	169.7 μg/m^3	AM, after swim
	plasma	0.7–2.3 μg/l	range
	alveolar air	9.3–76.5 μg/m^3	range AMs
Aiking et al., 1994	pool water	18.4, 24.0 μg/l	in/out door, respectively
	plasma levels	0.89 μg/l	mean
Levesque et al., 1994	pool water	365 μg/l	controlled levels
	pool air	1252 ppb	controlled levels
	exhaled breath	425 ppb	after 55-min swim
Weisel and Shepard, 1994	pool water	85 μg/l	mean
	pool air	87 μg/m^3	mean
	exhaled breath	720–1400 μg/m^3	after 30 min-swim
Cammann and Hubner, 1995	pool water	3.04–27.8 μg/l	range
	pool air	7.77–92.8 μg/m^3	range
	plasma levels	1.14–5.23 μg/l	range comp swim
	plasma levels	0.56–1.65 μg/l	range norm swim

Study	Medium	Chloroform levels	Comment
Swimming			
	plasma levels	0.56–1.65 $\mu g/l$	range norm swim
	plasma levels	0.13–2.45 $\mu g/l$	range attendants
Lindstrom et al., 1997	pool water	68, 73 $\mu g/l$	two samples only
	exhaled breath	371 $\mu g/m^3$	peak level
Matthiessen and Jentsch, 1999	pool air	approx. 300 $\mu g/m^3$	peak level

AM=arithmetic mean.

Table 10.5: Reported TCAA levels in different media in various studies (from Nieuwenhuijsen et al. 2000b)

Study	Medium	TCAA levels	Comment
Krasner et al., 1989	drinking water	4–6 $\mu g/l$	quarterly medians
LeBel et al., 1997	drinking water	4.3–15.6 $\mu g/l$	yearly means at different distances chlorine treatment
Williams et al., 1997	drinking water	5.8–56.7 $\mu g/l$	means for different treatments
Chen and Weisel, 1998	drinking water	5.5–7.5 $\mu g/l$	means cold season
		5.5–10 $\mu g/l$	means warm season
Kim and Weisel, 1998	swimming pool water	420 $\mu g/l$	AM
Kim et al., 1999	urine	0–30 ng/min	range excretion rate
Weisel et al., 1999	water	1.94 $\mu g/l$	AM low exposure
	urine	6.35 ng/min	AM low exposure
	water	27.6 $\mu g/l$	AM high exposure
	urine	10.3 ng/min	AM high exposure

AM=arithmetic mean.

Modelling work carried out by Whitaker et al. (2003) provides an approximate indication of how trichloromethane concentrations in water delivered to the home relate with actual uptakes, while taking into account individuals' activities (swimming, showering, bathing and ingesting tap water). Using figures in the literature, estimates were made for average uptake per microgram per litre trichloromethane in the water, per minute spent in the activity or per litre of drinking water consumed. Tables 12 to 14 show the input parameters and uptakes estimates for swimming, tap water ingestion and bathing respectively.

Table 10.6: Input parameters for swimming (Whitaker et al., 2003)

Parameter	Distribution/value
Pool chloroform concentration (µg/L)	Log-normal (3.95, 0.77) 5th percentile 15 µg/L, median 52 µg/L, mean 70 µg/L, 95th percentile 184 µg/L
Time spent in pool (min)	Log-normal (3.91, 0.4) 5th percentile 26 min, median 50 min, mean 59 min, 95th percentile 96 min
Proportion of women who swim	41%
Proportion of swimmers who swim occasionally	59%
Swimming rate for occasional swimmers (< once/month) for the 90-day period	Uniform (0, 3)
Swimming rate for regular swimmers for the 90-day period (once/month to 6.5 times/week)	Triangle (3, 84); maximum probability at 3; 0 probability at ≥ 84[a]
Number of swims in 90 days	Poisson (swimming rate)
Uptake (µg/min) per µg/L chloroform in pool	Uniform (0.00102, 0.004537)

[a]For those who swim > 6 times/week, 0.3%; 2–6 times/week, 21%; < 2 times/week, 79%.

Table 10.7: Input parameters for tap water ingestion (Whitaker et al., 2003)

Parameter	Distribution/value
Average daily tap water intake (L/day)	Log-normal (–0.15, 0.45) 5th percentile 0.41 L/day, median 0.86 L/day, mean 0.95 L/day, 95th percentile 1.8 L/day
Proportion of drinks made with boiled water	Uniform (0, 1); average, 50%
Chloroform loss from heating and pouring	85%
Uptake (µg) per µg/L chloroform in water per liter consumed	0.003676

Table 10.8: Input parameters for tap water ingestion (Whitaker et al., 2003)

Parameter	Distribution/value
Showering/bathing rate for the 90-day period	Uniform (60, 100); average, 0.9 showers or baths/day
No. of showers or baths for the 90-day period	Poisson (showering/bathing rate)
Percentage of mothers who take baths only	18%
Percentage of mothers who take showers only	61%
Proportion of baths taken for those who bathe and shower	Uniform (0, 1); average, 50%
Time spent showering (min)	Log-normal (1.9705, 0.3869) 5th percentile 4 min, median 7 min, mean 8 min, 95th percentile 14 min
Time spent bathing (min)	Log-normal (2.8, 0.65) 5th percentile 6 min, median 16 min, mean 20 min, 95th percentile 48 min
Uptake (µg/min) per µg/L chloroform in water for showering	Uniform (0.001114, 0.001524)
Uptake (µg/min) per µg/L chloroform in water for bathing	0.001384

- **Critical values**

  N/A

- **Normal range in populations (including power calculations)**

  N/A

- **Dose – response/effect relationships**

  N/A

☑ Time trend, geographical variation, susceptible groups

- **Geographical variation**

  Clearly the geographical variation and identification of susceptible groups both rely on the degree to which individuals carry out those activities presenting risk of exposure. Given that exposure sources are quite diverse and/or culturally/socially specific, there will be considerable degrees of geographic variation.

- **Susceptible groups**

  - *Competitive swimmers*

    Longer time spent in chlorinated water.

    Increased rates of respiration due to exercise level.

  - *Children*

Ingestion of pool water, in addition to dermal and inhalation uptake of DBPs.

Lower body mass and resultant greater relative dose.

- *Occupationally exposed*

  Swimming pool attendants and cleaners

  Laundry employees

12 FLUORINATED SURFACTANTS

☑ General information

Fluorinated surfactants include a divers group of different chemicals with properties, which make them suitable in a range of industrial purposes and consumer applications. The largest volumes of fluorinated organic compounds are used in fire-fighting foams, but considerable amounts are also used in the treatment of clothes, carpets and leather products. In addition they are used as lubricants, pesticides, paints and medicine. Some perfluorinated compounds, several perfluorinated cyclic alkylamines, are even used as blood substitutes (Golovanov and Tsygankova, 2001; Kissa, 2001). Several of the fluorinated chemicals have been used for over 50 years, but only recently this group of components have been regarded as a potential threat to the environment. Due to the carbon-fluorine bond, which has very high bond strength, these chemicals are extremely persistent towards degradation. Little information has been available about their presence in the environment, primarily due to analytical challenges. However, recent developments in chemical analysis, especially in LC-MS techniques, have revealed that several fluorinated compounds have become ubiquitously spread. Recently it was also discovered that selected fluorinated alkyl substances have a potential for bioaccumulation in organisms, which has raised concern about their harmful effects. Considerable amounts have even been detected in animals from the Arctic, such as polar bears, birds and marine mammals (Smithwick et al., 2005; Bossi et al., 2005; Verreault et al, 2005).

The fluorinated surfactants can be divided in three major groups, which include the perfluoroalkylsulfonic acids and derivates, the perfluorinated carboxylic acids and the fluorotelomers (Fig 1-4). The perfluorinated compounds are fully fluorinated in the hydrophobic tail, whereas the fluorotelomers contain non-fluorinated sites, typically methylene groups, near the head group. It is primarily the area tied to the head group that is subjected to degradation in the environment. The perfluoroalkylsulfonic acids derivates includes primarily the perfluorooctanesulfonic acids (PFOS) salts and sulfonamide derivates of PFOS, such as perfluorooctanesulfonamide (FOSA) and the alkylated perfluorooctanesulfonamidoethanol (FOSE). The PFOS derivates are prepared by electrochemical fluorination of octansulfonyl fluoride (Kissa, 2001).

The major perfluorinated carboxylic acid is perfluoroctanoic acid (PFOA), which is used in the aid of manufacturing PFTE and polyvinylidine fluoride (Lehmler, 2005). PFOA is also used as fire extinguisher agent, as insulator and in textiles (Kissa, 2001). The major manufacturer of PFOS derivates, 3M, has voluntarily ceased their production of PFOS and PFOA replaced these with shorter chain perfluorochemicals (Renner, 2001; Lehmler, 2005), which have characteristics that makes them less subjected to bioaccumulation (Renner, 2001). It is demonstrated that the bioaccumulation of PFAS is a function of carbon length. Other types of PFAS are the fluorotelomer sulfonates, fluorotelomer alcohols, fluorotelomer olefins and fluorotelomer iodide.

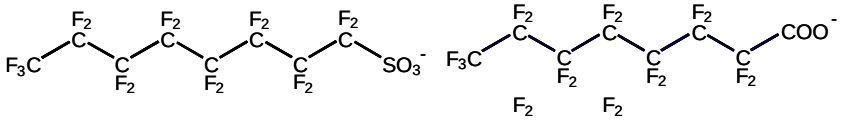


Fig. 11.1: Perfluorooctanesulfonate (PFOS) and perfluoroctanoate (PFOA)

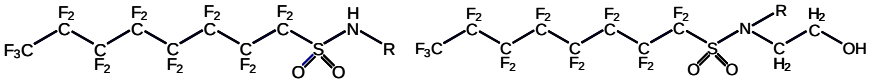


Fig 11.2: Perfluorooctanesulfonamide (FOSA) and perfluorooctanesulfonamidoethanol (FOSE)

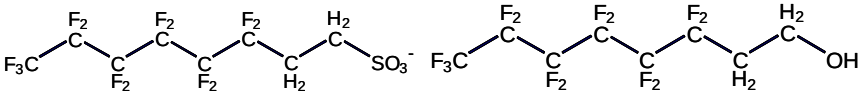


Fig. 11.3: Fluorotelomersulfonates (FTS) and fluorotelomeralcohol (FTOH)

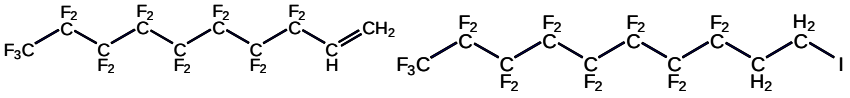


Figure 11.4: Fluorotelomeolefin (Ftolefin) and fluorotelomerjodid (FTjodid)

Due to its amphiphilic nature, PFOS, and most other fluorinated surfactants, behave differently in the environment than the more classical POPs such as the polychlorinated biphenyls. It has been attributed to debate how perfluorinated alkyl substances (PFAS), such as PFOS and PFOA, can be spread to such remote areas, since it is claimed that it is unlikely that these compounds is attributed to long-range atmospheric transport (Renner, 2001; Hekster et al., 2003; Simcik, 2005). Suggested theories are that major remote spread of PFOS is due to breakdown of more volatile precursors, such as sulfonamides, transport by ocean streams or transport by binding to particles (Simcik, 2005). The perfluorinated carboxylic acids are found in biota with different carbon length chain. In remote areas, such as the Arctic, it has been identified PFCA with both odd and even numbered carbon chains with varying carbon chain length. The origin of these components has challenged scientists. It is shown that the fluorotelomer alcohols may be metabolized to their carboxylic moiety, both by abiotic and biotic mechanisms. The fluorotelomer alcohols are, however, only produced in even chain length, but still reported to yield both even- and odd length PFCAs. Other potential sources are thermolysis of polytetrafluoroethylenes (PFTE), which may lead to PFCAs with different carbon chain length. In this review there will be most focus on PFOS and PFOA. These are the two dominating perfluorinated contaminants in the environment, and have reached a global distribution.

☑ Matrix

Martin et al., (2003a) analyzed tissue distribution of PFAS in rainbow trout, and in contrast to the lipid soluble POPs, PFAS primarily accumulate in blood, kidney, liver and gall bladder. Similar is also observed in rat experiments for PFCA (Vanden Heuvel et al., 1991ab). The knowledge that PFOS, or PFAS in general, often is associated with protein binding and in special plasma albumine (Jones et al., 2003), makes blood as a suitable matrix to investigate exposure to fluorinated surfactants. PFAS have been analyzed in human blood, both whole blood and serum, which appear as a good indicator matrix for exposure. There is evidence for PFAS in milk from mother exposed to PFOS in rat studies (Kuklenyik et al., 2004), but few studies have proven significant concentrations of PFAS in human milk (Kuklenyik et al., 2004). PFAS are proven as developmental toxicants and cord-blood has been used as an indicator matrix for exposure (Inoue et al., 2004).

☑ Kinetics

- **Uptake**

  Several PFAS chemicals have substantial water solubility (Simcik, 2005) and in fish, the major uptake route is across the gills through water, whereas accumulation through food appears to be less important (Martin et al, 2003ab). In human and higher animals the major pathway for exposure to PFAS is by food (Olsen et al., 2005). The low vapor pressure of PFOS and PFOA makes inhalation exposure unlikely, and according to the 3M company, dermal absorption of PFOS is limited (Olsen et al, 2005). Exposure of PFOS may however be a consequence of inhalation if associated to particles and aerosols, or due to inhalation of more volatile PFOS precursors, such as certain perfluorated sulfonamides or perfluorooctanesulfonyl fluoride (POSF). PFOS and PFCA is readily absorbed following ingestion. In a study performed by 3M (3M, 2003), rats were fed one single dose of PFOS and more than 95% was taken up. PFOS distributes predominantly to blood and liver, which may be due to its strong affinity to serum proteins. In both rats and monkeys there are shown that serum PFOS accumulation is linear to exposure (Seacat, 2002; 3M, 2003). In human and higher animals the expected major pathway for exposure to PFAS is therefore by food (Olsen et al., 2005). The assumption that the volatile fluorotelomers may be metabolized to their corresponding acids makes it also likely to believe that exposure by air may be a significant exposure pathway and source for PFCAs. There is almost no information about PFAS in human breast-milk. In a report by Kuklenyik et al., (2004) no PFAS was detected, and the authors suggested that milk is not a primary route for exposure.

- **Metabolism**

  The different PFAS substances are in general very inert and are not likely to degrade in significant amount. It is primarily their functional group at the head moiety that is subjected to metabolism and degradation, and the final products are usually a sulfonic acid derivate or a carboxylic acid.

- **Biological half-life**

  In both rats and monkeys there are shown that serum PFOS accumulation is linear to exposure (Seacat, 2002, 3M, 2003). The elimination half-life is species dependent and appears to be biphasic (3M, 2003). Serum elimination half-life for PFOS in male

rats was demonstrated to be 7.5 days after one single oral administration of 4.2mg PFOS. Whole body elimination half-life was more than 89 days in male rats after a single intravenous administration of 4.2mg/kg PFOS. Serum elimination half-life in both female and male cynomologus monkeys is approximately 200 days (Seacat et al., 2002; 3M, 2003). PFOS appears to have the longest elimination half-life in humans. In a study on retired employees from a PFOS manufacturing facility a serum elimination half-life was estimated to be 8.7 years, with a range between 2.3-21.3 years (3M, 2003). This is much higher than reported in laboratory animals, but may represent an estimate on human elimination rate following low-level exposure to PFOS.

- A striking observation is the sex and species differences in the clearance and elimination of PFOA (Kennedy et al., 2004). The female rat, male hamster and both sexes of rabbits rapidly eliminate PFOA. On the contrary in the male rat, both sexes of mice and the female hamster, PFOA is retained in the tissue. Also in dog and monkeys there appears to be sex difference and the female seem to eliminate PFOA faster than the male. Elimination half-life in rats is estimated 15 days and less than 1 day in male and female rats respectively after intra peritoneal administration (Van den Heuvel et al, 1991a). The major difference in elimination seems to be the rate of urinary excretion. The elimination half-life of PFOA in humans is estimated to 4.4 years by measuring plasma in retired fluorochemical production employees. This is much higher than reported in laboratory animals, even in monkeys. However, it is reasons to believe that this estimate represents elimination rate following low-levels exposure, and that exposure to high concentrations of PFOA may be followed by an initial higher rate of elimination. In a recent work by Fasano et al., (2006) rats were administrated the 8-2 Fluorotelomer alcohol (8-2 FTOH). The plasma elimination half-life was estimated to less than 5 hours with no sex related differences. Most of the FTOH was eliminated in feces of which 37-55% was identified as the parent compound. Identified metabolites were clucuronoide and glutathion conjugates

☑ Sampling conditions

PFOS and PFCA are not easily degraded and are not volatile compounds, and no particular precautions needs to taken regarding storage. Samples are in general stored frozen at -20ºC. The more volatile PFAS, such as the fluorotelomers, are very volatile chemicals and may also be subjected to metabolism to their sulfonic acids or carboxylic

acid moiety and should be frozen immediately after collection. The samples should be kept as cold as possible, and be attributed to analysis as soon as possible after the extraction and clean-up procedure. We are continuously surrounded by potential sources for sample contamination by our own clothing and laboratory equipment. One must therefore be very careful when handling the samples to avoid contamination.

☑ Analytical aspects

- **Analysis**

  Analysis of PFAS has been a challenging approach. Only recently by the development more advanced LC-MS techniques one has been able to make proper analysis. One major challenge is to avoid contamination of the samples, even from the analytical instrument itself. One important contamination source might be connection tubes covered with Teflon. The perfluorinated sulfonates are analyzed by LC-MS. The perfluorinated carboxylates are analyzed primarily by LC-MS, but GC-MS is becoming more popular. For GC-MS analysis the carboxylate moiety is derivatized by a proper reagent, such as diazomethane. The perfluorinated sulfonamides are primarily analyzed by LC-MS, but also by GC-MS. The fluorotelomer alcohols are extremely difficult to analyze due to its volatile properties. Both GC-MS and LC-MS have been used.

- **Sensitivity and specificity**

  The detection limits for the various PFAS are dependent on the type of matrix and chemical. In liver the approximate detection limit in liver by use of LC-HRMS-TOF is less than 1ng/g wet weight (Berger and Haukås, 2005). The instrumental detection limits were reported to be approximately 1pg injected.

- **Units**

  PFAS do not share similar accumulation properties as the more classical POPs. PFAS primarily accumulates in blood, kidney, liver and gall bladder, and not in lipid tissue. Each PAFS are generally individually expressed as weight unit per gram wet weight.

☑ Performance characteristics

Only one worldwide inter laboratory study on perfluorinated compounds is performed revealing large variations in analytical performance (van Leeuwen et al., 2005). It appeared that the extraction and clean-up procedure contributed to most of the variation

in the results. The variation in results increased by an increase in complexity of the matrix. Further, it was concluded that water analysis of PFAS was not under control. This preliminary report shows that further development and more experience are needed to assure the reliability in PFAS analysis. A work by Berge and Haukås, 2005 also showed that one has to be aware of matrix effects in order to make proper quantification and method validation. Especially when analyzing complex matrices, such as liver tissue.

☑ Validation

No particular guidelines for PFAS analysis in human tissues are available. The available literature on PFAS analysis is, however, increasing and there are several recognized methods in use.

☑ Confounding factors

In a human monitoring study of a broad selection of the United State population, performed by 3M, no influence of age on PFOS body burden was revealed (3M, 2003). There was, however, found a trend in higher serum concentration of PFOS in male. Geometric mean of serum concentrations of PFOS was reported to 0.038, 0.035 and 0.031 ppm (µg/ml) in children, adults and in elderly respectively. The same cohort was also attributed to serum PFOA monitoring. No influence of age on serum levels of PFOA was reported, and geometric mean of serum concentrations of PFOA was 0.004-0.005 ppm (Butenhoff et al., 2004a).

☑ Concentrations reported in the literature

Kannan et al (2004) did a survey of PFAS levels in human blood collected from several different countries (Table 1). Their findings are representative for similar studies performed by others (Inoue et al., 2004, Olsen et al., 2005).

Table 11.1: Data on PFAS levels in human blood collected from several countries. The results at presented as median concentration (ng/ml) of PFOS, PFHxS, PFOA, and PFOSA. The results are from Kannan et al (2004).

Country	Gender	PFOS	PFHxS	PFOA	PFOSA
Michigan (US)	Female	28.9	2.8	4.4	2.2
Michigan (US)	Male	26.2	3.3	4.4	2.9
Kentucky (US)	Female	81	1.1	20	3.1
Kentucky (US)	Male	72	2.2	38.1	4
New York City (US)	Unknown	42	2.9	25.2	NA
Colombia	Female	7.3	0.2	5.6	0.9
Colombia	Male	8.1	0.2	5.9	1.4
Brazil	Female	8.4	2.2	<20	0.7
Brazil	Male	12.7	0.8	<20	1.5
Italy	Female	3.5	1.3	<3	1.7
Italy	Male	4.2	1.7	<3	1.6
Poland	Female	33.8	1.2	23.2	1.6
Poland	Male	40.9	1.2	18.4	1
Belgium	Female	10.4	<1	2.4	<3
Belgium	Male	17.6	1.2	4.3	<3
India	Female	2.5	1.6	<3	<3
India	Male	1.3	1.5	3.5	<3
Malaysia	Female	12.7	2.3	<10	4.1
Malaysia	Male	13.1	1.4	<10	3.9
Korea	Female	11.3	2.9	30.9	1.1
Korea	Male	21.7	3.4	26.8	1.3
Japan	Female	18.3	3.3	12.3	5.8
Japan	Male	12.4	3.7	<6.8	5.8

☑ Dose-respons/effect relationship

- **Perfluorooctanesulfonic acids (PFOS) salts**

  The different PFAS substances are in general very inert and there are no evidences for PFAS to be chemical carcinogens or mutagens. What have gained concerns are their similarities to cellular phospholipids, with a long hydrophobic tail and a hydrophilic head moiety making it likely that they may affect cellular lipid homeostasis. Further, it is likely that PFAS may affect cellular membrane properties, which for example may have consequences for the distribution of oxygen in lung cells, or inter- or intracellular communication (Hu et al., 2002; 2003). Lethal oral concentrations of PFOS acutely administered to rats inducing 50% death are in the range of approximately 200mg/kg. It appears that the liver is the main target, and the rats tended to be hypoactive and depressed (OECD, 2002). PFOS is shown to be eliminated slowly from the organisms and the toxicity appears to be cumulative.

  Rats were administered repeatedly to PFOS for 90 days to 30-3000ppm in diet, corresponding to 2-200mg/kg/day. All animals receiving 300ppm or more died,

whereas 50% in the 100ppm (~4.5mg/kg/day) group died (OECD, 2002). Rhesus monkeys repeatedly exposed to PFOS showed similar sensitivity as rats with a cumulative dose associated with mortality of approximately 200mg/kg (OECD, 2002). Prior to their death, the monkeys showed symptoms such as gastrointestinal sufferings, decreased activity and convulsions. Although the animals suffered from symptoms of toxicity, all rats receiving 2 mg/kg/day and monkeys receiving 1.5mg/kg/day survived the experiments indicating a critical exposure dose (OECD, 2002; Seacat et al., 2003). In a study by Seacat et al. (2002), cynomolgus monkeys were exposed to 0.03-0.75 mg/kg/day for 182 days. Adverse effects were only observed in the high exposure group of which two animals died. The most profound findings were lower serum cholesterol levels, lower triiodothyronine levels (without evidence for hypothyroidism), and lower estradiol levels. Further, the monkeys experienced decreased body weight and increased liver weights. The authors suggested a no-observed-effect-level (NOAEL) of 0.15mg/kg/day.

The most profound effect of PFOS in rodent studies is as peroxisome proliferators. Characteristics for peroxisome proliferators are hepatomegaly, proliferation of smooth endoplasmatic reticulum and peroxisomes in association of enzyme induction, and inhibition of mitochondrial beta-oxidation. Biochemical characteristics are decrease in serum lipids, such as triglycerides and cholesterol and induction of CYP4A. Isseman and Green (1990) identified a receptor, which was activated by peroxisome proliferators. This receptor is known as the peroxisome profilator activated receptor (PPAR). This receptor belongs to the steroid/thyroid/retinoid superfamily of nuclear receptors, and is involved in the regulation of carbohydrate - and lipid-metabolism as well as in and cell-regulation (Suga, 2004). Endogenous ligands for PPAR are polyunsaturated fatty acid. There are 3 isoforms of the receptor, PPAR-α, -β , -γ, which are coded by 3 different genes. PFOS is known to be a PPAR- agonist. PARP inducers are recognized as non-genotoxic carcinogens, or tumour promoters. Only few studies have evaluated the carcinogenic potential of PFOS.

Alexander et al., (2003) performed an investigation of mortality of employees in a PFOS manufacturing facility. They found a small increase in mortality among highly exposed workers from bladder cancer. However, one could not rule out the possibility of chance and it is therefore difficult to make certain conclusions about

the findings. In a study performed by 3M, refereed in an OECD-report, male and female rats were exposed to PFOS in diet for 104 weeks (0.5ppm-20ppm). The study showed that PFOS induces a small increase in the incident of tumors in liver, and the thyroid and mammary glands (OECD, 2002). The NOAEL for male and female was considered to be 0.5 ppm and 2ppm in diet respectively, which corresponds to approximately 0.03mg/kg/day and 0.15mg/kg/day. In this study and in a work by Seacat, et al (2003) it has not been provided evidence for hepatocellular peroxisomal or cellular proliferation measured as hepatic palmiotyl-CoA activation, at the doses tested. However, similar to the monkey study by Seacat et al., (2002) the animals had increased liver weight and decreased serum cholesterol, which is indicative of PFOS induced alterations in protein synthesis and/or lipid metabolism. The knowledge that it is a species difference in hepatic response to PPAR-inducers, of which rodents are especially sensitive, it has been questioned if this mechanism of action is relevant to human exposure (Suga, 2004).

Recently it was discovered that PFOS induced high mortality among developmentally exposed rodents (Lau, et al., 2003). Pregnant Sprague-Dawley rats and CD-1 mice were given 1-20mg/kg/day from gestational day (GD) 2 to GD 20 and GD 1 to GD 17 respectively. The major findings on the mothers were a reduction in serum thyroxine (T4) and triiodothyronine (T3), without effects on thyroide-stimulating hormone (TSH). The maternal rats exposed to high dosages (>5mg/kg/day) experienced a reduction in serum triglycerides and cholesterol. The mice dam experienced a reduction in serum triglycerides and an elevation in liver weight already at 1mg/kg/day. The most pronounced effects were seen on the newborn rodents. At high doses (10mg/kg/day) an increase in the prevalence of birth defects, such as cleft palate, anasarca, ventricular septal defects and enlargement of the right atrium (Lau et al, 2003). Even more concerning was the observation that 50% of the newborn rats and mice died within 24 hours when prenatally exposed to 3mg/kg/day and 10mg/kg/day respectively. In a more detailed study by Luebker et al., (2005) it appears that maternal exposure to 1.6mg/kg/day is a critical dose leading to approximately 50% mortality among prenatally exposed pups within 4 days after delivery. No long-term permanent effects have been observed in pups, which survive the first 4 days after delivery. To indicate the most critical period in gestation Grasty et al., (2003) exposed pregnant rats at certain time intervals of 4

days to 25mg/kg/day. Mortality of offspring was observed independently of exposure period, but was highest when the dams were exposed late in gestation.

The mechanisms for the high mortality of pups are not elucidated, and appear unclear. Luebker et al., (2005) could not make evidence for that the high mortality was due to lipid status, utilization of glucose or thyroid hormones. The most plausible hypothesis is a PFOS induced effect on the lungs of the neonates. Grasty et al., (2005) showed that exposed neonates had morphological changes in lungs that were indicative of immaturity. However, by co-exposure of protective agents and a more detailed investigation of the pulmonary surfactant profile failed to make certain conclusions. Since Grasty et al, (2003) achieved mortality even when dams were exposed only two times at gestation day 19 and 20 it is reasonable to believe that PFOS may influence the surface properties of the lungs making them less efficient to absorb oxygen. Other possible mechanisms of PFOS toxicity is inhibition of gap junctional intercellular communication (Hu et al., 2002), disruption of calcium homeostasis by changing membrane surface properties (Harada et al., 2005) and its PPAR activating properties which may influence several processes in cells as stated above, but also induce oxidative stress and mitochondrial dysfunction.

- **Perfluorinated carboxylic acids (PFCA)**

As for PFOS, PFOA primarily accumulates in liver and plasma due to its high affinity to proteins (Martin, et al., 2003ab; Kennedy et al., 2004). In fish PFOA does not biomagnify, but bioconcentrate by uptake through water probably across the gills (Martin et al., 2003ab). In human and animals the major pathway for exposure is probably by food or inhalation. Bioaccumulation of PFCAs is a function of carbon chain length. PFCAs with less than 8 carbons were shown not to bioaccumulate in fish. Studies of PFCAs in polar bears confirm this assumption by the absence of the seven carbon chained perfluoroheptanoate (Smithwick et al. 2005ab). This knowledge has led to a shift in the production towards more short chain length perfluorinated compounds. There is no evidence for PFOA to be chemical carcinogens or mutagens and it is unlikely that PFOA represent any significant human cancer risk (Kennedy et al., 2004; Butenhoff et al., 2004a). Biegel et al., 2001 performed a two-year feeding study in rats and found increases in liver, Leydig cell and pancreatic acinar cell tumors in the PFOA treated rats. This observation was attributed to PFOA as a potent PPAR inducer.

Bringing in mind that human and primates are poor PPAR inducers makes it not likely believe that PFOA is carcinogenic by this pathway.

Lethal oral concentration of PFOA, acutely administrated to rats and mice, inducing 50% death is in the range of approximately 400-600mg/kg. Despite the sex differences in elimination the lethal concentration appears to be independent of sex. Approximate LD50 value for guinea pig is 200mg/kg. When administered repeatedly the effect is cumulative and male rats appears more susceptible than the female due to differences in the elimination rate (Kennedy et a., 2004). In feeding studies with rhesus monkeys Griffith and Long (1980) observed mortality at 100mg/kg (2-5 weeks) and at 30 mg/kg (7-12 weeks) indicating that monkeys are more susceptible than rodents. This was also shown in a later study by Butenhoff et al. (2002) who observed that cynomologus monkey poorly tolerated repeatedly exposure of 30mg/kg/day. The dose was adjusted to 20mg/kg/day, but still resulted in serious weight loss and increased liver weight which was followed by serious hepatocellular necrosis. Even at the lowest dose (3mg/kg/day) an increase in liver weight was observed, which was attributed to increased mitochondrial proliferation (Butenhoff et al., 2002). Also rats experience increased liver weight as a consequence of PFOA exposure, which is a typical effect for PPAR inducers. In a 13 week oral feeding study on rats by Perkins et al.,(2004) a no effect level of 0.06mg/kg/day was estimated.

The most profound effects on rat liver are influences on the fatty acid levels (Olson and Andersen, 1983), reduction in serum triglycerides and cholesterol (Haughom and Spydevold, 1992), increase in mitochondrial proteins and microsomal content of Cyp 450 (Permadi et al., 1992) and rat liver triglyceride accumulation (Kudo and Kawashima, 2003). There is reason to believe that several of the observed the effects on rats are a consequence of PPAR activation. Several investigations have further revealed that PFCAs with longer carbon chain length, such as the perfluorordecanoic acid, are even more potent PPAR inducers than PFOA. In a study by Biegel et al., 1995 PFOA was shown to reduce serum and testicular interstitial fluid levels of testosterone and increase estradiol levels in exposed rats after peroral exposure to 25mg/kg/day for 14 days. This effect on endocrine functions in rats led to a more thoroughly investigation of employees at 3M workplace, however no certain association between PFOA exposure and hormonal changes has been achieved

(Kennedy et al, 2004). There are, however, been reported a slight correlation between serum PFOS/PFOA levels and an increase in serum triglyceride level, alkaline phosphatase and T3 levels (Olsen, et al., 2003; Kennedy et al., 2004). Another PFOA induced effects not related to PPAR inductions are inhibition of gap junctional intercellular communication (Upham et al., 1998).

Bearing in mind the decreased survival of the developmentally PFOS exposed rat puppies, some concern was raised if PFOA acted in a similar way. The effect of PFOA on rodent is clearly species dependent and dependent on their ability to eliminate the compound. Pregnant CD-1 mice were exposed by gavage to PFOA daily from GD1 to GD17 (1, 3, 5, 10, 20 or 40 mg/kg/day) (Lau et al., 2006). Shortly after delivery approximately 25% of the litters in 5mg/kg/day group died, whereas only 25% of the pups in 10- and 20mg/kg dose groups survived. The observation was reported to be similar to the developmental effects previously observed for PFOS. A similar study was performed on rats of which only a small effect was observed on the post weaning mortality at the high exposure group (maternal exposure to 30mg/kg/day 70 days prior to mating until weaning) (Butenhoff et al., 2004b). The rat puppies in this studying the high dose group experienced reduction in body weight and a reduction in sexual maturity. No effects on offspring were observed in the lower dose groups.

☑ Time trend, geographical variations, susceptible groups

- **Time trends**

  PFOS and PFOA, which are the components that dominate in environmental samples, are now considered as ubiquitously spread. Substantial increase in environmental concentration of PFAS the last decade has been reported, especially in arctic environment. A recent study by Olsen et al., (2005) revealed an increase in serum concentration of PFAS in the period 1974 to 1989. No significant increase was found in the period 1989 to 2001, which can be attributed to less spread of human exposure to PFAS compounds attributed to bioaccumulation. The major manufacturer of PFOS derivates, 3M, has voluntarily ceased their production of PFOS and replaced this compound group with shorter chain perfluorochemicals (Renner, 2001; Lehmler, 2005), which have characteristics that makes them less subjected to bioaccumulation (Renner, 2001).

- **Geographical variations**

  Kannan et al., (2004) performed a monitoring survey of human blood samples collected from US, Colombia, Brazil, Belgium, Italy, Poland, India, Malaysia, Japan and Korea. Spatial trend were observed with highest level in samples collected from the US and Poland (>30ng/ml) and lowest in India (<3ng/ml) (Table 1). No age- or gender related differences were observed. The dominating compound was PFOS, followed by PFOA. It appeared that serum levels of PFAS were dependent on local- or regional sources of exposure, and historical and present use of PFAS in e.g. household products.

- **Susceptible groups**

  Concerns are raised about the developmental toxicity of PFOS and PFOA. No gender or age related differences in body burden of PFAS are seen, and there is reason to believe that the margins of safety between potential harmful doses and the actual concentration in the general population are high. Butenhoff et al (2004a) have performed a risk characterization for exposure to PFOA. The margin of exposure, which is an estimate for potential risk, was calculated as high even for occupationally exposed individuals. The calculation of MOE was based on a comparison between serum PFOA levels in exposed animals and the serum concentration level in the general population. However, one important aspect is the knowledge that certain people lives in an environment that imply higher susceptibility for exposure such as indoor environment with carpets treated with PFAS.

13 LEAD (PB)

☑ General information.

Pb is a widespread environmental pollutant that can cause neurological, haematological and renal effects in industrial workers and in environmentally exposed populations.

☑ Matrix

Blood is the best biological matrix to monitor Pb exposure in the environment or the industry.

☑ Kinetics

- **Absorption**

  Pb can enter the organism by inhalation (absorption of about 50 % or by ingestion (oral absorption rate < 10% in adults, children, around 50%).

- **Distribution**

  Absorbed Pb is mainly stored in the bones that contain more than 90 % of the total Pb body burden. The rest is stored in soft tissues such as kidneys, liver and muscles. Pb can cross the placental and the brain-blood barriers.

- **Excretion**

  Pb is mainly excreted via urine and the bile.

- **Biological half-life**

  Elimination of Pb follows the kinetics of a three compartment models with half-lives of 35 days (blood), 400 days (soft tissues) and 20 years (bones).

☑ Sampling conditions

Blood should be collected in containers free of any metal contamination. Samples can be stored at 4°C or frozen.

☑ Analytical aspects

- **Techniques**

  Atomic absorption spectrometry (AAS) or ICP-MS.

- **Sensitivity**

  0.05 to 0.1 µg/l

- **Units**

  µg/l for µg/100ml in blood

☑ Performance characteristics

- **Analytical reproducibility**

  1-2 %

- **Inter- and intralaboratory variability**

  5-10 %

☑ Validation

Through the participation to intercomparison programmes and the use of certified standards or reference materials.

☑ Confounding factors

Smoking, alcohol consumption, menopauses and hormone-replacement therapy.

☑ Concentrations reported in literature

- **Current ranges of mean values**

  In non-occupationally exposed populations in countries where leaded gasoline has been banned are:

  - In adults: 30 – 50 µg/l
  - In children: 10 - 30 µg/l

- **Critical values**

  - Action level in children: 100 µg/l
  - Occupational exposure limit: 300 µg/l

☑ Dose –response/effect relationships

Effects	Pb in blood (µg/l)	
	Children	Adults
Cognitive or hearing impairment	50-100	
Vitamin D3 reduction	100-150	
Erythrocyte porphyrin elevation	150-200	200-300
Reduced haemoglobin synthesis	250-300	500
Increased urinary delta-aminolevulinic acid	400	400
Frank anaemia	700	800
Encephalopathy	800-1,000	1,000-1,200

☑ Time trend, geographical variation, susceptible groups

In countries where leaded-gasoline has been banned, concentrations of lead in the blood of general population have rapidly decreased to levels that are now about 70-80% lower than those prevailing in the 1970s

14 PARABENS

☑ General information

Parabens are a group of chemicals widely used as preservatives in the cosmetic and pharmaceutical industries. They can be found in shampoos, shaving gels, cleansing gels, deodorants, topical pharmaceuticals etc.

Parabens are esters of para-hydroxybenzoic acid. Common parabens include methylparaben, ethylparaben (E214), propylparaben (E126) and butylparaben. The general chemical structure of a paraben is shown below, where R symbolizes an organic group such as methyl, ethyl, propyl or butyl.

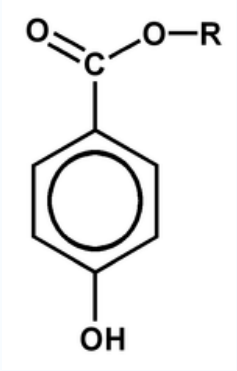


Figure13.1: General chemical structure of a Paraben (a *para*-hydroxybenzoate) where R = some organic group

Parabens have been linked to breast cancer, but so far there is no scientific evidence to support this claim. Parabens have been found in 20 samples of breast tumors (Darbre 2004), but it is unknown if this would be the same for healthy breast tissue. Further research is necessary to establish the significance of parabens in breast tumors and to establish a casual link between parabens in cosmetics and breast cancer. Tests on animals involving oral administration and injection of parabens have shown weak oestrogenic activity (Bayford 2002). Oestrogen is known to drive the growth of tumors. However, there is no evidence that underarm cosmetics containing parabens pose a health risk, because of the low doses involved and the fact that parabens are unlikely to penetrate into the tissue and to accumulate there (enzymes in skin and subcutaneous fat cells are capable of breaking down parabens).

These synthetic preservatives are frequently used in cosmetics and personal care products, such as shampoos, conditioners, hair styling gels, nail creams, foundations, facial masks, skin creams, and deodorants. Methyl and propyl parabens are also allowed for use as food preservatives in small quantities. They are considered "Generally Recognized As Safe" for food uses.

Parabens can cause skin irritation and contact dermatitis in individuals with paraben allergies. Additionally, less than 1% of all underarm products actually contain parabens. Parabens are extremely effective as preservatives in all types of formulas; for example, parabens can be used to preserve shampoo, lotions and personal lubricants, but a different preservative system would have to be used in each of those products in order to replace the parabens.

A recent review by Darbre (2003) published in *J. Appi. Toxicol*. 23: 89-95 has attracted public and scientific interest that requires perspective, particularly on the use of esters of *p*-hydroxybenzoic acid (parabens) as preservatives in underarm cosmetics. Although parabens are generally regarded as safe, recent reports suggest that they are oestrogenic in a variety of *in vitro* (including MCF7 and ZR-75-1 human breast cancer cell lines) and *in vivo* tests for oestrogenicity (uterotrophic assays in both rat and mouse). There are also recent reports of adverse reproductive and developmental outcomes in rodent toxicity studies.

A small study found laboratory evidence of low levels of substances called parabens in some samples of breast cancer tumors. Parabens are used in some underarm products as preservatives and may have entered the breast through the underarms in these cases. A possible concern is that in other studies, parabens have been found to have weak estrogen-like properties. Estrogen is a female hormone known to cause breast cells (normal and cancerous) to grow and divide, and some conditions that increase the body's exposure to estrogen (not having children, late menopause, obesity, etc.) have been linked to an increased risk of breast cancer. The study did not show that parabens caused or contributed to breast cancer development in these cases – it only showed that they were there. The significance of this is not yet clear. While parabens have weak estrogen-like properties, the estrogens that occur naturally in the body are hundreds to thousands of times more potent. Therefore, these natural estrogens (or those taken as hormone replacement) are much more likely to play a role in breast cancer development. This study did not contain any information useful in determining the source of parabens

found in breast tissue. The bottom line is that this is a very early finding, and larger studies will be needed to determine what effect, if any, parabens might have on breast cancer risk. This topic will undoubtedly receive a lot of scientific attention in the near future (ACS 2006).

☑ Matrix

- **Invasive**
  Breast tissue
- **Non-invasive**
  Urine, skin, high potential for hair as matrix
- **If different methods are available, compare these**
  Breast tissue only available for cancer cases

☑ Kinetics

The absorption, metabolism and excretion of parabens had been studied in rats, rabbits, dogs and humans. The methyl, ethyl and propyl esters of p-hydroxybenzoic acid appeared to be well absorbed and the ester linkage was assumed to be readily hydrolyzed. Urinary excretion of the unchanged esters was very low, usually less than 1% of the administered dose. Butyl paraben was suspected to follow a different metabolite pathway, but studies in dogs had shown no evidence of accumulation of either parent compound or metabolites in the tissues (SCCP/0873/05).

Of interest is the lack of activity by the oral route but clear activity by the subcutaneous and topical routes, which is of some relevance to the use of underarm cosmetics. There would seem to be a case now to supplement these emerging toxicity data with longer term regulatory standard tests examining other oestrogenic endpoints and at least to consider these findings in more up-to-date risk assessments specific for cosmetic use. Further, there are few data on the use of underarm cosmetics and the risk of breast cancer, and although one recent retrospective interview-based study found no association there is a need for more thorough investigation taking into account the type of chemicals used. Darbre has forwarded a hypothesis and called for further work to establish whether or not the use of underarm cosmetics (particularly containing oestrogenic formulants) contributes to the rising incidence of breast cancer. It would seem prudent to conduct this work because the current database is sparse and the effects

of long-term low-level exposures to weakly oestrogenic chemicals on human health, particularly their application to the underarm and the risks of breast cancer, are unknown.

However the ubiquious exogenous exposure to parabens will undoubtly cause an equilibrated level of endogenous exposure, resp. level.

☑ Sampling conditions

- **Analytical aspects**
  - *Human breast material and thin layer chromatography:* All glassware was pre-washed in 0.1 M NaOH and extractions were performed using sterile polycarbonate tubes. Samples of human breast tissue (1 g) were chopped finely with a sterile razor and homogenized in 5 ml of hexane using a hand-homogenizer. Samples were left in a sealed polycarbonate tube with mixing for 1 h and then spun at 1500 rpm in a bench centrifuge at room temperature for 2 min. The supernatant was placed in a sterile polycarbonate tube, 5 ml of 0.1 M potassium bicarbonate was added and the tube was inverted 40 times by hand. The mixture was spun at 1500 rpm at room temperature for 2 min to separate the phases. The upper yellow hexane layer containing phenolic compounds was placed in a new sterile polycarbonate tube, 5 ml of 0.1 M potassium carbonate was added and again the tube was inverted 40 times by hand. The mixture was spun at 1500 rpm at room temperature for 2 min to separate the phases. The lower aqueous layer containing the phenols as potassium salts was taken into a new sterile polycarbonate tube and acidified by the addition of 300 µl of concentrated hydrochloric acid to give a pH in the 1–3 range (checked with pH paper). The free phenolic compounds released on acidification were extracted into 5 ml of diethyl ether by inverting the tube by hand 40 times. The mixture was spun at 1500 rpm at room temperature for 2 min to separate the phases. The upper ether layer was removed and evaporated to dryness under nitrogen overnight in a fume hood. The extract was taken up in 50 µl of ethanol and aliquots were run against paraben standards (50–400 ng per track) on thin-layer chromatography plates using a solvent of 5% (v/v) ethanol–95% (v/v) chloroform. Parabens were visualized under ultraviolet light. For quantification,

the image under ultraviolet light was captured digitally and relative levels of bands were analysed by image analysis.

- *Extraction of parabens from human breast tumour material and analysis by HPLC-MS/MS:* Samples of human breast tumour material (0.25 g) were chopped finely with a sterile razor and homogenized in a mixture of 6.25 ml of ethanol and 6.25 ml of acetone. This mixture was left with periodic shaking overnight in a sealed glass Corex tube. The next day, the mixture was spun at 2500 rpm for 10 min on a bench centrifuge at room temperature. The supernatant was removed to a clean Corex tube. The pellet was re-extracted with a further 1.5 ml of ethanol and 1.5 ml of acetone, spun and the two supernatants pooled. The total supernatant was dried under nitrogen at room temperature. To the residue was added 6 ml of 70% (v/v) aqueous methanol; the mixture was vortexed and then incubated overnight at -20 °C. The next day, the mixture was spun at 3200 rpm for 20 min at 4 °C and the supernatant was removed to a clean Corex tube. The pellet was re-extracted with a further 1 ml of 70% (v/v) aqueous methanol by vortexing and spun again at 3200 rpm for 20 min at 4 °C. The two supernatants were pooled and dried under nitrogen for analysis by HPLCMS/MS. The extracts were dissolved in HPLC mobile phase (0.25 ml) and the paraben concentration determined by HPLCMS/MS. Samples (20 µl) of the final extracts were chromatographed on a Hypersil Elite C18 column (150 × 2.1 mm; 5 µm) at a flow rate of 0.3 ml min^{-1} and eluted with a linear binary gradient of 15 mM ammonium acetate pH 4.5 (A) and acetonitrile (B) *(*t = 0 min A 70%, t = 15 min A 40%, t = 16 min A 70%, t = 25 min next injection). The parabens were detected with a Sciex API 2000 triple quadrupole mass spectrometer equipped with a heated nebulizer probe operated in the negative ion mode. Optimal setting of the instrument for detection by mass reaction monitoring (MRM) was established empirically by infusion of paraben standards (1 µg ml^{-1}). Chromatographic peaks corresponding to individual parabens were detected automatically and the mass of analyte calculated after interpolation from calibration curves prepared over the working range 1–300 ng ml^{-1} using the Analyst™ (PE Biosystems) software package.
- *Urine:* A sensitive method using high-performance liquid chromatography-tandem mass spectrometry with peak focusing to measure the urinary

concentrations of methyl-, ethyl-, propyl-, butyl-, and benzyl parabens was developed (Ye et al.). A fully automated column-switching system was constructed using one autosampler, two HPLC pumps, and a 10-port switching valve. The parabens present in 100 µL of urine were retained and concentrated on the SPE column, 'back-eluted' from the SPE column, and diluted through a mixing Tee before being separated from other urine matrix components by HPLC and detected by tandem mass spectrometry. The method, validated using pooled urine samples, shows very good reproducibility and accuracy. The efficient preconcentration of the parabens by the SPE column and analyte peak-focusing by the dilution resulted in limits of detection of 0.1-0.2 ng/mL for all of the analytes. These values reflect very good sensitivity of the method compared with the LODs at 2-10 ng/mL from previously published papers. This newly developed method was used for measuring the concentrations of five parabens in 20 urine samples collected from anonymous adult donors. Methyl-, ethyl-, and propyl parabens were detected in all samples.

- *Skin:* Parabens sampled from human skin on forefinger was determined by GC/MS for 10 young subjects (Shibata 2002). The sampling of parabens from forefinger was performed with 75 ml of a 70% ethanol-aqueous solution in a 1.5 ml vial. After the solution was contacted with the skin for 2 min, the sample was dried under a vacuum, followed by GC/MS analysis after adding methyl benzoate as an internal standard. The amounts of methyl-, ethyl-, propyl-, and buthyl-parabens in human skin of forefinger were 3 to 1350, 0.3 to 196, 0.3~516, and 0~52.1 pmol/cm^2, respectively. High amounts of parabens were found for subjects who used a cosmetic liquid for hair and body make up. The increment of parabens during 1 hour was also studied. After several steps of a washing procedure (washing with tap water, immersed in 43°C hot water, washing with ethanol, and finally with distilled water), the first sampling was performed as a blank test; a second sampling was performed after one hour. The value of the increment of paraben was 2.0~6.9 pmol/cm^2, which was estimated from the difference in the amount of parabens at the first and second sampling. These increments are supposed to be derived by the migration of paraben from inside of the body. There is not much difference in the stability of parabens in the skin on

the short term (1 hour period). The present sampling method is very simple and was easily adopted for collecting chemical substrates in human skin.

☑ Performance characteristics

Estimates of recovery of parabens from the extraction procedure for brest cancer tissue were made by spiking samples with benzylparaben, because this was the only paraben not detected in any blank or tumour extract. Analysis by HPLCMS/MS of three extraction blank samples, each spiked with 200 ng of benzylparaben, gave an average recovery of this paraben of 48.5% ± 4.8%. Blanks are reported to occur from sample preparation steps. No information available on inter- and intralaboratory variability.

☑ Validation

Partly validated by internal measures in case of the breast tissue study.

☑ Confounding factors

It is likely that in general, young women are more likely than elderly women to shave their underarms, regardless of whether they later develop breast cancer. Males and children might exhibit different use pattern.

☑ Concentrations reported in literature

Testing of parabens has revealed to varying degrees that individual paraben compounds have weakly estrogenic activity in some in vitro screening tests, such as ligand binding to the estrogen receptor, regulation of CAT gene expression, and proliferation of MCF-7 cells. Reported in vivo effects include increased uterine weight (i.e., butyl-, isobutyl-, and benzylparaben) and male reproductive-tract effects (i.e., butyl- and propylparaben). However, in relation to estrogen as a control during in vivo studies, the parabens with activity are many orders of magnitude less active than estrogen. While exposure to sufficient doses of exogenous estrogen can increase the risk of certain adverse effects, the presumption that similar risks might also result from exposure to endocrine-active chemicals (EACs) with far weaker activity is still speculative. In assessing the likelihood that exposure to weakly active EACs might be etiologically associated with adverse effects due to an endocrine-mediated mode of action, it is paramount to consider both the doses and the potency of such compounds in comparison with estrogen. In this review, a comparative approach involving both dose and potency is used to assess

whether in utero or adult exposure to parabens might be associated with adverse effects mediated via an estrogen-modulating mode of action. In utilizing this approach, the paraben doses required to produce estrogenic effects in vivo are compared with the doses of either 17beta-estradiol or diethylstilbestrol (DES) that are well established in their ability to affect endocrine activity. Where possible and appropriate, emphasis is placed on direct comparisons with human data with either 17beta-estradiol or DES, since this does not require extrapolation from animal data with the uncertainties inherent in such comparisons. Based on these comparisons using worst-case assumptions pertaining to total daily exposures to parabens and dose/potency comparisons with both human and animal no-observed-effect levels (NOELs) and lowest-observed-effect levels (LOELs) for estrogen or DES, it is biologically implausible that parabens could increase the risk of any estrogen-mediated endpoint, including effects on the male reproductive tract or breast cancer (Golden 2005). Additional analysis based on the concept of a hygiene-based margin of safety (HBMOS), a comparative approach for assessing the estrogen activities of weakly active EACs, demonstrates that worst-case daily exposure to parabens would present substantially less risk relative to exposure to naturally occurring EACs in the diet such as the phytoestrogen daidzein.

Table 13.1: Concentrations reported in literature

Table —The HPLCMS/MS analysis of parabens in 20 human breast tumours[a]

Tumour extract	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Mean	SEM
Benzylparaben	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Isobutylparaben	5.2	0.1	5.2	3.3	2.8	3.5	2.5	1.9	4.9	2.2	1.7	3.9	2.2	2.3	1.2	2.0	1.7	0.0	1.0	0.0	2.4	0.4
n-Butylparaben	15.3	7.2	29.5	22.4	14.3	15.5	9.8	6.7	10.3	7.4	2.4	14.7	7.1	6.7	7.4	7.2	5.3	3.1	5.7	3.2	10.1	1.5
n-Propylparaben	6.5	7.9	18.6	18.4	10.9	17.9	10.0	10.5	11.6	6.9	9.1	16.5	10.2	17.2	10.1	15.4	4.2	5.3	5.5	5.6	10.9	1.1
Ethylparaben	7.2	3.0	9.6	6.3	4.6	2.3	1.9	1.8	7.0	3.2	1.8	6.4	2.1	4.5	1.1	2.2	1.8	0.7	2.0	2.1	3.6	0.6
Methylparaben	34.2	20.6	53.0	49.9	34.4	37.6	27.3	19.6	35.7	16.0	17.2	36.4	21.6	39.6	18.5	28.8	36.7	8.2	12.0	10.7	27.9	2.8
Total paraben	68.3	38.8	115.9	100.3	67.0	76.9	51.4	40.4	69.5	35.7	32.1	77.9	43.1	70.3	38.3	55.6	49.7	17.3	26.2	21.6	54.8	5.8
Blank value	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Mean	SEM
Benzylparaben	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Isobutylparaben	0.0	0.0	0.0	0.0	0.0	2.2	2.2	2.2	2.2	2.1	2.1	2.1	2.1	2.1	2.3	2.3	2.3	1.1	1.1	1.1	1.3	0.4
n-Butylparaben	6.5	6.5	18.0	18.0	18.0	9.3	9.3	9.3	9.3	4.0	4.0	4.0	4.0	4.0	8.2	8.2	8.2	2.2	2.2	2.2	8.0	2.3
n-Propylparaben	2.0	2.0	13.4	13.4	13.4	12.0	12.0	12.0	12.0	6.8	6.8	6.8	6.8	6.8	8.4	8.4	8.4	4.8	4.8	4.8	7.9	1.8
Ethylparaben	1.9	1.9	2.2	2.2	2.2	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	0.0	0.0	0.0	1.6	1.6	1.6	1.6	0.3
Methylparaben	10.1	10.1	27.8	27.8	27.8	20.5	20.5	20.5	20.5	10.3	10.3	10.3	10.3	10.3	11.6	11.6	11.6	9.9	9.9	9.9	15.0	3.0
Total paraben	20.5	20.5	61.4	61.4	61.4	45.9	45.9	45.9	45.9	25.0	25.0	25.0	25.0	25.0	30.6	30.6	30.6	19.6	19.6	19.6	33.8	6.8
Tumour less blank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Mean	SEM
Benzylparaben	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Isobutylparaben	5.2	0.1	5.2	3.3	2.8	1.3	0.3	-0.3	2.7	0.2	-0.4	1.8	0.1	0.2	-1.1	-0.3	-0.6	-1.1	-0.1	1.1	0.9	0.4
n-Butylparaben	8.8	0.7	11.5	4.4	-3.7	6.2	0.5	-2.6	1.0	3.4	-1.6	0.7	3.1	-2.7	0.8	-1.0	-2.9	1.0	3.5	1.0	2.3	1.0
n-Propylparaben	4.5	5.9	5.2	5.0	-2.5	5.9	-2.0	-1.5	-0.4	0.2	2.3	9.7	3.4	10.4	1.7	7.0	-4.3	0.5	0.7	0.8	2.6	0.9
Ethylparaben	5.3	1.2	7.4	4.1	2.4	0.5	0.0	-0.1	5.1	1.3	-0.2	4.5	0.2	2.6	1.1	2.1	1.8	-0.9	0.4	0.5	2.0	0.5
Methylparaben	24.1	10.5	25.2	22.1	6.6	17.1	6.8	-0.9	15.2	5.7	6.9	26.1	11.3	29.3	6.9	17.2	25.1	-1.8	2.1	0.8	12.8	2.2
Total paraben	47.9	18.4	54.5	38.9	5.6	31.0	5.5	-5.5	23.7	10.7	7.1	52.8	18.1	45.3	7.7	25.0	19.1	-2.3	6.6	2.0	20.6	4.2

[a] Paraben extractions were performed in small groups such that each group contained between two and five tumour samples together with one corresponding blank extraction. The blank extraction was performed with all procedures identical except for the omission of tumour material. Results are shown in ng g-1

tumour for the 20 extractions and for the corresponding blank values. The concentrations of parabens in the 20 tumours were then each corrected by subtraction of the corresponding blank value.

☑ Dose –response/effect relationships

Competitive inhibition of [3H]oestradiol binding to MCF7 cell oestrogen receptors could be detected at 1,000,000-fold molar excess of n-butylparaben (86%), n-propylparaben (77%), ethyl-paraben (54%) and methylparaben (21%). At concentrations of 10(-6)M and above, parabens were are able to increase expression of both transfected (ERE-CAT reporter gene) and endogenous (pS2) oestrogen-regulated genes in these cells (Byford 2002). They could also increase proliferation of the cells in monolayer culture, which could be inhibited by the antiestrogen ICI 182,780, indicating that the effects were mediated through the oestrogen receptor. However, no antagonist activity of parabens could be detected on regulation of cell proliferation by 17 beta-oestradiol at 10(-10)M. Molecular modelling has indicated the mode by which paraben molecules can bind into the ligand binding pocket of the crystal structure of the ligand binding domain (LBD) of the oestrogen receptor alpha (ERalpha) in place of 17beta-oestradiol; it has furthermore shown that two paraben molecules can bind simultaneously in a mode in which their phenolic hydroxyl groups bind similarly to those of the meso-hexoestrol molecule. Future work will need to address the extent to which parabens can accumulate in hormonally sensitive tissues and also the extent to which their weak oestrogenic activity can add to the more general environmental oestrogen problem.

☑ Time trend, geographical variation, susceptible groups

A carefully-designed epidemiologic study of this issue published in 2002 compared 813 women with breast cancer and 793 women without the disease found no relationship between breast cancer risk and antiperspirant use, deodorant use, or underarm shaving.

Another study published in 2003 reported that women who used underarm antiperspirant and shaved their underarms tended to be diagnosed with breast cancer at a younger age than women who do not. However, the study design did not include a control group of women without breast cancer and has been criticized by experts as not relevant to the safety of these underarm hygiene

practices. It is likely that in general, young women are more likely than elderly women to shave their underarms, regardless of whether they later develop breast cancer.

Parabens can be an ingredient in baby lotions, shampoos, and other personal care products for children.

15 PESTICIDES

☑ General information

Pesticides are a heterogeneous class of chemicals, having in common that they have been specifically designed for the control of pests, weeds or organisms causing plant diseases. This implies that pesticides are purposely toxic, although intended to be harmful selectively against certain organisms, without causing adverse effects to non-target ones.

However, a perfect selectivity is a very difficult goal to achieve, and most pesticides present a significant toxic risk to ecosystems and also to humans.

As a high number of pesticides are currently marketed (about 890 active ingredients, in more than 20000 commercial products, only in the USA), or, being persistent toxicants (presently banished in developed countries) have been heavily used in the past, and as they still constitute an essential factor to maintain the modern agricultural productivity, all people are exposed to them, through environmental contamination or occupational use.

The general population is exposed to pesticides, or their degradation products, through air, water, and particularly food. Moreover, pesticides are the most frequent method of self-poisoning in the developed world.

Occupational exposure occurs during formulation, manufacture or application, and often involves contact with complex mixtures of chemicals, including by-products, solvents, and even degradation compounds produced during storage.

Biomonitoring of the exposure to pesticides may be approached through direct analysis of the pesticide itself, or its metabolites, in biological matrices, or by the modification of certain enzymatic activities; the toxic effects are assessed by means of general biomarkers, as, for instance, genotoxicity assays.

We will briefly review the most relevant of these biomarkers.

15.1 INHIBITION OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE

☑ General information

For organophosphate insecticides (OP), a number of well-established measurements exist to assess exposure and early biological effects. These include measurements of OP metabolites in the urine, of cholinesterase (BChE) activity in plasma, of AChE activity in red blood cells, and of NTE in lymphocytes

Organophosphate and carbamate insecticides inhibit the activity of a group of enzymes known as cholinesterases (ChE). The toxicity of these chemicals is mainly induced through the inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) (Costa, 2006). This enzyme hydrolyses the neurotransmitter acetylcholine in synapses of the CNS, the autonomic nervous system and at neuromuscular junctions of the voluntary nervous system. When AChE is inhibited, acetylcholine is not hydrolyzed and accumulates at cholinergic sites, causing alterations in the normal nervous system function. In addition to binding to and inhibiting synaptic AChE, OPs and carbamates also inhibit red blood cell acetylcholinesterase and a different enzyme, butyrylcholinesterase (BChE, EC 3.1.1.8), located in the plasma of vertebrates. Inhibition of these easily accessible enzymes has been proposed as biomarkers of exposure and, in some circumstances, of effect in the biomonitoring of populations occupationally exposed to these insecticides (Wilson et al, 2005; Nigg & Knaak, 2000; Lotti, 1995).

OPs have been the major insecticides in number and market share for many decades, and they are still essential tools in crop protection and public health. Their selective toxicity is based on specificity differences in the AChE targets, more rapid detoxification in mammals than insects, and the use of pro-insecticides undergoing preferential activation in insects as compared with mammals. Their facile biodegradation and low environmental persistence are coupled with toxic effects more likely due to acute rather than chronic exposure.

OPs react covalently with AChE resulting in irreversible inhibition and the formation of a phosphorylated enzyme. Inhibition of AChE in the synapse results in a build up of acetylcholine (ACh) in the synaptic cleft and in the vicinity of the ACh receptor. This, in turn, causes repetitive firing of cholinergic neurons that subsequently leads to clinical symptoms of cholinergic intoxication such as salivation, tremors, ataxia, and lethargy. Over stimulation at muscarinic synapses results in hypersalivation, excess lacrimation, miosis, intestinal cramps, vomiting, diarrhea, urinary and fecal incontinence, bronchorrhea and bronchoconstriction. Over stimulation at nicotinic synapses results in muscle cramps, fasciculation, weakness, paralysis and pallor. Central nervous system (CNS) effects include anxiety, restlessness, dizziness, confusion, ataxia, convulsions and respiratory and circulatory depression. Effects may be immediate or delayed by hours or days. Carbamate insecticides act in a similar way; however, the inhibition of AChE is reversible and, normally, the margin between doses that produce first cholinergic symptoms and doses with potential lethal effects is wider than for OPs.

The severity and onset of signs of intoxication are determined by the compound, the dose, frequency and duration of exposure, location of different cholinergic synapses within an organism, the timing of their inhibition, and the pharmacokinetics involved in the activation and detoxification of organophosphates by different organs in the body. In addition, the build up of ACh in the synaptic cleft can alter receptor binding, density, and choline uptake kinetics.

OP compounds can be grouped into direct and indirect inhibitors of AChE. Direct inhibitors are effective without any metabolic modification after absorption into the body. Indirect inhibitors need to be transformed into the body to be effective. For instance, since thiono OP pesticides contain a P-S bond (phosphorothioates and phosphorodithioates), they cannot act as direct inhibitors of AChE, and they require activation by oxidation of the P-S to the P-O group (”oxon”), thus producing the corresponding oxygen analogues. The reaction is catalyzed by multiple cytochrome P450 enzymes (CYPs). Although the liver has the greatest capacity to perform the reaction, this bioactivation may also occur in other tissues,

including lung and brain. The practical importance of this classification is that signs and symptoms caused by direct inhibitors appear quickly, immediately during or after exposure, so providing an early warning, whereas the onset of symptoms and signs because of indirect inhibitors is slower and the effects last longer after exposure. Catalytic hydrolysis of the OPs by phosphotriesterases known as A-esterases (which are not inhibited by OPs) also plays an important role in the detoxication of certain OPs. One example is the enzyme paraoxonase (PON1) which hydrolyzes the oxons of chlorpyrifos and diazinon, and, at least in vitro, also of parathion. Genetically determined variations in biotransformation enzymes, target molecules or cellular repair processes can modify the individual response to OPs.

The use of AChE and plasma BChE inhibition as biomarkers in case of OP exposure has been debated for some time. Some major issues can be summarized in the five following points: (1) Different OPs may inhibit AChE or BChE to a different degree. For example, the oxygen analogues of malathion, diazinon, chlorpyrifos or dichlorvos are stronger inhibitors of plasma BChE than of RBC AChE. (2) There is a high degree of variability of enzyme activity (particularly of BChE) among individuals (in part due to genetic differences; see below). This requires establishment of a baseline value for each individual, or in case this is not available, of repeated post-exposure measurements to determine possible changes back toward baseline. (3) RBC AChE activity is better correlated with target tissue AChE, than is plasma BChE activity, as indicated by a few animal studies. (4) Neither measurement is specific for a certain OP, and indeed, other insecticides such as carbamates also inhibit AChE and BChE. (5) A 30% or greater decrease from pre-exposure baseline requires health and workplace surveillance and removal of the worker from exposure, yet the toxicological significance of such a decrease is unclear. Despite these caveats, measurements of plasma BChE and, particularly of RBC AChE, remain a very valid way to determine exposure to, and early biological effects, of OP exposure.

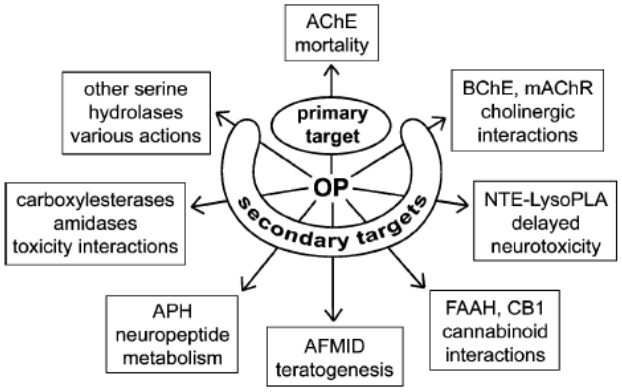
BChE has no known physiological role in neurotransmission and differs from AChE in substrate affinity, chemical structure and binding sites. In human serum,

BChE is the predominant (99%) cholinesterase, whereas in rat serum there is an approximate equal distribution of the two enzymes. BChE is not involved in the neurotoxicity of OP insecticides. BChE can be significantly inhibited in humans without causing any clinical signs of cholinergic toxicity. By scavenging OPs, BChE can guard against their toxicity, as the OP would be unavailable for reaction with the primary target AChE. In a human study, where volunteers were given a single oral dose of 0.5 mg/kg of chlorpyrifos, plasma BChE activity was depressed by 85% from control values, and yet no signs of cholinergic toxicity or changes in RBC AChE activity were observed (Chen et al, 1999; Nolan *et al.*, 1984). The high efficiency of BChE to bind OPs and carbamates and the ease of obtaining human plasma makes BChE an enzyme that can be conveniently monitored to confirm exposure to cholinesterase inhibitors, even though there is no evidence that inhibition of BChE is related to any relevant pharmacological or toxicological adverse effect. The lack of relevancy to the neurotoxicity of OPs and carbamates makes inhibition of plasma BChE a poor choice as an indicator of OP toxicity. RBC ACHE, which is the same molecular target as that responsible for acute OPs and carbamates toxicity in the nervous system, is a more specific indicator than BChE.

Organophosphates also bind to a wide variety of other esterases present in blood and/or tissues; the specific esterases involved and the affinity of binding being dependent upon the species and specific organophosphate. Such binding is not directly related to the neurotoxic consequences of these compounds but may affect their overall potency. In some cases (e.g. parathion) nonspecific binding actually protects against neurotoxicity because it prevents compounds from reacting with neuronal AChE. Most safety evaluations of OP insecticides are based on the premise that AChE inhibition is the principal action for both acute and chronic toxicity of individual compounds or mixtures. Clearly, OPs act on non-AChE systems raising questions on the contribution of these alternate targets to the acute lethal action and secondary effects of short- or long-term exposure. What are the safety aspects of non-AChE secondary targets? Some are well known, as the

delayed neuropathy related to neuropathy target esterase (NTE) phosphorylation; other are subject of current research (Casida and Quistad, 2004).

Figure 14.1: Primary and secondary toxicity targets of organophosphates (Casida and Quistad, 2004)



Some organophosphates are undergoing increasing scrutiny and restriction because of their propensity to elicit developmental neurotoxicity (Slotkin, 2006; Aluigi et al, 2005; Barone et al. 2000; Casida and Quistad, 2004; Landrigan 2001; Rice and Barone,2000; Slotkin 2004). Originally, it was thought that the adverse effects on brain development reflected the same basic mechanism that underlies systemic toxicity, namely, cholinesterase inhibition and consequent cholinergic hyper stimulation. However, evidence accumulating over the past decade implicates a host of other mechanisms that depend instead upon the direct targeting of events specific to the developing brain (Barone et al. 2000; Rice and Barone 2000; Slotkin 2004). Chlorpyrifos, the most-studied organophosphate, has been shown to disrupt the basic cellular machinery that controls the patterns of neural cell maturation and the formation and activity of synapses, exclusive of the effects on cholinesterase, which are mediated instead by its metabolite, chlorpyrifos oxon (Barone et al.2000; Casida and Quistad 2004). These

mechanisms are likely to be shared by other organophosphates, but these have not been evaluated in detail.

Whether young humans are more sensitive than adults to the cholinesterase inhibition and neurotoxicity produced by the organophosphate and carbamate pesticides is a question with implications for risk assessment. The answer to this question will guide the decision as to whether an additional safety factor is required for age-related effects. This issue has been addressed by dosing young and adult laboratory animals according to the same protocol, and looking for changes in behavior, acetylcholinesterase (AChE) activity, and other relevant endpoints. However, to use these data for human risk assessment, it is important to validate that the postnatal model is a good one for predicting responses in the postnatal human.

Basically, three approaches have been applied to detect exposure to an OP compound (Aprea et al, 2002):

- The oldest method comprises measurement of RBC AChE or BChE activity. The original colorimetric Ellman procedure is generally used for occupational health screening and therapeutic monitoring of pesticide-poisoned patients. It suffers from serious drawbacks, since (i) it does not identify the OP compound, (ii) the specificity of the method is low, i.e., the interference with various unrelated chemicals is high,(iii) it does not provide reliable evidence for exposures at inhibition levels less than 20%, which is due to both substantial intra- and interindividual variations while control activity levels are often not available, and (iv) it is less suitable for retrospective detection of exposure due to de novo synthesis of enzyme.
- A second family of methods is based on measurement of the hydrolysis products of nerve agents, e.g., *O*-alkyl methylphosphonic acids. Methods for analysis of these compounds are based on GC-MS, GC-MS-MS or LC-MS. The advantage of determination of hydrolysis products, compared to the first method, is the partial identification of the nerve agent. A serious drawback, however, is the rather rapid elimination rate of the hydrolysis product from the

organism (within several days), which limits its use for retrospective detection of exposure. These biomarkers are reviewed in other section of this text.

- The third method relies on analysis of modified BChE (Fidder et al, 2002) . Nerve agents react rapidly with the serine-198 residue in the active site, under formation of a phosphate or phosphonate ester. Rather long half life times for this enzyme have been reported, ranging from 5-16 days, and its concentration in plasma is approximately 80 nM, making it a persistent and abundant source for biomonitoring of exposure to OPs. Several procedures for analysis of phosphylated BChE in plasma or serum samples have been developed, which are reviewed in other section of this paper. The advantages of these methods are: 1) in contrast with the determination of intact agent or its metabolites, the larger in vivo lifetime of phosphyl moieties bound to BChE; 2) in contrast with the inhibition of ChE, a major sensitivity in detecting low levels of exposure.

☑ Matrix

Blood (AChE: whole blood ; BChE: plasma or serum)

☑ Kinetics

- **Uptake**

  Not applicable

- **Metabolism**

  Not applicable

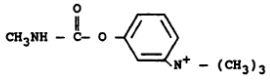
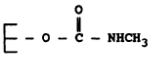
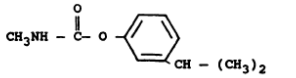
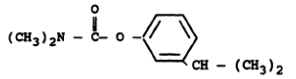
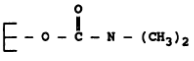
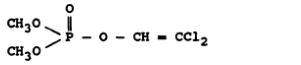
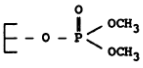
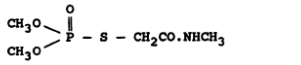
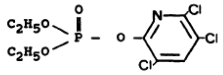
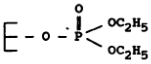
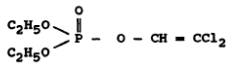
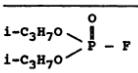
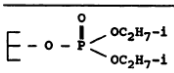
- **Biological half-life**

  Inhibition of cholinesterases is reversed by two mechanisms: a) hydrolysis of the inhibitor – serine bond, and b) neo-synthesis of ChE.

  Both substrate and inhibitors react covalently with the esterase in essentially the same manner because acetylation of the serine residue at the AChE catalytic center is analogous to phosphorylation and carbamylation.In contrast to the acetylated enzyme, which rapidly yields acetic acid and restores the catalytic center, the phosphorylated enzyme is stable. Carbamylated AChE restores its catalytic activity more slowly than acetylated AChE but more

rapidly than the phosphorylated enzyme. However, inhibited AChE might also reactivate spontaneously when inhibited by OPs. Reactivation requires several hours, as with dimethoxyphosphorylated AChE, or does not occur at all, as with AChE phosphorylated by OPs with secondary y or tertiary alkyl groups. For these reasons, carbamates and dimethyl organophosphates are considered reversible inhibitors, whereas other OPs are irreversible. The loss of one alkyl group, through a nonenzymatic process called aging, further enhances the stability of the phosphorylated enzyme.

Figure 14.2 (Lotti M, 1995)

	Chemical	Inhibition[a] (AChE I_{50}, M)	Inhibited AChE	Spontaneous reactivation ($t_{½}$ hours)	Aging ($t_{½}$, hours)
1		1.2×10^{-11}		0.5	NO
2		6.4×10^{-8}			
3		1.1×10^{-5}		0.9	NO
4		9.5×10^{-7}		0.85	3.9
5		1.4×10^{-4}			
6		7×10^{-9}		58	41
7		4.1×10^{-7}			
8		8.3×10^{-7}		no reactivation at 6	4.6

When AChE is irreversibly inhibited by OPs in erythrocytes, the recovery toward normal values depends on new cells entering the bloodstream, and has been calculated for most OPs to correspond to 1%/day (Gallo & Lawryk,

1991). The half-life of AChE resynthesis in the nervous system has been estimated from animal data to be 5-7days (Lotti, 1992). Therefore, the enzyme is restored in brain more rapidly than in erythrocytes. When AChE is inhibited by carbamates, its recovery is faster because of spontaneous reactivation in the nervous system and in erythrocytes.

☑ Sampling conditions

As a result of the natural variations of RBC ACHE and BChE levels in healthy people, these tests have some limitations when used to monitor human OP exposure. The sensitivity of detection of low-level inhibition can be increased by adopting individual pre-exposure values as a reference. Three sequential samples are recommended to establish a baseline value of CHE activity (WHO, 1982c). Post-exposure sampling should be performed not later than 2 h after the end of exposure. Venipuncture should be preferred, although this requires trained personnel because capillary blood from a finger or ear-lobe can be contaminated by pesticide residues present on the skin.

☑ Analytical aspects

- **Techniques**

  Several analytical methods are available to determine ACHE and BChE in blood. They have been extensively reviewed by Coye et al. (1986b), Dillon and Ho (1987), Jaeger (1987) and Gallo and Lawryk (1991). Most of these methods yield comparable results, but their accuracy and precision may vary. In fact, some methods have a different sensitivity scale, so that a given percentage of inhibition may not coincide, in terms of absolute activity, with the same percentage of inhibition measured with a different technique.

  Methods for the detection of cholinesterase activity can be divided into four groups: electrometric and colorimetric, which are used in field surveillance and research in developed countries; titrimetric and tintometric, mainly used in developing countries. Titrimetry is extremely accurate and precise but is rarely used because of its high cost and complexity. Electrometric method is less sensitive than colorimetric method and less accurate than titrimetric methods.

Tintometric method is main-used in the field. The colorimetric method developed by Ellman et al.(1961) measures cholinesterase activity on substrates such as acetylcholine and butyrylthiocholine. Thiocholine released through hydrolysis reacts with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) to form a yellow anion (5-thio-2-nitrobenzoate), which is measured by spectrophotometric analysis at 405 nm. This method (available in two kits, for laboratory and field use) has been evaluated by various authors.

According to the WHO, the Ellman spectrophotometric determination (Ellman et al., 1961) should be considered as the reference method. A modified version of this method was used in a multinational epidemiological study on neurotoxic effects of OP exposure conducted in Europe under the co-ordination of the Office for Europe of WHO and the sponsorship of the United Nations Development Programme. By means of these methods, CHE activity can be measured in whole blood and plasma; CHE activity of RBC is calculated considering the difference between whole blood and plasma activities. Under field conditions, whole blood CHE can be measured using a field kit, such as the Tintometer method. More recently, paper-test methods have also been developed for screening purposes and field use in agriculture. These methods, though only semiquantitative, have the advantages of low cost and ease of application.

- **Sensitivity and specificity**

  As a result of the natural interindividual variations of RBC ACHE and BChE levels, these biomarkers are insensitive for monitoring of general non-occupationally exposed population.

  In the “Reference values” section, factors that can modify cholinesterase activity are described.

- **Units**

  Enzymatic activity, expressed as mol (product)/min/ml (plasma, serum, whole blood, RBC volume). RBC AChE activity can also be expressed normalized to Hb. As in any enzymatic assay, the activity is very dependent of the conditions of the assay (substract, pH, temperature, etc.).

☑ Performance Characteristics

- **Analytical reproducibility**

  The reference methods are those based on that of Ellman (1961). Different improved versions have been proposed, and their reproducibility is variable.

- **Inter and intra-laboratory variability**

  A laboratory kit showed a RSD of 3.8 (precision between series) in detecting erythrocyte cholinesterase. The mean RSD between series of the field kit was 4.1% for erythrocyte cholinesterase and 5.6% for blood cholinesterase (Aprea et al, 2002). In the standard operating procedure developed by Deutsche Forschungsgemeinschaft (Lewalter, 1991), RSD values of 5.8 and 10.6% within-series imprecision are reported for AChE and BChE respectively, and 14.0% (between-day imprecision) for BChE; LOD was 235 U/ l.

☑ Concentrations reported in the literature

See the *refererence values* section

- **Reference values**

  ACHE and BChE levels may vary in healthy people according to interindividual differences or specific physio-pathological conditions (Table I). In the general population, interindividual coefficients of variation are about 15–25% for PCHE and 10–18% for erythrocyte ACHE. Corresponding figures of intraindividual variation over time are 6 and 3–7%, respectively. A significant portion of these variations may be attributed to the laboratory technique, instrument, and analyst. There is no significant difference in ACHE activity associated with sex, whereas BChE activity is significantly higher in males than in females. ACHE and BChE activities do not change with age in adults. Infants aged less than 6 months have ACHE values lower than adults. Significant positive correlations have been found between BChE activity and body mass or serum cholesterol. In females, lower values of BChE activity are measured during menstruation and pregnancy. BChE activity drops significantly during the first trimester of pregnancy and then rises, but slightly and non significantly, in the third trimester. BChE activity has been reported to

be lower in the black race than in the white race although it is not known whether genetic or nutritional factors are responsible for this difference. Some pathological conditions may affect cholinesterase activity: ACHE can be depressed by certain cancers, leukaemias, and in paroxysmal nocturnal hemoglobinuria. If a correction for hematocrit is not carried out, the apparent ACHE activity determined in whole blood may decrease in the presence of anaemia. Low values of BChE may be found in liver diseases, uraemia, neoplasms, heart failure, allergic reactions and acute infections. Increased values of ACHE may be measured in polycythemia, thalassemia and other blood dyscrasias, in hyperthyroidism and other conditions characterized by high metabolic rate. Since most of these conditions are not compatible with work, they rarely contribute to the depression of CHE observed among occupationally exposed subjects. Several drugs, such as chloroquine and other antimalarian drugs, caffeine, xanthine-related compounds, and narcotic analgesics depress BChE activity. Also certain chemicals, namely organic mercury compounds, carbon disulphide, and benzalkonium salts, can depress BChE. ACHE can be depressed by a few drugs, including echothiophate, quinine and other antimalarian drugs (Dillon and Ho, 1987).Table xxx reports normal ACHE and BChE values measured in healthy unexposed subjects using the most common and widely used analytical methods.

As a result of the natural variations of RBC ACHE and BChE levels in healthy people, these tests have some limitations when used to monitor human OP exposure. The sensitivity of detection of low-level inhibition can be increased by adopting individual pre-exposure values as a reference. WHO (1982c) recommended calculating an individual pre-exposure baseline as the average of three values. After exposure, CHE activities have to be compared with the individual baseline values and expressed as percentage change. When the pre-exposure CHE levels are not known, the values after exposure have to be compared with the CHE activity of a reference population. In this instance, the diagnosis of CHE inhibition based on a single post-exposure value may be difficult because of the wide variation in normal CHE activity. In fact, values at the

upper limit of the normal range may be 200% greater than those at the lowest one. Thus, patients with originally high values may lose half of their CHE activity, but the activities may still be above the lowest normal limit.

Table 14.1: Validation of ACHE and PCHE activities in healthy subjects and in specific physio-pathological conditions. Cholinesterase activities of healhty subjects without exposure to OP measured with selected methods.(WHO, 1982c)

Variation of ACHE and PCHE activities in healthy subjects and in specific physio-pathological conditions

Condition	ACHE	PCHE
Healthy subjects		
Interindividual variation	10–18%	15–25%
Intraindividual variation	3–7%	6%
Sex differences	No	10–15%↑ male
Age	↓ up to 6 h month old	
Body mass		Direct correlation
Serum cholesterol		Direct correlation
Seasonal variation	No	No
Circadian variation	No	No
Menstruation		↓
Pregnancy		↓
Race		↓ black person
Pathological conditions		
Reducing activities	Leukemia, neoplasm	Liver disease, uraemia, cancer, heart failure, allergic reactions, acute infections, anemias
Increasing activities	Polycitemia, thalassemia, other congenital blood dyscrasias	Hyperthyroidism, conditions of high metabolic rate

Cholinesterase activities of healthy subjects without exposure to OP measured with selected methods[a]

Method (unit)	ACHE	PCHE	Reference
Michel			Laws et al., 1967
Man (ΔpH/h)	0.77 ± 0.08	0.95 ± 0.19	
Woman (ΔpH/h)	0.75 ± 0.08	0.82 ± 0.19	
Titrimetric			Laws et al., 1967
Man/woman (μmol/min per ml)	13.2 ± 0.31	4.90 ± 0.02	
Ellman's modified			Alcini et al., 1988
Man (UI/ml)	4.01 ± 0.65	3.03 ± 0.66	
Woman (UI/ml)	3.45 ± 0.61	3.03 ± 0.68	

[a] Values are mean ± S.D.

- **Critical values**

  Given the large inter- and intraindividual variation of erythrocyte AChE activity, a marginal inhibition is impossible to assess unless pre-exposure values have been determined on each subject. The intraindividual CV of erythrocyte AChE is-10%, whereas the interindividual CV is 10-40%.Gallo

and Lawryk *(1991)* reported that one measurementof erythrocyte AChE before exposure must detect a minimum of 15% inhibition to be statistically significant.

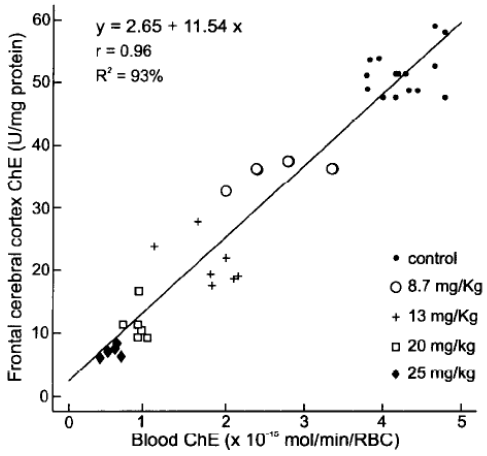
☑ Dose/effect relationships

Cholinesterase inhibition in either the nervous system or muscle is accepted as an adverse effect because cholinesterase activity in these target tissues is known to participate in neurotransmission. Cholinesterase activity in the blood however, apparently does not take part in neurotransmission and therefore, inhibition of circulating cholinesterase activity is typically classified only as a marker of exposure to organophosphate or carbamate compounds. Consequently, the risk assessor is faced with at least two very important questions: (1) does the level of cholinesterase inhibition in the blood reflect the inhibition in target tissues? and (2) do the levels of cholinesterase inhibition in the blood or target tissue correlate with clinical or behavioural alterations?

Some works indicate a good correlation between blood AChE and brain AChE activity (Fossi et al. 1992; Padilla et al. 1994) and seem to contradict earlier studies that pointed to a non dependable correlation (Jimmerson et al. 1989). The compound tested and the time between the toxicant administration and the analysis in blood and tissues are factors that seem to have a relevant influence in the results. Several factors may contribute to the apparently contradictory results found in the literature. First, differences in the experimental methodology, since the enzymes measured are plasma ChE in some cases, erythrocyte ChE in others and whole blood ChE in others. It is generally reported that ChE activity in whole blood and in erythrocytes correlates better with the activity in target tissues than plasma ChE (Padilla et al. 1994). Second, besides the common anti-cholinesterase effect, differences in the mechanisms of activation and/or detoxification among organophosphates exist (Costa 1988). Since the results reported in the literature were obtained with different compounds, this may lead to differential conclusions. Third, the different periods between the administration of the tested compound and the enzymatic determinations may also explain contradictory results found in the literature, since the maximum AChE inhibition does not occur at the same

time for all the organophosphates (Gordon 1994). Furthermore, the recovery rate of inhibited enzymes depends of the tested compound and for the same compound may differs with the species (Chaudhuri et al. 1993). Finally, inter-specific differences in susceptibility to organophosphates (Quadri et al. 1994) may contribute to different results.

Fig. 14.3: ChE activity in frontal cerebral cortex as a function of ChE activity in whole blood, 24 hours after the administration of several doses of parathion. Since there was no significant differences in blood ChE activity and in frontal cerebral cortex ChE activity between non-treated animals and rats injected with the vehicle, both groups were considered as control and pooled together. The doses indicated in the figure are expressed as mg of parathion per Kg of body weight. (Guilhermino L, 1998)



There appears to be a threshold level of AChE inhibition required before any clinical signs or symptoms occur. When rats are given a single oral dosage of chlorpyrifos (10 mg/kg), RBC AChE activity is inhibited by more than 90%,and brain AChE is inhibited by more than 40%; yet there are no observable functional effects on motor activity in these animals (Nostrandt *et al.*, 1997).Changes in gait, body temperature, and motor activity are not observed in rats having less than 60% inhibition of brain AChE following administration of chlorpyrifos, even though these are the most sensitive behavioral measurements of cholinergic

toxicity (Bushnellet *al.*, 1993; Nostrandt *et al.*, 1997). Only at the higher dosages of chlorpyrifos, where brain AChE is inhibited by greater than 60%, are overt signs of cholinergic toxicity expressed.

There is a poor correlation between the time course for onset and recovery of AChE inhibition and the clinical and/or behavioral expression of cholinergic toxicity. When animals are administered large doses of chlorpyrifos which result in functional deficits in working memory and motor function, these functional parameters of cholinergic toxicity occur as much as 2 days after the inhibition of AChE activity. In addition, recovery from these functional deficits occurs long before AChE activity is fully restored (Nostrandt *et al.*, 1997).This lack of correlation between the onset of inhibition of AChE activity and the onset and recovery of clinical or behavioral cholinergic effects are indicative of pharmacokinetic processes that moderate the toxicity of chlorpyrifos. Studies have shown that animals can rapidly develop functional tolerance to OP compounds, due to neurochemical compensation for the inhibited enzyme. This compensation may involve down regulation of central muscarinic receptors, or other compensatory mechanisms operating, even in the presence of significant inhibition of brain AChE (Bushnell *et al.*, 1993). Down regulation of central muscarinic receptors following exposure to acutely toxic dosages of chlorpyrifos is believed to increase the tolerance of animals to chlorpyrifos and serve as a protective mechanism (Bushnell *et al.*, 1993, 1994; Costa *et al.*, 1982; Moser and Padilla, 1998).

In conclusion, the correlation between AChE inhibition in erythrocytes and that in nervous system depends on the pharmacokinetics of the compound, i.e., how effectively it crosses the blood-brain barrier, as well as on how long after exposure the inhibition is measured.

In workers moderately exposed to OP compounds for several weeks, clinical signs of intoxication may appear only at an ACHE inhibition level of 85–90% of the pre-exposure values. On the other hand, a previously unexposed individual may develop symptoms after a sudden exposure and experience a rapid drop in cholinesterase activity of less than 30%. Based on these findings, the severity of

illness following repeated exposures may not be proportionally related to the degree of ACHE inhibition. This can be partly explained by the ability of the body to adapt to increased concentrations of acetylcholine, developing a sort of tolerance at nerve endings, attributable to an increase in the cholinesterase production in response to OP inhibition (Gallo and Lawryk, 1991).

Potency is also affected by AChE binding reversibility. Recovery of AChE activity is fast in the case of carbamates because of the reversible non-covalent binding. For OPs recovery occurs in some cases via hydrolytic removal of the inhibiting phosphoryl moiety. Like the rate of binding, the rate of hydrolytic removal varies depending upon the specific compound. Some phosphorylated–AChE complexes are characterized by a process termed 'aging' in which the rate of reactivation declines after exposure due to dealkylation of the phosphorylated–AChE (Ballantyne andMarrs, 1992). When this happens, reactivation of phosphorylated AChE is not possible. De novo synthesis of the AChE enzyme itself, which occurs within 12 and 24 h depending on the anatomical location, also leads to recovery of AChE activity (Chambers, 1992).These toxicodynamic factors alone, however, are not the only determinants of organophosphate potency. Also important are their toxicokinetic characteristics. For most organophosphates, absorption is rapid and complete, or nearly complete, via dermal, oral or inhalation exposure, and distribution is usually extensive. Metabolic reactions, which both detoxify and toxify these compounds, however, are often highly complex and variable. Elimination half-lives, which vary from minutes to days, are also highly variable. The overall balance between metabolic generation and elimination of more versus less potent toxic derivatives is increasingly recognized as a major factor determining organophosphate potency. Additionally, differences in the overall balance between generation and elimination of more versus less potent toxic derivatives following oral exposures compared to inhalation exposures, due to first pass hepatic metabolism, can contribute to difference sin potency for the same compound when administered by different routes.

Despite these considerations, since RBC AChE inhibition involves the molecular target also responsible for the neurotoxic effects of organophosphates, significant

inhibition of RBC AChE indicates the potential for an adverse effect and also function as a biomarker for exposure (Padilla et al., 1994; Padilla, 1995; EPA, 1998a; Mileson et al., 1998).Therefore, RBC AChE inhibition is regarded as a defensible common endpoint for organophosphate health risk assessment. For occupational exposures this is reflected in the recommendation of the American Conference of Governmental Industrial Hygienists (ACGIH) that RBC AChE activity in workers exposed to organophosphates be monitored (ACGIH, 1991). When RBC AChE activity decreases to 70% of an individuals baseline, the ACGIH has concluded that the potential for overexposure to organophosphates exists and adverse effects may occur. Consequently, ACGIH advises that organophosphate exposures of workers experiencing this degree of RBC AChE inhibition be prevented until RBC AChE activity returns to baseline.

It must be noted that ACHE inhibition is relevant only to acute OP toxicity; thus, the assessment of ACHE inhibition is not predictive of chronic or delayed effects. However, most recent expert reviews tend to conclude that the balance of evidence does not support the existence of clinically significant neuropsychological effects, neuropsychiatric abnormalities, or peripheral nerve dysfunction in humans chronically exposed to low levels of OPs (Costa, 2006; Ray & Richards, 2001).

☑ Time trend, geographical variation, susceptible groups

- **Geographical variation**

  There are no differences in cholinesterase associated with race, but BChE in North American black races has been reported to be lower than in whites of the same sex. To date, only one genetic variant of human AChE has been described, which is not associated with any abnormality in activity (Bartels et al, 1993). A large number of genetic polymorphisms have been described for BChE. In addition to the wild-type allele, there are at least 39 identified genetic variants with nucleotide alterations in the coding region. Several of these variants are silent (i.e., they have 0–10% of normal activity), but they are rare; most common variants (e.g., atypical variants) have a reduced activity and are far less efficient scavengers of positively charged cholinesterase inhibitors

[52]. Due to the protective role of plasma cholinesterase, individuals with genetic variants of BChE with no or low activity would be predicted to be more susceptible to OP toxicity.

15.2 ORGANOPHOSPHATE INSECTICIDES

☑ General information

As previously signalled, organophosphate (OP) insecticides inhibit the activity of cholinesterases (ChE), which constitutes its main mechanism of toxicity (Costa, 2006). We have already stressed the importance of OPs in crop protection, even nowadays. Their selective toxicity is based on specificity differences in the AChE targets, more rapid detoxification in mammals than insects, and the use of pro-insecticides undergoing preferential activation in insects as compared with mammals. Their facile biodegradation and low environmental persistence are coupled with toxic effects more likely due to acute rather than chronic exposure. About 75% of registered organophosphate pesticides will be metabolized to measurable dialkyl phosphate metabolites (Table II). In contrast to the organophosphates, the dialkyl phosphate metabolites do not inhibit acetylcholinesterase enzymes. Dialkyl phosphates themselves are not considered toxic, but they are markers of exposure to organophosphates. Dialkyl phosphate metabolites can be present in urine after low-level organophosphate exposures that do not cause clinical symptoms (Davies and Peterson, 1997; Franklin et al., 1981). Measurement of these metabolites reflects recent exposure that has occurred predominantly in the last few days.

Table 14.2: Dialkyl phosphate metabolites of organophosphate pesticides.

Dialkyl phosphate metabolites	**CAS number**
Dimethylphosphate (DMP)	813-79-5
Dimethylthiophosphate (DMTP)	1112-38-5
Dimethyldithiophosphate (DMDTP)	756-80-9
Diethylphosphate (DEP)	598-02-7
Diethylthiophosphate (DETP)	2465-65-8
Diethyldithiophosphate (DEDTP)	298-06-6

Dialkyl phosphates may also be present in the environment from the degradation of organophosphates (Lu et al., 2005). Therefore, in addition to reflecting exposure to the parent pesticides, the level of the metabolite in a person's urine may reflect exposure to the metabolite itself, if it was present in the person's environment. Generally, health-related guidelines for urinary levels of these metabolites have not been established.

Specific metabolites of Organophosphate insecticides differ from the dialkyl phosphates because each specific metabolite derives from one or only a few parent pesticides. Table III shows the parent organophosphate pesticides and their metabolites. Figure 4 shows the chemical structure of some OP.

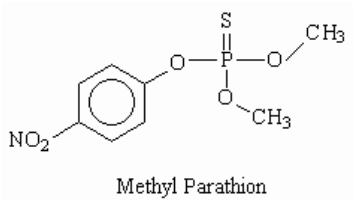
Sources of potential exposure to organophosphates vary, depending on the chemical. Some organophosphates (including malathion and chlorpyrifos) are commonly used in agriculture, whereas the use of other organophosphates (such as parathion and methyl parathion) have been restricted significantly in the United States. Some organophosphates (malathion, naled) are used for the control of mosquitoes in order to public health.

The organophosphates share a common mechanism of toxicity that occurs through inhibition of the enzyme acetylcholinesterase in the nervous system. The specific metabolites do not inhibit acetylcholinesterase enzymes but rather are an indicator of exposure to the parent compounds.

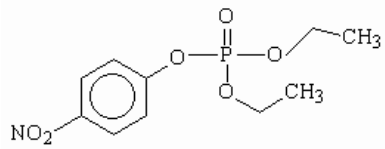
Table 14.3: Organophosphate pesticides: specific metabolites

Organophosphate pesticide (CAS number)	**Primary urinary metabolite** (CAS number)
Malathion (121-75-5)	Malathion dicarboxylic acid (1190-28-9)
Parathion (56-38-2)	*para* -Nitrophenol (100-02-7)
Methyl parathion (298-00-0)	*para* -Nitrophenol (100-02-7)
Chlorpyrifos (2921-88-2)	3,5,6-Trichloro-2-pyridinol (6515-38-4)
Chlorpyrifos methyl (5598-13-0)	3,5,6-Trichloro-2-pyridinol (6515-38-4)
Diazinon (333-41-5)	2-Isopropyl-4-methyl-6-hydroxypyrimidine (2814-20-2)
Pirimiphos methyl (29232-93-7)	2-(Diethylamino)-6-methylpyrimidin-4-ol/one
Coumaphos (56-72-4)	3-Chloro-7-hydroxy-4-methyl-2H-chromen-2-one/ol

Figure 14.4: Chemical structure of methyl parathion and ethyl parathion.



Methyl Parathion



Ethyl Parathion

☑ Matrix

- **Invasive**

  Several studies on OP detection have been carried out on blood or amniotic fluid, and also post-mortem, on brain tissue of exposed animals.

  Dialkyl phosphate can be analyzed in serum. Dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylphosphate, diethylthiophosphate and diethyldithiophosphate have benn also detected in samples of kidney and liver, by means of GC-FPD (Richardson and Seiber, 1993).

- **Non-invasive**

  Specific metabolites of OP are generally analyzed in urine. Several studies analyzing 3,5,6-tricholoro-2-pyridinol in umbilical cord tissue samples have also been reported.

  Dialkyl phosphate are generally analyzed in urine. Total dialkyl phosphates have also been analyzed in samples of meconium, that begins to accumulate at approx. 16 weeks of gestation.

- **If different methods are available, compare these**

  Measurement of urinary metabolites is less invasive and logistically simpler than determination of blood cholinesterase activity to assessing the exposure of workers to OPs.

☑ Kinetics

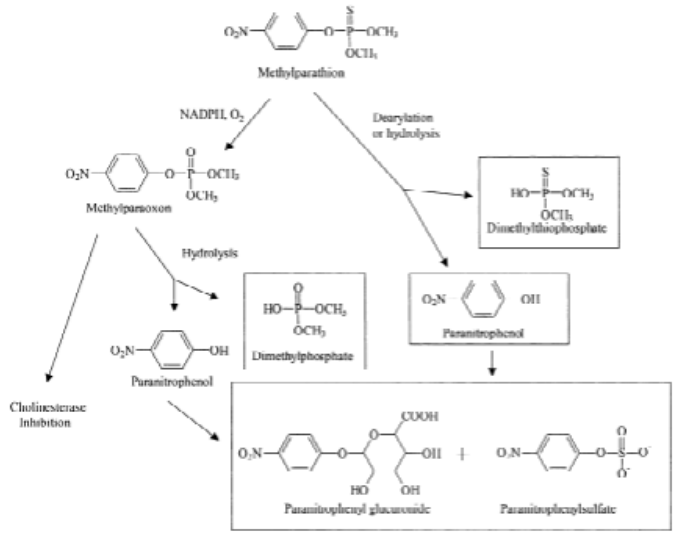
- **Uptake**

  Exposure to organophosphates may occur by ingestion, inhalation, or dermal contact.

- **Metabolism**

  Acephate is an OP pesticide that do not metabolize to dialkyl phosphate compounds. It is metabolized to relatively little extent in the human body, 73–77% of the absorbed dose being excreted unchanged in urine. Methamidophos is an acetyl-cholinesterase inhibitor, but 3,5,6-tricholoro-2-pyridinol don't inhibit acetylcholinesterase enzymes. 3-methyl-4-nitrophenol is a metabolite produced by esterase cleavage of fenitrothion. The organophosphate pesticide fenitrothion, in turn, shows androgen receptor-antagonist activity. Malathion a-monocarboxylic acid and malathion dicarboxylic acid derive by hydrolysis from diethylsuccinic ester in the lateral chain, and are the main urinary metabolites of malathion. p-nitrophenol, present in pesticides such as Parathion-methyl, Parathion or EPN, may also derive from non-pesticide chemicals used in industrial processes.

Figure 14.5: Metabolic pathway of methyl parathion in the body representative of organophosphate pesticide metabolism (Barr and Needham, 2002)



- **Biological half-life**

  The excretion is usually quite rapid (80-90% of the total dose within 48h). The detection of Dialkyl Phosphate Metabolites in urine reflects recent exposure that has occurred predominantly in the last few days.

☑ Sampling conditions

- **Operational aspects**

  In occupationally exposed subjects, blood sampling for measuring biological indicators should be done at the end of exposure. Since a significant number of compounds may be present in the general 'unexposed' population, pre-exposure sampling is recommended for comparison with post-exposure levels.

  If analysis is aimed at assessing the total absorbed dose of pesticide, it is advisable to collect a 24-h urine sample (in a single container) or to carry out intermittent sampling throughout the day. An alternative strategy is to obtain spot samples. In this case the right time has to be determined according to the specific kinetics of the biological marker and its urinary excretion speed. For

monitoring exposure to compounds characterised by slow absorption and excretion, 24–48 h sampling of urine may be needed, starting from the beginning of exposure (azinphos-methyl, chlorpyrifos). Exposure of agricultural workers is mainly dermal, thus absorption is quite slow and steady over time. In such cases, collection of a single sample of urine at the end of the work-shift may not be significant. A 24-h sample or intermittent collection of different samples (during the work-shift, and from the end of the work-shift to the beginning of the work-shift next day) has been performed in different studies. It was found advisable to continue collection of samples from the same subject for a definite period, based on the half-life of the substance. The number of samples to be collected should be determined according to the final use of the data and the required level of statistical reliability, etc. With regard to field exposure, variability may be more accurately assessed by increasing the number of subjects instead of repeatedly monitoring the same subjects. When using spot urine samples, creatinine or specific mass should also be determined, in order to normalise results for concentration and rule out over-diluted or over-concentrated samples. For 24-h samples and 1-day intermittent samples (taken at scheduled times), the total volume of urine excreted should be measured to permit the absolute quantity of metabolites in the sample to be determined.

Urine should be placed in plastic containers shielded with aluminium foil to prevent light-induced breakdown of metabolites. If analysis is not carried out immediately, samples should be frozen. In these cases, it is strongly suggested to consider sample stability.

☑ Analytical aspects

- **Techniques**

  Measurement of urinary metabolites is less invasive and logistically simpler than determination of blood cholinesterase activity to assessing the exposure of workers to Ops.

  Techniques used for dialkyl phosphates (DAP) in urine include:

- HRGC-FPD
- HRGC-MS
- GC-MS-MS
- GC-FPD
- GC-MS (ion trap)
- GC-AFID
- Isotope dilution – GC – MS

Other techniques used for for organophosphate insecticides (OP) detection are:

GC-NPD

- GC-MS
- GC-MS-MS (developed by CDC to quantify the non-specific di-alkyl phosphate OP metabolites)
- isotope dilution – GC – MS – MS
- GC–MS EI SIM
- GC-MS and quantified using isotope dilution calibration
- Conversion of MCA (malathion a-monocarboxylic acid) into alkylphosphates (DMTP and DMDTP), by alkaline hydrolysis. The compounds are then derivatized with thion pentafluorbenzylbromide.

- **Units**

  µg/L

☑ Performance characteristics

Dimethyl DAP metabolites were selected as the focus for comparisons across studies because their levels were substantially and consistently higher than those of diethyl metabolites in all studies.

Comparing pre- and post-exposure levels of blood ACHE activity is the recommended test for biological monitoring of human exposure to methamidophos.

Methamidophos was selected as the index chemical for the Preliminary OP Cumulative Risk Assessment. Sensitivity and Specificity (detection range)

Laboratory quality assurance for acephate analysis included repeat analysis of spiked matrix pools (10 μg/L of each analyte) and inclusion of these spiked pools in each run.

☑ Validation

Urinary PNP (p-nitrophenol) has been accepted as a biologic exposure index for MP by the American Conference of Governmental Industrial Hygienists.

☑ Confounding factors

One of the disadvantages of urinary analisis is that urine output varies. Many factors influence daily urinary output, such as water intake, urea and salt contents, specific gravity and osmolality. Consequently, the concentration of toxicants or metabolites may vary, even if the internal dose remains constant.

☑ Dose –response/effect relationships

Most OPs are rapidly absorbed, metabolized, and excreted as non-toxic derivatives and appear in urine within hours.

In volunteer studies with 1 mg oral doses of chlorpyrifos, diazinon and propetamphos the mean peak values were 160, 750 and 404 mol/mol creatinine, respectively, and were not associated with any reduction in blood cholinesterase activity.

After administration of combined doses of methamidophos and acephate, the highest levels of CHE depression were observed in volunteers treated with a methamidophos: acephate ratio of 1:9. The administration of a total dose of 0.2 mg:kg bw of 0.24 methamidophos:acephate combination was not associated with depression of CHE activity. A NOEL of 0.04 mg methamidophos: kg bw in humans was observed (WHO, 1993a).

MacIntosh et al. (2001) found that dietary chlorpyrifos levels were significantly correlated with mean urinary TCPY excretion in the National Human Exposure Assessment Survey in Maryland and that the dietary chlorpyrifos accounted for approximately 7% of the urinary TCPY.

Methamidophos may have the potential to affect male fertility and to produce transmisible adverse embryonic effects after an acute paternal germline exposure.The presence of methamidophos is protective at low doses and neuropathic at high doses.

The urinary levels in spot samples collected from spraymen after a 1-week exposure ranged from 2.2 to 25.2 g:l, whereas the urinary concentrations of 3-methyl-4-nitrophenol varied from 0.5 to 6.6 mg:l in weighers and supervisors not directly exposed to the insecticide. Urinary concentrations decreased from an average of 10.5 to 0.67 mg:l, about 60 h after exposure to fenitrothion. No significant inhibition of whole blood CHE was observed in workers or residents with urinary MNP (3-methyl-4-nitrophenol) concentrations up to 3 mg:l, values which are likely to correspond to an oral dose of 7 mg.

Urinary MDA (malathion dicarboxylic acid) levels during the latter part of pregnancy were also associated with significant increased risks for preterm delivery.

There is strong evidence from animal studies indicating a role for PON1 (paraoxonase) polymorphisms in modulating the toxicity of certain OPs. However, evidence in humans is very limited. Battuello et al. (2004) have concluded that current knowledge on the risk associated to PON1 variability is not sufficiently established to recommend a widespread genetic screening of PON1 status in OP-exposed workers. Additional epidemiologic data with documentation of exposure are needed to assess the role of PON1 polymorphisms as a susceptibility factor in the risk assessment of OP human exposure.

☑ Time trend, geographical variation, and identification of susceptible groups

Higher levels of TCPY (3,5,6-tricholoro-2-pyridinol) were found among urban children than among those living in nonurban areas.

The groups mainly at risk are farm workers, pesticide applicators and manufacturers.

15.3 PARAOXONASE (PON1)

☑ General information: Biomarkers of susceptibility to Organophosphate insecticides

Genetic differences in the enzymes involved in the bioactivation and detoxification of organophosphate insecticides and in the enzymes that are targeted by these compounds can greatly influence their toxicity. Polymorphisms of the general xenobiotic-metabolizing enzymatic systems, as cytochromes P450 (CYP) and glutathione-S-transferases (GST), as well as of the "target" BuChE and AChE, can modify the bioactivation-detoxication balance. Furthermore, some more specific polymorphic enzymes are implicated in the biotransformation of some OPs; the most studied is paraoxonase (PON1). This genetic variability could be of interest as possible biomarkers of individual susceptibility (Costa, 2001).

CYPs catalyze the oxidative desulfuration of organothiophosphates to the corresponding oxons and also mediate detoxication of several OPs. Both bioactivation and detoxication of OPs are mediated by multiple CYPs, some of which present polymorphisms that can confer differences in catalytic activity or level of expression of various substrates. However, little is known to date on the role that such polymorphisms play in the metabolism and toxicity of OPs. Similar considerations also apply to GSTs, which may play a role in the detoxication of certain OPs. Indeed, the potential significance of human GST polymorphisms as determinants of individual differences in human susceptibility to OPs has not been investigated.

A large number of polymorphisms have been described for BuChE, a secondary target, but also an enzyme that metabolizes OPs. In addition to the wild-type allele, there are at least 39 identified genetic variants with nucleotide alterations in the coding regions, several of them with null or very low activity. A genetic variant of human AChE has been described, which is not associated with alterations in enzyme activity.

Paraoxonase (PON1, EC 3.1.8.1) is a member of a family of proteins that also includes PON2 and PON3, the genes of which are clustered on the long arm of human chromosome 7 (q21.22). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoprotein (HDL) particles. PON1 is an esterase that hydrolyzes the active metabolites of several other OP insecticides (e.g. chlorpyrifos oxon, diazoxon) as well as nerve agents such as soman, sarin or VX. However, PON1 does not hydrolyze directly the parent compounds of such insecticides (i.e. parathion, chlorpyrifos, diazinon), nor several other OPs (e.g. malaoxon, dichlorvos). PON1 exhibits considerable variation among individuals due to a coding region polymorphism that alters its activity and to differences among individuals in plasma enzyme levels, which also vary temporally during development. Two polymorphisms are present in the PON1 coding sequence: a Gln(Q)/Arg(R) substitution at position 192, and a Leu(L)/Met(M) substitution at position 55. The Q/R polymorphism at position 192 significantly affects the catalytic efficiency of PON1. Gene frequencies of PON1Q192 range from 0.75 for Caucasians of Northern European origin, to 0.31 for some Asian populations. Recently, the three-dimensional protein structure of a hybrid form of PON1 has been elucidated (Harel et al, 2004)

The L/M polymorphism at position 55 does not affect catalytic activity, but has been associated with plasma PON1 protein levels, with PON1M55 being associated with low plasma PON1. However, this appears to result primarily from linkage disequilibrium with the low efficiency—108T allele of the T- 108C promoter region polymorphism. Four additional polymorphisms have been found in the 5V-regulatory region of PON1, but they have a lesser effect on PON1 protein level. Recent complete sequencing of PON1 from several individuals has led to the identification of more than 160 new single nucleotide polymorphisms, some in the coding regions and others in introns and regulatory regions of the gene.

Animal experiments indicate that the role of PON1 in modulating the toxicity of OPs varies markedly depending on the compound. PON1. Knockout mice, which

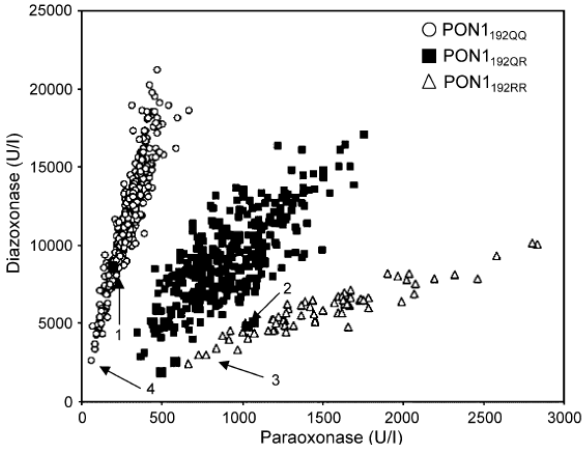
have no detectable plasma and liver hydrolytic activity toward paraoxon and diazoxon and very limited chlorpyrifos-oxonase activity, have dramatically increased sensitivity to the toxicity of chlorpyrifos oxon and diazoxon, and a small increased sensitivity to their respective parent compounds. Surprisingly, they did not show an increased sensitivity to paraoxon (Shih et al, 1998).

Catalytic efficiency with which PON1 degrades toxic OPs determines the degree of protection provided by PON1; in addition, higher concentrations of PON1 provide better protection. Thus, it is important to know PON1 levels and activity. In a given population, plasma PON1 activity can vary up to 40-fold, and differences in PON1 protein levels up to 13-fold are also present within a single PON1192 genotype. Recent studies investigating the role of PON1 in cardiovascular disease have indeed provided evidence that PON1 "status" (encompassing genotype and activity levels) is a much better predictor of disease than PON1 genotype alone. Although it might be possible to determine all of the PON1 SNPs (single nucleotide polymorphisms) for a given individual, doing so would not provide an accurate assessment or prediction of the individual's PON1 status (Costa et al, 2005; Furlong et al, 2005). Furthermore, alcohol, smoking, certain drugs, diet and certain physiological and pathological conditions can indeed increase or decrease PON1 activity (Costa et al, 2005b).

Two approaches have been applied to characterize the inter-individual PON1 variability:

- Spectrophotometric determination of PON1 activity on different substrates. A high-throughput enzyme assay involving two PON1 substrates, paraoxon and diazoxon has been proposed to determine the PON1 "status" in human populations. This two-substrate assay provides an accurate inference of $PON1Q_{192}$genotype (QQ, QR or RR, as confirmed by PCR), as well as the level of plasma PON1 activities (PON1 status) (figure 1) (Jarvik et al, 2003).
- Genotyping by standard PCR techniques to identify the PON1192Q/R polymorphism, or/and other SNPs in the intron, exon or regulatory regions.

Figure 14.5: Relashionship between Paraxonase -Diazoxonase-PON1 (Costa et al, 2005)



☑ Matrix

1st approach: Any DNA containing sample.

2nd approach: plasma

☑ Sampling conditions

For the second approach, don't use EDTA as anticoagulant (PON1 activity is Ca dependent).

☑ Analytical aspects

- **Techniques**

  1st approach: standard PCR techniques on DNA SNPs characterization.

  2nd approach: The two-substrate assays are carried out using a Molecular Devices SPECTRAmax PLUS Microplate Spectrophotometer or equivalent instrument. Initial rates of substrate hydrolysis are determined, and rates of diazoxon hydrolysis (y-axis) are plotted against the rates of paraoxon hydrolysis (x-axis). Hydrolysis of paraoxon produces p-nitrophenol, monitored at 405 nm, while hydrolysis of diazoxon produces 2-isopropyl-4- methyl-6-

hydroxypyrimidine, monitored at 270 nm. This method separates individuals into the three phenotypes of PON1192 activity, PON1192Q/Q, PON1192Q/R and PON1192R/R (Fig.1) (Costa et al, 2005).

☑ (Dose/effect relationships) PON1 "status"/susceptibility relationships

There is strong evidence from animal studies indicating a role for PON1 polymorphisms in modulating the toxicity of certain OPs. However, evidence in humans is very limited. Battuello et al. (2004) have concluded that current knowledge on the risk associated to PON1 variability is not enough well established to recommend a widespread genetic screening of PON1 status in OP-exposed workers. Additional epidemiologic data with documentation of exposure are needed to assess the role of PON1 polymorphisms as a susceptibility factor in the risk assessment of OP human exposure.

15.4 INHIBITION OF NEUROPATHY TARGET ESTERASE (NTE)

☑ General information

Some OP compounds induce delayed neurotoxic effects (so called 'delayed neuropathy' or OPIDN) after acute overexposure. Clinically, this neuropathy takes the form of an ascending paralysis of the lower limbs, caused by degeneration of the axons of the motor nerves. It becomes evident 1±3 weeks after exposure to those OPs which covalently modify a neuronal protein, now known as neuropathy target esterase (NTE). Although neuropathic OPs paralysed many thousands of people during the 20th century, mainly by a contamination of alcohol beverage by triorthocresyl phospate, this syndrome is now very rare. In part this is because, despite the worldwide use of very large quantities of OP pesticides, those with neuropathic potential have been screened out of the market.

Identification of NTE and evidence for a direct involvement of this enzyme in the delayed neuropathy were achieved through experiments in hens (Johnson, 1982). In humans, NTE is present in the nervous tissue, liver, lymphocytes, platelets and other tissues. Its physiological function, if any, is still unknown.

The sequence of biochemical events leading to the development of delayed neuropathy has been only partially elucidated. The initial biochemical reaction is represented by the phosphorylation of NTE, while the second step is the transformation of the phosphorylated target into an 'aged' form. The ageing reaction depends on the chemistry of OP pesticides and may only occur with phosphate, phosphonates and phosphoramidates. Compounds, such as phosphonates, sulphonates and carbamates are not able to age and, if they are linked to NTE before an axonopathic OP compound is administered, they block the receptor preventing the development of the neuropathy. In point of fact some classic protective inhibitors, such as carbamates, form an inhibited NTE which apparently does not age and yet produces neuropathy (Lotti and Moretto, 1993). Thus, all NTE inhibitors would be able to cause neuropathy. In analogy with the pharmacological models of drug-receptor interaction, NTE inhibitors may be

characterized by a variable intrinsic potential to initiate neuropathy once attached to the protein. Strong neuropathic chemicals require about 75% NTE inhibition, others 80–90% and the least potent almost 100%. In a concurrent multiple OP exposure, the least active compounds bind NTE and prevent the most active compounds from binding, thus exerting a protective effect against delayed neuropathy (Lotti, 1986). The hen is the animal model mainly used to investigate OP-induced delayed neuropathy. In this species, a minimum NTE inhibition of 75% in vivo must be reached in order to cause delayed neuropathy. Whether a threshold exists also in humans and what the no-observed effect level is, are at present unresolved questions. Moreover, little is also known of the biochemical and cellular reactions taking place in the neurones as a consequence of the linkage of OP pesticides to NTE. NTE activity per se is not an indispensable biochemical agent for trophism and good function of neurones, as it may be suppressed without the onset of neuropathy. Furthermore, clinical symptoms of neuropathy develop despite restoration of NTE activity in the nervous system. Defective protein synthesis or impaired axonal flow have been conjectured as putative mechanisms for delayed neuropathy, but these hypotheses are not supported by experimental evidence. After administration of axonopathic OP pesticides to hens, inhibition of NTE in blood lymphocytes proved to be correlated with NTE inhibition in central and peripheral nervous tissue. Accordingly, NTE inhibition levels have been proposed as a tentative indicator for biological monitoring of exposure/effect to OP compounds able to cause delayed neuropathy.

An assay for neurotoxic esterase (neuropathy target esterase, NTE) was developed by Johnson (1,2) to assess the delayed neurotoxic potential of organophosphorus compounds. NTE activity is calculated from the rate of phenyl valerate hydrolysis resistant to paraoxon and sensitive to mipafox inhibition under specified conditions of inhibitor concentrations, pH, temperature, and incubation times with inhibitors and substrate. The amount of phenol produced is measured colorimetrically after its oxidative coupling with 4-aminoantipyrine to yield 4-N-(1,4-benzoquinoneimine)-antipyrine, a chromophore with a wavelength of maximum absorbance (lambda m) 510 nm and corresponding molar absorptivity

(molar extinction coefficient, epsilon) equal to 13,900 M-1cm-1. The assay was improved and simplified later by Johnson (3) without any change in the lambda m or epsilon, even though the chromophore solvent was altered by adding the detergent, sodium dodecyl sulfate (SDS).

Unexposed subjects show a rather rich inter-individual variation of lymphocyte and platelet NTE activity (Table 1.6). The occurrence of variations in NTE activity in physiological or pathological conditions has not been investigated. Little information is available on lymphocyte NTE activity in exposed subjects. No study on platelet NTE activity in exposed subjects has been found in literature. In a case of suicidal poisoning with chlorpyrifos, the inhibition levels of the peripheral blood lymphocyte NTE were correlated with those measured in the peripheral nerves (Osterloh et al., 1983). Furthermore, in another case of attempted suicide with the same OP compound, inhibition of NTE activity in peripheral lymphocytes was followed by the onset of delayed neuropathy (Lotti, 1986). Two studies have been carried out using NTE for biomonitoring of occupational exposure to OP compounds. In one of the studies, NTE was measured after an occupational exposure to the cotton defoliant *S,S,S*-tributylphosphorotrithioate, an OP pesticide which may cause delayed neuropathy without overt cholinergic symptoms. Inhibition of NTE was observed, but no changes were found in the peripheral nervous system, as assessed via standard electrophysiological tests. Possible explanations of this finding include an effect below the threshold, or the production of a highly reactive metabolite of *S, S, S* tributyl-phosphorotrithioate which reacts with the lymphocytic rather than the nervous system NTE (Lotti et al., 1983). In the second study, NTE activity in peripheral lymphocytes was measured after occupational exposures to a mixture of OP compounds which were used as resin additives (triphenylphosphate, *meta*-, mono-, di- and triisopropyl triphenylphosphates). No effect on NTE activity was observed; in fact, according to the structure–activity relationship, these compounds do not yield either NTE inhibitory products or cause delayed neuropathy (Emmett et al., 1985).

At present, the available data are insufficient to permit the use of NTE measurement in peripheral blood for biological monitoring of subjects exposed to axonopathic OP compounds. Further research on this topic is needed.

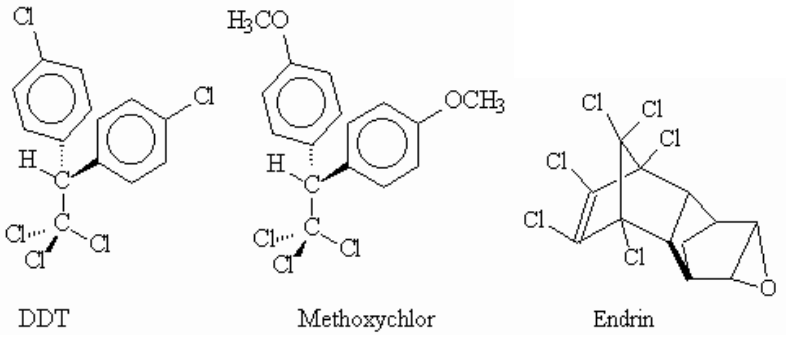
15.5 ORGANOCHLORINE INSECTICIDES

☑ General information

Organochlorine insecticides (Figure 6) are a broad class of pesticides that were widely used as insecticides in the 1950s and 1960s. Their use was subsequently discontinued in many countries due to persistent contamination of the environment. They can be divided into three groups: benzene hexachloride isomers (e.g. lindane), cyclodienes (aldrin, dieldrin, endrin, chlordane, heptachlor, endosulfan) and DDT and analogues (methoxychlor, dicofol, chlorobenzylate). Because of their persistence in the environment, most Organochlorine pesticides (Ocs) are ubiquitous pollutants and can usually be detected in biological samples from the general population.

Nine of the organochlorine pesticides as well as polychlorinated dibenzo-*p*-dioxins, furans, and biphenyls are the subject of the Stockholm Convention on Persistent Organic Pollutants (POPs), which was held in May 2001; this treaty calls for an immediate ban on the production, import, export, and use of most of these POPs as well as disposal guidelines (United Nations Environment Program, 2001). DDT has a health-related exemption for the control of malaria-carrying mosquitoes. These nine pesticides are aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, and toxaphene.

Figure 14.7: Chemical structure of some Organochlorine insecticides.



☑ Matrix

- **Invasive**

  Biological monitoring of OC exposure can be carried out by determination of intact compounds or their metabolites in blood, fatty tissue and liver.

- **Non-invasive**

  Biological monitoring of OC exposure can be carried out by determination of intact compounds or their metabolites in urine.

☑ Kinetics

- **Uptake (by different routes)**

  Inhalation, dermal or ingestion routes are possible.

- **Metabolism**

  After absorption, aldrin is rapidly converted to dieldrin. Exposure to both compounds has been assessed by measuring dieldrin concentrations in blood, serum, fatty tissue and milk.

  Technical chlordane is a mixture of α- and γ- chlordane, nonachlor and heptachlor. Biological monitoring of human exposure has been based on measurement of concentrations of chlordane and related compounds (oxychlordane, nonachlor, heptachlor-epoxide) in blood, fatty tissue and milk.

  Endrin is rapidly metabolized to 12-hydroxy-endrin, and excreted as sulphate and glucuronide conjugates. Intact pesticide is usually undetectable in blood,

fatty tissue or milk of occupationally exposed workers and of the general population.

After absorption, DDT is largely transformed to DDE, and several intermediate metabolites have been measured in body tissues.

The induction of cytochrome P450 (CYP) activates these pesticides to genotoxic species; in that sense, detoxifyng metabolism may be considered an epigenetic mechanism for indirect genotoxicity in the case of these compounds.

☑ Sampling conditions

- **Operational aspects**

  In occupationally exposed subjects, blood samples for measuring biological indicators should be at the end of exposure. Since a significant number of compounds (e.g. organochlorines) may be present in the general 'unexposed' population, pre-exposure sampling is recommended for comparison with post-exposure levels.

  As has been previously mentioned, different sampling strategies are recommended, depending on the purposes sought (see the section on OPs); the same is valid for storage conditions.

☑ Analytical aspects

- **Techniques**

  Typically, serum or plasma is extracted using a liquid partitioning or solid-phase extraction (SPE) and the extract is analyzed using capillary gas chromatography (GC) with electron-capture detection (ECD). These methods are reliable and use affordable instrumentation. However, GC–ECD analyses have a higher potential for detecting interfering components than do more selective analysis techniques. Other methods for analysis of serum extracts include mass selective detection (MSD aka MS) and high-resolution mass spectrometry some with isotope dilution quantification. These analyses are typically more selective and sensitive than GC–ECD analyses; however, the

high cost of instrumentation and isotopically labeled standards and the complex operation and maintenance of these instruments often preclude their routine use in most laboratories.

Methods similar to those employed with serum and plasma are used to measure OCs in other lipid rich matrices such as adipose tissue and breast milk. These methods may involve some modifications in the sample preparation procedures to accommodate the change in matrix properties.

Some OCs are metabolized more readily than others and their polar metabolites are excreted in urine. The most common OCs whose metabolites are measured in urine are endosulfan and lindane (γ-HCH). Endosulfan and its polar metabolites endo- sulfan-lactone, endosulfan-ether, and endosulfan-sulfate have been measured in the low-ng/ l level in urine using SPE with analysis by GC–tandem mass spectrometry (GC–MS–MS) A metabolite of dicofol, 4, 49-dichlorobenzilic acid, was in pesticide applicators by GC. Lindane metabolites, primarily chlorinated phenols, have been measured in urine using several methods, all of which employ some deconjugation technique (e.g.,acid or enzyme hydrolysis) to liberate glucuronide and sulfate-bound chlorinated phenols. In most instances, the chlorinated phenols are extracted, derivatized, and analyzed using GC– ECD (Angerer et al., 1981) or GC–MS–MS (Holler et al., 1989; Hill et al., 1995). One novel method employs an on-line cleanup or supercritical fluid extraction (SFE) and pre-concentration followed by separation and analysis using micellar electro-kinetic chromatography with UV detection (Mardones et al., 2000).

☑ Dose –response/effect relationships

Organochlorine insecticides are linked with STS, non-Hodgkin's lymphoma (NHL), leukemia, and, less consistently, with cancers of the lung and breast.

Affect fertility mechanisms at relatively low levels (0.41 ng/mL or 0.41 ppb).

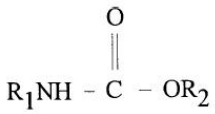
15.6 CARBAMATE INSECTICIDES

☑ General information

Like OP, carbamate insecticides (Table IV) are inhibitors of ChE. The inhibition is labile, of short duration and reversible, unlike that induced by OP compounds. Blood samples must therefore be obtained and analyzed as soon as possible after exposure. The analytical methods and sampling procedures have already been discussed. Carbamate insecticides are semi-volatile and are readily detectable in indoor and personal air samples.

N-methyl carbamate insecticides (Figure 6) are widely used as insecticides in the United States and throughout the world. The estimated annual worldwide use for all carbamates ranged from 20,000 to 35,000 tons (International Program on Chemical Safety, 1986). In the United States, uses of carbamate insecticides include agricultural crops, residential lawns and gardens, nurseries, and golf courses.

Figure 14.8: General structural formula of carbamate pesticides.



where R_2 is an aromatic or aliphatic substituent. According to the nature of R_1 three classes of compounds may be identified:

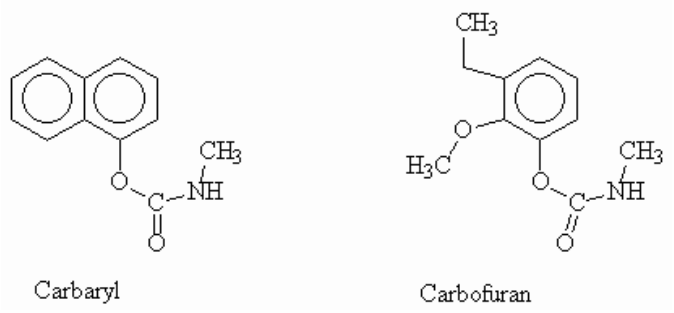
1 carbamate insecticides, where R_1 is methyl group
2 carbamate herbicides, where R_1 is an aromatic moiety
3 carbamate fungicides, where R_1 is a benzoimidazole moiety

Carbamate insecticides do not persist long in the environment, so they have a low potential for bioaccumulation (NCEH, 2005). Figure 7 shows the chemical structure of Carbaryl and Carbofuran.

Table 14.4: Carbamate insecticides and their metabolites

Carbamate insecticides (CAS number)	**Primary urinary metabolite** (CAS number)
Propoxur (114-26-1)	2-Isopropoxyphenol (4812-20-8)
Carbofuran (1563-66-2)	Carbofuranphenol (1563-38-8)
Benfuracarb (82560-54-1)	Carbofuranphenol (1563-38-8)
Carbosulfan (55285-14-8)	Carbofuranphenol (1563-38-8)
Furathiocarb (65907-30-4)	Carbofuranphenol (1563-38-8)

Figure 14.9. Chemical structure of Carbaryl and Carbofuran



☑ Matrix

- **Invasive**

  Measurement of unmodified carbamate insecticides in blood has often been performed to confirm exposure in acute poisoning cases.

  Amniotic fluid collected during amniocentesis is the only medium available to characterize direct fetal exposures early in pregnancy (~18 weeks of gestation).

  In fatal cases, unmodified compounds may be measured in various organs (liver, stomach). Carbofuranphenol has also been analyzed in stomach contents.

- **Non-invasive**

  Measurement of unmodified carbamate insecticides in urine has often been performed to confirm exposure in acute poisoning cases.

☑ Kinetics

- **Uptake (by different routes)**

  Exposure to carbamate insecticides may occur by ingestion of food products, inhalation of aerial spraying, or dermal contact.

- **Metabolism**

  Carbamate insecticides are rapidly eliminated from the body. Carbofuran measured in urine of occupationally exposed farmers showed that about 7% of the total absorbed dose (dermal and inhalation) is excreted in urine in 24 h (Hussain et al., 1990).

  The main group of carbamate metabolites in urine is the neutral glucuronide conjugates.

☑ Sampling conditions

- **Operational aspects (e.g. retrieval conditions, transport and storage conditions)**

  Carbamates are unstable in blood, so their levels may decrease during storage, producing false negative results.

☑ Analytical aspects

- **Techniques**

  We can find different analytical procedures for the determination of intact carbamate pesticides in biological samples, as RP HPLC–UV for blood, urine, liver, stomach (Duck and Woolias, 1985), GC–FPD and LC–MS–MS (Driskell et al., 1991) or GC–NPD for quantitation in blood and TLC and GC–MS to confirm the presence of the pesticide in stomach contents (Lee et al., 1999).

  We can find different analytical procedures for the determination of 1-naphthol (1NAP): HRGC MS–MS in PCI $(CH)_4$ in urine samples (Hill et al., 1995), HPLC UV (210 nm) in plasma or urine samples (Ward et al., 1987) or GC ECD in urine samples (Shafik et al., 1971).

  The procedure of Hill et al. (1995a) was used to analyse urine samples of subjects participating in the NHANES III study. 1NAP was detected in 86% of

subjects. The highest concentration found was nearly 1400 µg/g creatinine; 95% of the results were less than 36 µg/g creatinine (Hill et al., 1995b).

The analytical procedure for the determination of carbaryl in plasma or urine is HPLC UV (210 nm) (Ward et al., 1987).

The analytical procedure for the determination of carbofuran in urine is HPLC–fluorescence detector (Hussain et al., 1990).

☑ Performance characteristics

- **Analytical reproducibility**

  In some studies, laboratory quality assurance included repeat analysis of spiked matrix pools (10 µg/L of each analyte) and inclusion of these spiked pools in each run.

☑ Dose –response/effect relationships

Accidental or intentional ingestion of carbofuran can produce a life-threatening syndrome that requires prompt diagnosis and treatment. After acute overexposure, carbamate insecticides inhibit acetylcholinesterase enzymes. This inhibition leads to an increase of acetylcholine in the nervous system, resulting in symptoms including nausea, vomiting, cholinergic signs, weakness, paralysis, and seizures.

Carbamate pesticides decreased estrogen - or progesterone responsive reporter genes at concentrations of 10–7 M in breast (MCF-7) and endometrial (Ishikawa) cancer cells.

15.7 PYRETHROIDS

☑ General information

Pyrethrins are naturally-occurring chemicals that are produced by chrysanthemums which exhibit a toxic effect on insects. Natural pyrethrins are comprised of many isomeric forms and are usually classified as the pyrethrin I and II isomer sets. Synthetic pyrethroids are man-made chemicals that are produced to mimic the effective action of natural pyrethrins. Their chemical structures are typically comprised of a chrysanthemic acid analogue that is esterified most often with a ringed structure. Pyrethroids are non-systemic pesticides that have contact and stomach action. Some pyrethroids also have a slight repellent effect. In most formulations, piperonyl butoxide is added as a synergist. In the past several years, the use of synthetic pyrethroids has escalated as the use of the more toxic OP and carbamate insecticides has been curtailed. Many products such as Raid brand pesticides that are commonly found in retail stores for home use contain pyrethroids such as permethrin and delta-methrin for eliminating household pests such as ants and spiders (Barr and Needham, 2002).

Pyrethroids are a group of insecticides largely used in agriculture and public health because of their relatively low toxicity to man and mammalian species at the usual application rates and because of their short environmental persistence.

☑ Matrix

- **Invasive**

  Blood

☑ Kinetics

- **Uptake (by different routes)**

  Oral, dermal, inhalation.

- **Metabolism**

  During metabolism of the pyrethroids, the chrysanthemic acid ester is usually cleaved via esterase or mixed function oxidase activity and any resulting

alcohol moieties are converted to their corresponding acids. These metabolites are partly conjugated to glucuronide and both the conjugates and free acids are excreted in the urine.

PYR insecticides are rapidly metabolized by mammalian species to their inactive acids and alcohol components, which are excreted mainly in urine. A minor portion of the absorbed compounds is excreted unchanged.

- **Biological half-life**

  5h-8h

  Detectable in urine for no more that 5 days

☑ Sampling conditions

- **Operational aspects:**

  For blood sampling, see above, organophosphates section.

  Synthetic pyrethroids have also been measured in serum and plasma. Leng et al. (1997) observed a dramatic decrease in the concentrations of permethrin and several other pyrethroids in spiked serum (60 γg/ l) when stored at 4 ºC over 8 days. By adding 1% formic acid before storing the spiked serum, the deterioration, presumably due to esterase activity, was diminished for permethrin and markedly reduced for the other pyrethroids. Barr et al. did not observe this decrease in permethrin concentrations in spiked serum (50 and 15 pg/g) stored at -70 ºC over 4 months (Barr et al., in press). However, more varimonly ability was observed in the analysis of permethrin isomers in these stored serum samples after about 1 month and other pesticides that are metabolized via esterase activity (i.e., carbamates and some reactive OPs) did show marked decreases. This area warrants further investigations if serum measurements will continue to be made.

☑ Analytical aspects

- **Techniques**

  No methods exist to measure natural pyrethrins or piperonyl butoxide in human matrices. This may be due to the logistically difficult task of measuring multiple isomers for exposure assessment of one pesticide product or it may be

the lack of priority due to its inherently low human toxicity or the relative security individuals feel when using a ‘‘natural’’ product.

Several methods exist for the measurement of synthetic pyrethroid metabolites in human urine: GC–FID, GC–MS, GC–ECD, HPLC–APCI-MS–MS, HPLC.

☑ Concentrations reported in literature

- **Reference value per age group / sex**

  The average daily intake for a man weighing 70 kilograms is estimated at about 3.2 micrograms per day.

☑ Dose –response/effect relationships

Most adverse effects are related to the action of pyrethroids on the nervous system, where these chemicals open sodium channels when a nerve cell is excited.

Cases of systemic poisoning are rare and usually result from accidental or intentional ingestion of pyrethroid insecticides. Signs and symptoms of acute pyrethroid poisoning include tremor, salivation, choreoathetosis and seizures.

15.8 PHENOXYACETIC HERBICIDES

☑ General information

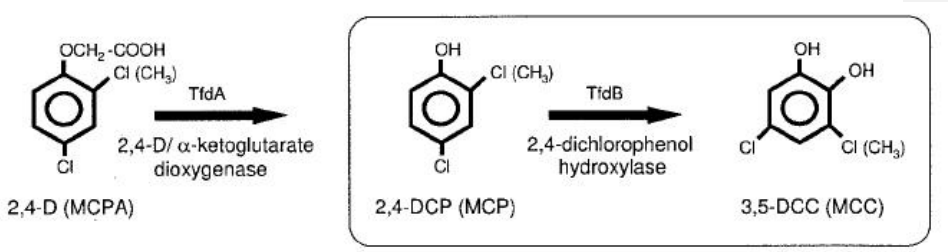
Phenoxyacid herbicides are post-emergence inhibitors used to eliminate unwanted foliage or weeds. The most common phenoxyacid herbicides are 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). These two herbicides were combined in equal proportions to make Agent Orange, the herbicide applied in the jungles of Vietnam, Laos, and Cambodia along with agricultural regions of Vietnam in the late 1960s and early 1970s during the Vietnam War. Because it is contaminated with the highly toxic and persistent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin along with other chlorinated dioxins and furans, 2,4,5-T has been banned for most applications. Although 2,4-D also contains small amounts of persistent chlorinated dioxins and furans, it is still the most abundantly applied residential pesticide. In its ester or salt forms, it is commonly found in home and garden stores in combination with other herbicides such as dicamba or mecoprop for application on lawns (Barr and Needham, 2002).

Phenoxyalkanoic compounds are used worldwide as herbicides. Bacteria play a fundamental role in the degradation of many chlorinated aromatic pollutants. *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134(pJP4) was isolated for its ability to use 2,4-dichlorophenoxyacetate (2,4-D) as a sole carbon and energy source (Don and Pemberton, 1981). This strain has been the focus of intensive studies because of its ability to degrade a wide spectrum of aromatic compounds, including 4-chloro-2-methylphenoxyacetate (MCPA) (Pieper et al., 1988), 2,4,6-trichlorophenol (Clement et al., 1995), 4-fluorobenzoate (Schlömann, 1990), and 3-chlorobenzoate (Don and Pemberton, 1981), among many others.

Cupriavidus necator JMP134(pJP4) catabolizes 2,4-dichlorophenoxyacetate (2,4-D) and 4-chloro-2-methylphenoxyacetate (MCPA), using *tfd* functions carried on plasmid pJP4 (Figure 1). TfdA cleaves the ether bonds of these herbicides to produce 2,4-dichlorophenol (2,4-DCP) and 4-chloro-2-methylphenol (MCP), respectively. These intermediates can be degraded by two chlorophenol

hydroxylases encoded by the *tfdB*I and *tfdB*II genes to produce the respective chlorocatechols (Ledger et al., 2006).

Figure 14.10: Chlorocatechol-producing peripheral reactions for 2,4-D and MCPA are carried out by enzymes encoded by the tfdA and tfdB genes in plasmid pJP4 (Ledger et al., 2006).



☑ Matrix

- **Invasive**
  Studies on mecoprop have been carried out in blood and other tissues
- **Non-invasive**
  Urine.

☑ Kinetics

- **Uptake**
  By different routes
- **Metabolism and biological half-life**
  2,4-Dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), pichloram, mecoprop, dichlorprop are poorly metabolized and are excreted largely unchanged in urine (IARC, 1986). Excretion is slow with a maximum on the first and second days after oral administration of 2,4-D; 40% of MCPA is excreted within 24 h and 80% within 5 days of administration. For dermal absorption, maximum

urinary excretion is detected on the first and second days after application of 2,4-D and 48 h after application of MCPA (Kolmodin-Hedman et al., 1983).

☑ Analytical aspects

- **Techniques**

  The most common techniques involve conversion of the free acid to its protected ester form (usually methyl or pentafluorobenzyl esters) then analysis using GC–HECD (Libich et al., 1984), GC–ECD, GC–FID, GC–MS, or GC–MS–MS. It has also been measured using HPLC–diode array detection (DAD). In addition to 2,4-D, mecoprop, dichlorprop, MCPA (2-methyl-4-chloro-phenoxyacetic acid), and 2,4,5-T have been measured using some of these methods (Erickson et al., 1979; Vural and Burgaz, 1984; Draper, 1982; Edgerton and Moseman, 1978; , De Felip et al., 1989).

☑ Concentrations reported in literature

- **Critical values**

  A LOD of 15 µg/ l makes the HPLC method useful only for occupational exposure. The GC method (LOD 1 µg/ l) is sensitive enough to monitor the general population. At concentrations above 15 µg/ l, the results of the two methods coincide The HPLC procedure has been widely tested in occupationally exposed subjects engaged in weed control spraying with 2,4-D and MCPA on wheat (Aprea et al., 1995). The GC–ECD method was used for 2,4-D and MCPA in a group of 100 children aged 6–7 years: 2,4-D was detectable in 20% of samples, and the maximum detected value was 2.5 µg/ l, MCPA was never detectable (Aprea et al., 1997). The standard operating procedure of Deutsche Forschungsgemeinschaft for 2,4-D, MCPA, mecoprop and dichlorprop have LODs (10 µg/l) too high for monitoring the general population. The GC method with MS–MS detection is less practicable for routine analyses.

In the USA, 2,4-D was only detected in 0.3% of urine samples in the NHANES II study were LOD was 30 µg/ l. Urinary 2,4,5-T concentrations did not reach the LOD of 10 µg/ l (Aprea et al., 2002).

The procedure of Hill et al. (1995) for 2,4-D in urine was used in NHANES III: it was detected in 12% of samples and never exceeded the concentration of 15 µg/g creat.; 95% of the results were below 1.5 µg/g creat.

Biological exposure limits are not available for 2,4-D and MCPA but some authors (Kolmodin-Hedman et al., 1983) suggest that urinary MCPA levels up to 0.5 µg/ml may be observed under good working conditions.

☑ Dose –response/effect relationships

Non Hodgkin's lymphoma (NHL) is associated with farm herbicide use, especially phenoxyacetic-acid herbicides (Hoar et al., 1986).

An investigation was conducted to examine the hepatotoxicity of two commercial forms of phenoxyacetic-acid, 2-methyl-4-chlorophenoxyacetic-acid (MCPA) and 2,4-dichlorophenoxyacetic-acid (2,4-D), and how they affect drug metabolizing enzyme activities in the chicken embryo liver. The authors suggest that phenoxyacetic herbicides have some effects on hepatic drug metabolizing enzymes of the chick embryo which can not be easily interpreted. They suggest that biliary retention, produced in particular by MCPA, may be partly responsible for the results (Santagostino et al., 1991).

The effects of phenoxyacetic acid herbicides were investigated on the induction of chromosome aberrations in human peripheral lymphocyte cultures in vitro and in lymphocytes of exposed workers in vivo. Pure 2,4-dichlorophenoxyacetic acid (2,4-D; 0.125, 0.150, 0.200 and 0.350 mM) did not increase the number of aberrations, whereas the commercial 2,4-D formulation (0.125, 0.250, 0.500, 1.000 and 1.250 mM, with respect to phenoxyacetic acid concentration) significantly increased the number of chromosome aberrations in vitro (without exogenous metabolic activation). The phenoxy acid levels in the breathing zone of the workers varied between 0.3 and 0.4 mg/m3, and the concentrations of phenoxyacetic acids in the urine of the workers after exposure varied from 0.000

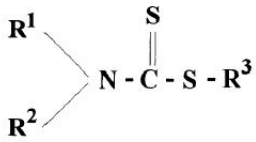
to 0.055 mmol/l. There were no increases in chromosome aberrations in peripheral lymphocytes of the exposed subjects (Mustonen et al., 1986).

15.9 DITHIOCARBAMATE FUNGICIDES

☑ General information

Dithiocarbamate (DTC) pesticides are mainly used in agriculture as fungicides and, to a lesser extent, as insecticides and herbicides. Additional uses are as biocides for industrial or other commercial applications and in household products. Some DTC are used for vector control in public health. The general formula of DTC pesticides is shown in Figure 10.

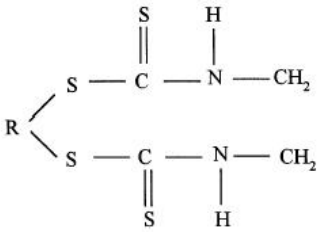
Figure 14.11: General structural formula of dithiocarbamate pesticides.



The Dithiocarbamate pesticides (DTC) can be sub-grouped into thiurams (thiram, methiram, disulfiram), dimethyldithiocarbamates (ferbam, ziram), and unsaturated alkyldithiocarbamates [ethylene-bisdithiocarbamates (EBDTCs) and propylene-bisdithiocarbamates].

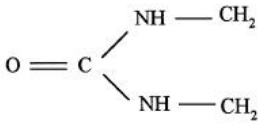
No methods for biological monitoring of exposure to DTC compounds are commonly accepted at present. Determination of DTC metabolites in urine has been carried out in exposed subjects for few compounds. Since DTC are mainly metabolized to carbon disulphide (CS_2), measurement of urine levels of this metabolite has been suggested when monitoring high-level DTC exposure (WHO, 1988). Some studies have investigated ethylenethiourea (ETU) concentrations in urine of workers exposed to EBDTC fungicides. ETU is a metabolite of EBDTC pesticides and it is also the most important substance from a toxicological point of view (Figure 11).

Figure 14.12: Chemical structure of ethylene-bis-dithiocarbamate pesticides and their main metabolites, ethylenethiourea (ETU) and carbon disulphide (CS_2) (Maroni et al., 2000).

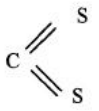


Ethylenebisdithiocarbamates

R = Mn, Zn, $(Na)_2$, $(NH_4)_2$



Ethylene thiourea (ETU)



Carbon disulphide

The measurement in urine of the metals present in the DTC molecule has been proposed as an alternative approach to monitor DTC exposure. In particular, urinary excretion of manganese has been investigated in workers exposed to mancozeb. DTC are mainly metabolized to CS_2, which is partially biotransformed to 2-thiothiazolidine-4- carboxylic acid (TTCA) (WHO, 1988d); CS_2 is mainly excreted via the exhaled air and, to a lesser extent, the urine. While very few data are available on the use of urinary TTCA for biological monitoring of exposure to DTC, some studies suggest monitoring high level exposures to DTC by means of CS_2 determination in urine: samples should be collected the morning following exposure (Liesivuori and Savolainen, 1994). Determination of CS_2 prior to exposure was recommended to establish individual baselines, since CS_2 is commonly found in the urine of non-exposed subjects (Brugnone et al., 1992).

ETU is one of the metabolic products deriving from EBDTC biotransformation in mammals, plants, and lower organisms. It may also be present as an impurity in EBDTC technical products. Moreover, EBDTC residues in food may be partly transformed into ETU during cooking. This compound shows goitrogenic effects as a result of an impairment in the synthesis of thyroid hormones that is followed by an enhancement of the TSH incretion (WHO, 1988). Teratogenic and carcinogenic effects have also been observed in rats and other animal species treated with this compound (IARC). An ADI of 0.004 mg/kg bw was established in 1994 by JMPR.

DTC are chemically characterized by the presence of metals in the molecule (iron, manganese, zinc, etc.); therefore, measurement of these metals in urine has been proposed as a tool to monitor exposure.

Biological monitoring of exposure to metal-containing DTCs may be performed by measuring the metal concentrations in biological fluids. This application represents a promising approach for biological monitoring, since determination of metals in biological fluids can be performed with very sensitive AAS methods which are of lower complexity than those required for DTC metabolite determination.

An increased urinary excretion of manganese was observed in workers exposed to mancozeb (Canossa et al., 1993). However, the available data are at present insufficient to convalidate the use of metals as biomarkers of human exposure to DTC.

☑ Matrix

- **Invasive**
  Blood
- **Non-invasive**
  Urine

☑ Kinetics

- **Uptake**
  By different routes

- **Metabolism**

  The metabolic pathway of DTC is very complex, producing a great number of metabolites (see above). One is carbon disulfide, which is further partially metabolised to 2- thiazolidinethione-4-carboxylic acid (WHO, 1988). These compounds have both been determined in urine of occupationally exposed and unexposed subjects, though they are not specific indicators of exposure to DTC. Ethylenethiourea, on the contrary, is the specific metabolite of ethylenebisdithiocarbamates (EBDC) (mancozeb, zineb, maneb, etc.) and is the more promising indicator of exposure for biological monitoring.

- **Biological half-life**

  Biological monitoring of occupational exposure to mancozeb has been carried out by determining the urinary concentrations of ETU and manganese. Urinary excretion of ETU was measured in some groups of low-level exposed workers. After 1-day exposure, a prolonged urinary excretion of this compound was observed in potato farmers. Detectable urinary concentrations of ETU were still present in urine specimens collected 22 days after exposure ceased. The half-life in urine was calculated to be 100 h (Kurttio et al., 1990). However, since ETU half-life is about 28 h in monkeys and 9–10 h in rats (WHO, 1996), this half-life is abnormally long and the possible presence of confounding factors has to be taken into account.

  The urinary excretion of ETU and manganese was investigated in farm workers with estimated absorbed doses of mancozeb (mean: 5.5 mg/kg bw) lower than its ADI. No urinary concentrations of ETU were detected in most subjects (analytical detection limit: 0.9 mg/l), while a significant increase in manganese concentrations was observed in some spot urine samples collected soon after the end of exposure. Urinary levels comparable to those measured in pre-exposure samples were found in specimens collected the morning after exposure was over (Canossa et al., 1993).

☑ Sampling conditions

- **Operational aspects**

  The sampling pattern and conditions of storage for blood are similar to those previously described for other pesticides.

  Urine should be placed in plastic containers shielded with aluminium foil to prevent light-induced breakdown of metabolites. Ethylenethiourea is transformed into ethyleneurea when exposed to light or in the presence of specific activators, such as chlorophyll and organic solvents. If analysis is not carried out immediately should be frozen and stored frozen. In these cases, it is strongly suggested to consider sample stability.

☑ Analytical aspects

- **Techniques**

  The analytical procedure for measurement of CS_2 in blood is GC–MS SIM. The same method has been used in the analysis of the metabolite in urine (Brugnone et al., 1992).

  The analytical procedures for the measurement of TTCA in urine are: HPLC–UV (van Welie et al., 1991; Krstev et al., 1993), GC–MS SIM (Weiss et al.., 1999), GC–FPD (van Welie et al., 1991).

  Most analytical procedures for measurement of Ethylenethiourea involve HPLC, only a few GC with FPD detection (Camoni et al., 1984).

  HPLC of purified urine extracts is the technique of choice for assessment of occupational and non-ccupational exposure.

- **Sensitivity and specificity (detection range)**

  The procedure that involves HPLC–TSP-MS is quite sensitive and selective, but is too expensive for routine analyses (Aprea et al., 2002).

☑ Dose-response/effect relationship

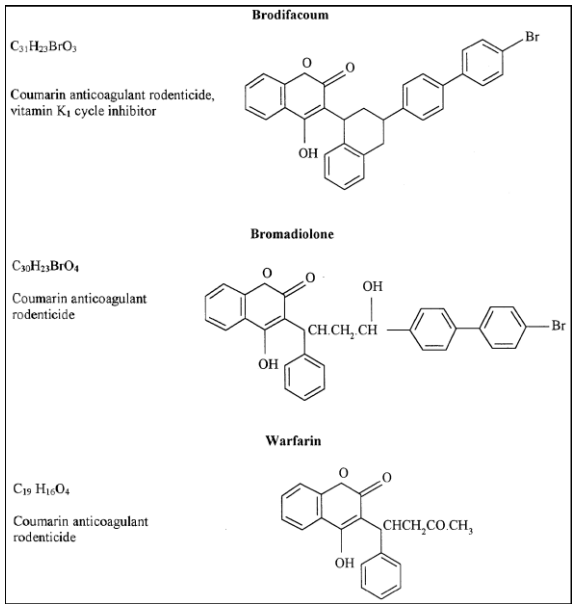
NIOSH classifies ethylenethiourea (ETU) as carcinogenic for humans (Aprea et al., 2002).

15.10 COUMARIN RODENTICIDES

☑ General information

Coumarin derivatives are used in human drug treatment as anticoagulants. Since they are active in mammalians, they are also used as rodenticides against various species, including rats and mice. Coumarin rodenticides can be grouped into so called first generation (e.g. warfarin) and second generation (e.g. brodifacoum) compounds, the later being characterised by their very long biological half-lives (100–200 days).

Fig. 14.13: Chemical structure of some representative compounds of first and second generation (Maroni (2000)



Coumarin rodenticides are vitamin K antagonists.

The biochemical mechanisms involved in the anticoagulant action of coumarin derivatives are common to all mammalian species. Coumarin derivatives act by inhibiting both the enzymes vitamin K epoxide (KO)-reductase and vitamin-K reductase of the vitamin cycle in the liver. In this way the recycling of vitamin K

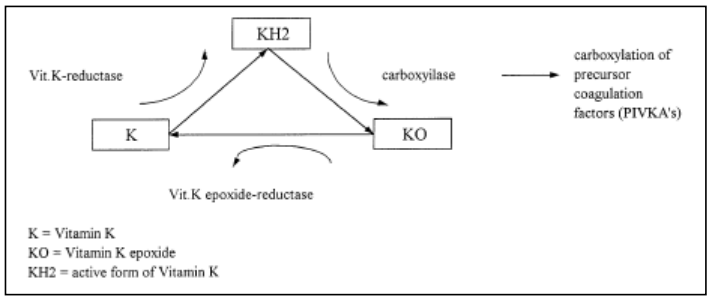
is blocked, thereby depleting the supply of vitamin KH2, the active form of vitamin K.

Administration of coumarin derivatives results in a decrease of the synthesis rate of vitamin K-dependent clotting factors II, VII, IX, and X and eventually in a decrease in their plasma levels. The rate of decay is dependent on the half-lives of the clotting factors in plasma, which in humans is about 60 h for prothrombin, 45 h for factor X, 14 h for factor IX and 6 h for factor VII. Anticoagulant effects occur when plasma levels drop approximately below 20% of normal levels. Some authors describe teratogenic effects.

The measure of clotting properties / clotting factors concentration are the most widely used biomarkers of effect (and exposure?). Recently, some analytical techniques are able to measure unaltered compounds, mostly from 2nd generation, in biological fluids. This may be used as biomarker of exposure.

At the research level, the use of the increased levels of PIVKA (Protein Induced by Vitamin K Antagonist / Absence) as rodenticide effect biomarker is actually under study. This is widely used in hepatic illnesses diagnostic and surveillance.

Figure 14.14: Vitamin K cycle in the mammalian liver (M. Maroni 2000)



PROTHROMIN TIME

☑ General information

It means the measurement of the activity of Factor II or Prothrombin in the blood plasma. In the workplace and environmental practice it is used for exposure

monitoring, but it is also useful as effect marker, as well as in the medical practice.

☑ Matrix

- **Invasive**

  Blood plasma (citrate) is exclusively used.

☑ Sampling Conditions

The prothrombin time can be measured roughly on whole blood (which is done in neonates), but is more commonly measured from blood plasma. Blood is drawn into a test tube containing liquid citrate. Citrate acts as an anticoagulant by binding the calcium in the sample. The blood is mixed, and then centrifuged to separate blood cells from plasma. It is strongly recommended the use of fresh (not frozen) plasma to carry out the test. Sampling should be carried out at the end of exposure.

☑ Analytical Aspects

The plasma is put through a "coagulation machine", which takes a sample of the plasma. An excess of calcium is added (thereby reversing the effects of citrate), which enables the blood to clot again. For an accurate measurement the proportion of blood to citrate needs to be fixed. Tissue factor (also known as *factor III* or *thromboplastin*) is added, and the time the sample takes to clot is measured optically.

☑ Results and units

The reference range for prothrombin time is usually around 12-15 seconds. The prothrombin ratio, usually expressed as a percentage) is the prothrombin time for a patient, divided by the result for control plasma as a reference in the same batch series. Specially adequate if the circulating factors drops below 20% of normal levels.

Because of differences between different batches and manufacturers of tissue factor (it is a biologically obtained product), the INR was devised to standardise the results. Each manufacturer gives an ISI (International Sensitivity Index) for

every tissue factor they make. The ISI value indicates how the particular batch of tissue factor compares to an internationally standardized sample. The ISI is usually between 1.0 and 1.4. The INR is the ratio of a patient's prothrombin time to a normal (control) sample, raised to the power of the ISI value for the control sample used. The normal range for the INR is 0.8-1.2.

$$INR = \left(\frac{PT_{test}}{PT_{normal}}\right)^{ISI}$$

☑ Confounding factors

Some substances, such as alcohol, can affect the PT/INR test. Antibiotics, aspirin, and cimetidine can increase the PT/INR. Barbiturates, oral contraceptives and hormone-replacement therapy (HRT), and vitamin K - either in a multivitamin or liquid nutrition supplement - can decrease PT. Certain foods (such as beef and pork liver, green tea, broccoli, chickpeas, kale, turnip greens, and soybean products) contain large amounts of vitamin K and can alter PT results. Liver disease, intravascular clothing, pharmacological treatments with coumarinics or heparin, genetic alterations, malabsortive state, vitamin k deficiency, can also alter the expected results of the test.

☑ Dose/response

As usual, most of the exposure data comes from the workplace. In exposed workers, a decrease of 10% above the baseline values indicates the suspension of work. The return takes place after the re-assumption of baseline values.

This test is adequate to monitor clinically depressed coagulation, that is, when individual clotting factors decrease to approximately 20% of normal plasma levels, and is used to monitor anti-coagulation treatment or, in the occupational setting, to assess the severity of an existing over-exposure. However, the test is not suitable to detect early effects following exposure when circulating clotting factors decrease to 70–80% of normal plasma levels. For that purpose the determination of the prothrombin concentration (factor II) in plasma is recommended.

PROTHROMBIN CONCENTRATION

☑ General information

As cited before, the measurement of prothrombin plasma concentration is adequate when circulating clotting factors decrease to 70–80% of normal plasma levels, although the data in the literature are scarce.

☑ Matrix

- **Invasive**

  Blood plasma (citrate) is exclusively used

☑ Sampling Conditions

As in PT test, blood is drawn into a test tube containing liquid citrate. Citrate acts as an anticoagulant by binding the calcium in the sample. The blood is mixed, and then centrifuged to separate blood cells from plasma. In this case is possible to use of fresh or frozen plasma to carry out the measure. Sampling should be carried out at the end of exposure.

☑ Analytical Aspects

We can found some descriptions of their use for clinical purposes. Basically the analytical method is bases in a transformation of prothrombin in thrombin by a reactive molecule, normally from snake venoms; the produced thrombin cleaves a chromogenic substrate and produces the p-nitroaniline liberation. Among others, two methods are mainly used to transform prothrombin in thrombin, the activator Ecarin from *Echis carinatus* or Carinactivase-1 (CA-1), a calcium-dependent activator from *Echis Leucogaster*. The second one is advantageous because is specific for normal (fully carboxylated) prothrombin, while in the Ecarin method normal and undercarboxylate prothrombin are indiscriminately evaluated.

PROTEIN INDUCED BY VITAMIN K ANTAGONIST / ABSENCE (PIVKA)

☑ General information

PIVKAs are coagulation factor precursors, normally detectable in blood, which are released into the blood stream in case of blockage of the vitamin K cycle by coumarins. So far, this test has only been frequently used for research purposes.

High plasma PIVKA concentrations were observed in acute bromadiolone poisoning cases characterized by PT values higher than 120 s. In a study carried out in workers occupationally exposed to 4-hydroxy-coumarins, high concentrations of vitamin K1 2, 3-epoxide were found despite normal clotting factor activities. According to the authors, investigation of vitamin K metabolism might provide a more sensitive assessment of exposure than monitoring PT or its plasma concentration.

☑ Matrix

- **Invasive**

  Blood plasma (citrate) or serum

☑ Sampling Conditions

We can use citrated plasma or serum. The blood, after withdrawal, is mixed with the appropriate amount of sodium citrate as in Prothrombin Time method, after centrifugation, the plasma is removed from the packed cells. If citrate is not added, after clothing and centrifugation, blood serum can be removed from the cloth.

☑ Analytical aspects

We can find references to several methods; all of them are based on immunological (ELISA) techniques, after different kind of preparation procedures. One of them is developed in the work of Grosley et al (1996) and it seems to increase substantially the specificity and the practice aspects of the former methodologies. To increase the assay sensitivity, a mAb (P1-2-B9) specific

for the hypocarboxylated prothrombin forms was developed This mAb has been used for the development of a PIVKA-II ELISA technique. This study demonstrates the optimization of this immunoassay as well as the preparation of a reproducible calibrator for standardizing it. The PIVKA-II concentrations are now reported in nanograms per milliliter. This constitutes an important advancement, because up to now concentrations were expressed as arbitrary units.

UNALTERED COMPOUNDS

☑ General information

Little information is available on biological monitoring of human exposure to coumarins by means of intact compound determination in blood. Available data concern the application of these biomarkers to confirm coumarin exposure in acute poisoning cases or during anticoagulant therapy in clinical trials. The measurement of parent compounds in blood has found limited application because the analytical techniques required for their determination are much more complex than those required for clotting factor assay. Moreover, there is a lack of information about the relationship between blood concentrations and effects on the human coagulation system for most compounds.

Information on biological monitoring of exposure to coumarin derivatives is confined to a few compounds and mainly concerns acute poisoning from attempted suicide or murder cases.

☑ Matrix

- **Invasive**

  Blood plasma (citrate) is mostly used. Some references are available about the use of urine as working material.

☑ Sampling conditions

The time of sampling is not relevant due the long 1/2t (mostly for second generation molecules).

☑ Analytical aspects

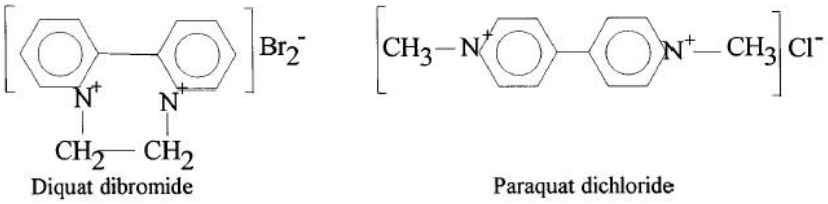
HPLC, more or less sophisticated and coupled to a variety of detectors are in the literature. For instance, we can find LC-ESI-MS (Liquid chromatography-electrospray ionization-mass spectrometry) or HPLC-FD.

15.11 QUATERNARY AMMONIUM COMPOUNDS

☑ General information

Diquat and paraquat are widely used as contact herbicides and crop desiccants. They are mainly used as aqueous solutions of their salts (Figure 14.15).

Figure 14.15: Structural formula of the quaternary ammonium herbicides.



Diquat dibromide

Paraquat dichloride

☑ Matrix

The determination in blood and urine of the unchanged compounds has been used to monitor human exposure to diquat and paraquat.

☑ Kinetics

- **Uptake**

  Different routes

- **Metabolism**

  When systemic absorption occurs, paraquat and diquat are rapidly distributed in the body. Paraquat mainly accumulates in the lungs and kidneys, while the highest diquat concentrations were found in the gastrointestinal tract, liver and kidneys. Urine is the major route of excretion of both compounds, which are mainly eliminated unmodified.

☑ Sampling conditions

Operational aspects (e.g. retrieval conditions, transport and storage conditions)

Under field conditions, it has been recommended that QAC compounds should be measured in 24-h urine samples collected after the end of exposure. Alternatively, when this is impractical, spot samples should be collected at the end of exposure. Blood should be tested soon after a QAC poisoning is suspected. To assess prognosis of the intoxication, it is important to accurately establish the time between blood sampling and exposure.

☑ Analytical aspects

- **Techniques**

  Several methods, based on colorimetric, gas chromatographic and radioimmunoassay techniques, are available for analysis of diquat and paraquat in blood and urine (Summers, 1980).

☑ Concentrations reported in literature

Little information is available on the relationship between occupational exposure levels and internal doses of QAC pesticides. QAC doses and plasma levels were measured specially after intentional poisoning following ingestion of large amounts of active ingredients. In a total of 20 fatalities that occurred as a consequence of paraquat and diquat absorption reported to US Poison Centers in the period 1983–1992, exposure was by ingestion in 19. Inhalation and dermal exposure occurred in addition to ingestion in one occupational accident, and inhalation and ingestion were involved in two cases. Only one case was attributed to both dermal and inhalation exposure. Under field conditions, no detectable urinary concentration of diquat was found in workers following good agriculture practices, while urinary paraquat concentrations have often been observed at levels below 0.01 mg/l.

☑ Critical values

Key information on the quaternary ammonium compounds (Maroni et al., 2000)

Compound	WHO classification (class of acute toxicity)	ADI (mg/kg bw); JMPR (year of evaluation)	Lowest possible lethal dose in human
Diquat	II	0.002 (1993)	6–12 g/subject
Paraquat	II	0.004 (1986)	35 mg/kg

☑ Dose –response/effect relationships

The oral lethal dose of diquat dibromide in man is in the order of 6–12 g (Vanholder et al., 1981).

The herbicide paraquat (PQ) can adversely affect dopamine systems (Cory-Slechta et al., 2005).

15.12 BIOMARKERS FOR GENOTOXICITY IN PESTICIDES BIOMONITORING

Specificity is considered, in most situations, a desirable characteristics for biomarkers; however, if a general alarm is required for the exposure to a complex (and often unknown) mixture of chemicals, or when the sought objective is the detection of a particular effect potentially attributable to several compounds, the use of unspecific biomarkers becomes essential.

Pesticides are an extremely heterogeneous set of chemicals, only identified by the purpose for which they have been designed. Experimental studies show that a variety of products used in agriculture posses the potential to cause mutations, chromosomal alterations or DNA damage. In such a scenario, biomonitoring, using one (or a battery) of the currently accepted genotoxicity assays, provide a useful tool for risk assessment.

In general terms, genotoxicity due to pesticides occurs in association with highly intensive exposure, misuse, or lack or failure of protection measures; and appears to be transitory in acute or discontinuous exposure, but persistent and cumulative in chronic, long term exposure conditions. Consequently, although some studies on consumers exposure and on specially sensitive groups (children) are available, most of the monitoring campaigns have focused on occupationally exposed populations (manufacturing workers, floriculturists, farm workers).

We will briefly review some of the genotoxicity biomarkers most frequently used in pesticide biological monitoring. Two recent, comprehensive (67 and 144 references) surveys of the topic may be found at Bolognesi and Morasso (2000) and Bolognesi (2003).

CHROMOSOMAL ABERRATIONS

☑ General information

Lymphocyte cultures are prepared according to standard techniques. Heparinized whole blood (0.8 mL) is added to 5mL of culture medium F10, supplemented to

18.5% with fetal calf serum, with 0.2mL, phytohemagglutinin, and with antibiotics (10,000 IU/mL penicillin and 10,000 IU/mL streptomycin).

Lymphocytes are cultured in the dark for 72 h and metaphases blocked during the last 1.5 h with colcemid at final concentration of 0.2 mg/mL. The mitotic index is calculated as the proportion of metaphases among the total cell population by counting a total of 1000 cells.

Chromosomal aberrations are evaluated in 72-h whole-blood cultures according to standard protocol. The cells are collected by centrifugation, resuspended in a prewarmed hypotonic solution (KCl,0.075 M) for 15 min, and fixed in methanol/acetic acid (3:1, v/v) solution (Carnoy's fixative). Air-dried preparations are then made, and the slides stained by the "fluorescence plus Giemsa" (FPG) procedure

Generally, for each donor, 100 cells at metaphase are analyzed for chromosome damage. Cells including either numerical or structural chromosomal aberrations are recorded as aberrant cells.

Chromatid breaks, chromosome breaks, fragments, dicentric and acentric chromosomes, deletions, and duplications are evaluated as structural chromosomal aberrations.

☑ Matrix

- **Invasive**

  In all the studies reported up to date in scientific literature, in our knowledge, chromosome aberrations for pesticides monitoring in humans have been measured in peripheral blood lymphocytes. As a very small sample (0.8 mL of heparinized whole blood) is required, the procedure results minimally invasive.

☑ Sampling conditions

- **Operational aspects**

  Venous blood is collected in heparinized sterile test-tubes and stored at 4 ºC for a maximum of 24 h. Lymphocyte enriched plasma is then obtained by centrifugation of whole blood for 40-50 min at 1000 rpm.

☑ Analytical aspects

- **Techniques**

  Although staining with Giemsa alone is also possible, the combined method "fluorescence plus Giemsa (FPG)" is advisable for the scoring of chromosomal aberrations, thus allowing an easier identification of chromosomes, and the possibility of carrying out simultaneously the Sister Chromatid Exchange test (see below). This method is based, with minor modifications (Tease, ; Hoffmann *et al*., 2002; Raposo *et al*., 2004), on the protocol established by Perry and Wolf (1974),

  In brief, the method consists on a 30 min staining with Hoechst 32258 in the dark, followed by a photodegradation period of 10 min under ultraviolet light (252 nm) and a final 10 min staining with 2% Giemsa.

- **Sensitivity and specificity**

  Chromosome Aberrations Test is considered more sensitive than Micronucleus Test (Bolognesi, 2003). The scarcity of available data with other techniques makes difficult to establish a more precise scale of sensitivity.

  Genotoxicity testing, as a monitoring tool, is based on the assumption of an association with increased cancer risk; such a conclusion has been established for Chromosome Aberrations (Hagmar *et al*., 1994, 1998), while only indirect evidence has been reached for other genotoxicity assays

- **Units**

Chromosome Aberrations results are expressed as the percent of aberrant cells. Under this term are included both the aneugenic cells and those presenting structural chromosomal aberrations.

For comparison purposes, the increment fold with respect to controls is generally used.

☑ Validation

The accepted protocol for experimental performance of this assay is presented in the OECD Guideline Nº 473 (adopted July 21st 1997), that is easily adapted to human biomonitoring use. Other related official guidelines are:

- ICH (S2A y S2B)
- FDA Redbook II
- EEC Guideline 92/96 (Annex V. Method B10)
- EPA Part 798, Sec 798.5375
- MAFF Guideline 4200
- MHW Notification n° 24

☑ Confounding factors

No consistent, significant differences have been observed in chromosomal aberrations regarding age, gender, or alcohol or coffee consumption.

Smoking may potentiate the genotoxic effects of pesticides, increasing the oral exposure during the agricultural practices (Lander *et al*., 2000) or having an additive effect in inducing chromosomal damage (Rupa *et al*., 1988, 1989).

☑ Data reported in literature

As many studies combine two or more genotoxicity assays, specially chromosome aberrations (CA), micronucleus test (MNT) and sister chromatid exchange (SCE), a summary of published data will be given below for the three mentioned tests.

☑ Dose-response relationships

Many studies (reviewed in Bolognesi, 2003) show a positive, significant correlation between exposure and percent of aberrant cells in CA test.Thsi correlation has been observed both in cases of acute intoxication (accident or attempted suicides) and in chronic occupational exposure, with higher levels during the spraying season when pesticides are used intensively, in individuals working exclusively in greenhouses with respect to those working in open-fields, and in poorly-protected workers when compared with those using masks and gloves.

☑ Time trend, geographical variation, susceptible groups

General population is exposed to pesticides through air, water and food

The main risk of exposure, however, occurs in the occupational framework

- Chemical plant workers
- Pesticide users

- Pesticide sprayers

Children are a specially sensitive group (Neri et al., 2006)

MICRONUCLEUS TEST

☑ General information

Micronucleus Test (MNT) (Schmid, 1973; von Ledebur i Schmid, 1973; Borràs, 1986; Ashby et al., 1990; Tucker i Preston, 1996; Sato i Tomita, 2001) is a widely accepted technique for the detection of clastogenic or aneugenic effects, as well as for spindle poisons.

The assay is generally performed on circulating erythrocytes. In the final phases of its maturation process, these cells expulse its nucleus; however, the fragments produced by clastogenesis, or due to a partially incomplete separation of the chromatids, are not extruded, and appear as little spherical bodies. These bodies are produced physiologically in a proportion of less than three per thousand, and are called Howell-Jolly bodies. When, by the action of a genotoxic agent, these bodies significantly exceed this level, they are called "micronuclei", and their percent is considered to be proportional to the genotoxic activity of the compound.

The assay may also be conducted in other cell types, such as circulating lymphocytes or buccal epithelial cells, provided a cell culture is carried out, and the separation or daughter cells in mitosis is prevented by the addition of cytochalasin B. The number of bi-nucleated cells that present micronuclei is then the end-point.

☑ Matrix

- **Invasive**

  In many reported studies for pesticides monitoring in human, micronuclei have been measured in peripheral blood lymphocytes or erythrocytes. As a very small sample (0.8 mL of heparinized whole blood) is required, the procedure results minimally invasive.

- **Non-invasive**

  Buccal epithelial cells (Lucero et al., 2000; Pastor et al., 2001)

☑ Sampling conditions

- **Operational aspects**

  Blood is obtained by venipuncture using heparinized vacutainers.

- **MN analysis in erythrocytes**

  A drop of blood is smeared over a slide, and fixed in methanol. Although Giemsa stain may be used, it is advisable, to avoid confounding artifacts, the use of the fluorescent dye Acrydine Orange (AO). The coverslips must be sealed, and the slides read within 3 hours, to prevent fading.

- **MN analysis in lymphocytes**

  Lymphocyte cultures are set up by adding 0.5 ml of whole blood to 4.5 ml of RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1% antibiotics_penicillin and streptomycin.and 1%L-glutamine. Lymphocytes are stimulated by 1% phytohaemagglutinin Gibco.and incubated for 72 h at 37ºC.

  A cytochalasin B solution is then prepared in dimethylsulphoxide at a concentration of 6 µg/ml and stored at -20ºC. This solution is thawed and added to the cultures 44 h later to arrest cytokinesis. At 72 h of incubation, the cultures are harvested by centrifugation at 800 rpm for 8 min. Next, to eliminate red cells and to preserve cytoplasm, blood cultures are washed once in RPMI 1640 medium and a mild hypotonic treatment (2–3 min in 0.075 M KCl at 4ºC) is carried out thereafter. Cells are centrifuged and a methanol–acetic acid_3:1 vrv.solution is gently added. This fixation step is repeated twice and the resulting cells resuspended in a small volume of fixative solution and dropped onto clean slides. Finally they are stained with 10% Giemsa.in phosphate buffer (pH 6.8) for 10 min.

- **MN analysis in buccal cells**

  Buccal cell samples are obtained by rubbing the inside of the cheeks with a toothbrush. The cells are collected in a conical tube containing 20 ml buffer solution (0.1 M EDTA, 0.01 M Tris–HCl and 0.02 M NaCl, pH 7). After three washes in this buffer solution, by centrifugation at 1500 rpm during 10 min, 50 ml of an adequate cell suspension density are dropped onto preheated (55ºC)

slides and allowed to air-dry for 15 min on a slide-warmer. The slides are fixed in 80% cold methanol for 30 min, air-dried overnight at room temperature, and stored at -20ºC until use. Finally, they are stained with a 4',6-di-amidino-2-phenylindole dihidrochloride (DAPI) solution and a total of 2000 cells/donor are scored under a fluorescence microscope.

☑ Analytical aspects

- **Sensitivity and specificity**

  Chromosome Aberrations Test is considered more sensitive than Micronucleus Test (Bolognesi, 2003). The scarcity of available data with other techniques makes difficult to establish a more precise scale of sensitivity.

  Genotoxicity testing, as a monitoring tool, is based on the assumption of an association with increased cancer risk; such a conclusion has been established for Chromosome Aberrations (Hagmar *et al*., 1994, 1998), while only indirect evidence has been reached for other genotoxicity assays

- Units
  - *Erythrocytes:*Percent of micronucleated polychromatic erythrocytes with reference to the total number of polychromatic erythrocytes (approximately 2000 scored polychromatic cells per subject)
  - *Lymphocytes:*To determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of MN, a total of 1000 binucleated cells with well preserved cytoplasm (500 per replicate)..are scored for each subject. In addition, 500 lymphocytes are scored to evaluate the percentage of cells with one to nuclei and the cytokinesis-block proliferation index (CBPI) is calculated.
  - *Buccal cells*: Micronuclei in 2000 cells/donor

☑ Validation

The accepted protocol for experimental performance of this assay is presented in the OECD Guideline Nº 474 (adopted July 21st 1997), that is easily adapted to human biomonitoring use. Other related official guidelines are:

EPA Health Effects Test Guidelines OPPTS 870.5395: Mammalian Erythrocyte Micronucleus Test (August 1998).

☑ Confounding factors

Data on differences in MNT results regarding age, gender, or alcohol or coffee consumption are contradictory and non-conclusive (Lucero et al., 2000).

Smoking may potentiate the genotoxic effects of pesticides, increasing the oral exposure during the agricultural practices (Lander *et al*., 2000) or having an additive effect in inducing chromosomal damage (Rupa *et al*., 1988, 1989).

☑ Data reported in literature

As many studies combine two or more genotoxicity assays, specially chromosome aberrations (CA), micronucleus test (MNT) and sister chromatid exchange (SCE), a summary of published data will be given below for the three mentioned tests.

☑ Dose-response relationships

See the considerations exposed for Chromosome Aberrations Test.

☑ Time trend, geographical variation, susceptible groups

General population is exposed to pesticides through air, water and food

The main risk of exposure, however, occurs in the occupational framework

- Chemical plant workers
- Pesticide users
- Pesticide sprayers

Children are a specially sensitive group

No clear conclusions may drawn from the study of the genotypic variability in the glutathion-s-transferase (GST) family (Lucero et al., 2000); on the contrary, Liu et al., 2006, report a positive association bwtween GSTP1 genetic polymorphism and the risk of DNA damage in pesticide-exposed fruit growers.

SISTER CHROMATID EXCHANGE

☑ General information

Sister chromatid exchanges (SCEs) involve breakage of both DNA strands, followed by an exchange of whole DNA duplexes. This occurs during S phase and is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication. The formation of SCEs has been correlated with recombinational repair and the induction of point mutations, gene amplification and cytotoxicity.

SCE occurs normally in cells during mitosis, or cell division, but when a cell's DNA is damaged by genotoxic agents, the rate of SCE increases. It is thought that SCE is an attempt by the cell to fix the DNA damage caused by genotoxic agents; therefore, you would expect a more potent genotoxic agent to generate a higher rate of SCE.

While DNA damage-repair pathways are usually quite efficient, too many damage events will cause a cell to give up and die. That is, after a certain point, the cell is unwilling or unable to try and fix all of the damage. Here's another way to think about it: If you wrecked you car, would you want to fix it? If the car sustained only minimal damage, then you probably would try to repair it. But if the damage is extensive, then you might think it's not worth the effort to save the car -- or even if you think it's worth it, you might not be able to. That is very similar to the "reasoning" process the cell uses to decide whether to try to fix its damaged DNA.

SCE must be considered as a biomarker of exposure, since sister chromatid exchanges do not correspond to mutations

☑ Matrix

- **Invasive**

  Sister Chromatid Exchange for pesticides monitoring in humans are measured in peripheral blood lymphocytes.

- **Sampling conditions**

  As stated for other genotoxicity tests previously described.

☑ Operational aspects

Venous blood is collected in heparinized sterile test-tubes and stored at 4 ºC for a maximum of 24 h. Lymphocyte enriched plasma is then obtained by centrifugation of whole blood for 40-50 min at 1000 rpm.

☑ Analytical aspects

- **Techniques**

  The SCE test is usually performed on human peripheral blood lymphocytes. As peripheral lymphocytes are in the resting G0 stage of the cell cycle, they have to be stimulated to divide by an aspecific antigen, like phytohaemagglutinin. To collect a sufficient number of mitotic cells, a spindle inhibitor like colcemid may be added shortly before fixation (at 72 hours) , to block cells in (pro)metaphase of the second mitosis.

  To allow for a differential staining that enables the researcher to distinguish both chromatids, BrdU (bromo-deoxy-uridine) is added to the culture medium for the duration of two complete cell cycles. Chromatids in which only one strand of DNA incorporated BrdU show a normal dark Giemsa staining, whereas those with two substituted strands, stain less darkly. If an exchange occurred, this can be seen as the dark part changes to the other arm: "harlequin chromosomes".

- **Units**

  Endpoint: detection of "reciprocal" sites, symmetrical or asymmetrical exchange between the sister chromatids of a single chromosome, probably related to recombinational repair

  The number of cells analyzed should be based on the spontaneous control frequency of SCE. Usually, at least 25 well-spread metaphases per culture are analyzed. Only metaphases containing 46 chromosomes are considered for scoring.

☑ Validation

The accepted protocol for experimental performance of this assay is presented in the OECD Guideline Nº 479 (adopted October 23st 1986), that is easily adapted to human biomonitoring use. Other related official guidelines are:

- ICH (S2A y S2B)
- EPA OPPTS 870.5915
- EPA OTS 798.5915

☑ Confounding factors

Smoking is considered the main confounding factor in SCE test.

☑ Data reported in literature

As many studies combine two or more genotoxicity assays, specially chromosome aberrations (CA), micronucleus test (MNT) and sister chromatid exchange (SCE), a summary of published data will be given below for the three mentioned tests.

☑ Dose-response relationships

Many studies (reviewed in Bolognesi, 2003) show a positive, significant correlation between exposure and percent SCE.

☑ Time trend, geographical variation, susceptible groups

General population is exposed to pesticides through air, water and food

The main risk of exposure, however, occurs in the occupational framework

- Chemical plant workers
- Pesticide users
- Pesticide sprayers

Children are a specially sensitive group (Neri et al., 2006)

Table 14.5.-Cytogenetic effects on populations exposed to pesticides – chemical plant workers. Chromosomal aberrations, SCE and micronuclei in peripheral blood lymphocytes (Bolognesi, 2003)

Study subjects (exposed/controls)	Exposure	Duration (years)	Analysed biomarker	Result	Reference
44/30	Novozir Mn80 (mancozeb- contained fungicide)	Up to 2	CA	Pos (+1.83)	Jablonika et al. [37]
			SCE	Pos (+1.17)	
14/50, nine formulators, five packers	Azynphos methyl, dimethoate, malathion, methyl parathion	N.D.	SCE	Pos (+1.21)	Laurent et al. [40]
19/36	2,4,5-T, 2,4-D	10–30	CA	Pos (+2.05)	Kaioumova and Khabutdinova [38]
20/20	Pesticide mixture; most commonly used pesticides: 2,4-D, atrazine, alachlor, cyanazine, malathion	4–30 (sampling carried out after 8 months high exposure period)	CA	Pos (+6.10)	Garaj-Vrhovac and Zeljezic, Zeljezic and co-workers
			MN	Pos (+3.63)	
			SCE	Pos (+2.23)	Zeljezic and Garaj-Vrhovac
135/111	Organophosphates	1–24	SCE	Pos(+1.85smokers) (+1.63non-smokers)	Padmavathi et al.

Table 14.6.- Cytogenetic effects on populations exposed to pesticides – pesticide sprayers (Bolognesi, 2003) [a] Chromosomal aberrations, SCE and micronuclei in peripheral blood lymphocytes. [b] The MN frequency was also evaluated in buccal mucosa cells.

Exposure	Duration (years)	Analysed biomarker	Result	Reference
(a) Exposure to single pesticide				
35/15 Forestry workers: 2,4-D, MCPA	N.D.	SCE		Linnainmaa
Before spraying			Neg	
During spraying			Neg	
After spraying			Neg	
19/15 Forestry workers: 2,4-D, MCPA; after spraying season	6–28 (days)	CA	Neg	Mustonen et al.
60/42 Papaya workers: ethylene dibromide	5 (average)	CA	Neg	Steenland et al.
		SCE	Neg	
24/24 Fumigant appliers (open-field): phosphine and other pesticides	Discontinuous use of phosphine, at least 8 months	CA	Pos (+3.58)	Garry et al.
18/26 Fumigant appliers (open-field): phosphine and other pesticides	Discontinuous use of phosphine, at least 8 months	CA	Pos. (+3.4)	Garry et al.
31/21 Fumigators: phosphine	N.D.	MN	Neg	Barbosa and Bonin
38/16 Medfly eradication program: malathion	After spraying season	MN	Neg	Titenko-Holland et al.
31/30 Ethylenbis (dithiocarbamate) *Fungicide sprayers*	N.D.	CA	Pos (+1.32)	Steenland et al.
13/30 Tomato farmers	N.D.	CA	Neg	
31/30 Fungicide sprayers	N.D.	SCE	Pos (+1.12)	Steenland et al.
31/27 Fumigant appliers: methylbromide	0.3–22	MNb	Neg	Calvert et al.
12/9 Pesticide applicators: 2,4-D	Discontinuous use	MN	Neg	Figgs et al.

Exposure	Duration (years)	Analysed biomarker	Result	Reference
(b) Exposure to pesticide mixture				
109/57 No data	2–20	CA	Pos (+1.68)	Nehez et al.
80/24 Pesticide mixture (80 formulations): carbamates, dithiocarbamates, heterocyclic compounds, nitro-compounds, organochlorines,phenoxy-acetic acids, phthalimides, pyrethroids, sulphur and copper containing chemicals	1 to >15	CA	Pos (+2.69 to +3.89)	Paldy et al.
15/10 Vineyard workers: copper sulfate, DDT, dichlorvos, dieldrin, dithane, lindane, metasystox, parathion, quinalfos	5–12	CA	Pos (+4.16)	Rita et al.
55/60 Greenhouse workers: pesticide mixture; insecticides (carbamates, organophosphates); pyrethroid fungicides, acaricides	2–15	CA	Pos(+1.18–1.52)	Nehez et al.
25/30 (male Vegetable garden workers: BHC, smokers) DDT, dimethoate, fenitrothion, gromor, malathion, parathion, urea	5–38	CA	Pos(+1.72–2.08)	Rupa et al.
		SCE	Pos (+1.43–1.64)	
		SCE	Neg	
		MN	Pos (+7.67)	
22/16 Pesticide mixture: captan, cyfluthrin, cypermethrin, deltamethrin, dichlorvos, diazinon, endosulfan, fenitrothion, fenvalerate, linuron, magnesium–aluminium phosphide, methamidophos, methomyl, methyl bromide, parathion pentachlorphenol, propoxur Chlorinated hydrocarbons, carbamates (propoxur), organophosphates (dichlorvos, dimethoate, malathion), pyrethroids (cypermethrin, d-allethrin, deltamethrin, sumithrin)	7	MN	Neg	Venegas et al.
		CA		Amr
39/20 *Formulators*	5–25		Pos (+1.61)	
32/20 *Applicators*	5–15		Pos (+2.38)	

Table 14.7: Cytogenetic effects on populations exposed to pesticides – floriculturists (taken from Bolognesi, 2003) [a] Chromosomal aberrations, SCE and micronuclei in peripheral blood lymphocytes.

Study subjects (exposed/control)	Exposure	Duration (years)	Analysed biomarker	Result	Reference
36/15 sprayers and not sprayers	Greenhouse workers: pesticide mixture—carbamates, organochlorines, organophosphates	At least 10	CA	Pos (+1.02) (+4.3 exchange type aberrations)	Dulout et al.
14/13	Symptomatic group/asymptomatic group	At least 10	SCE	Pos (+1.18)	Dulout et al.
38/32	*Plant breeders: pesticide* mixture—organophosphates, carbamates, organochlorines	At least 10	CA	Neg	Dulout et al. [92]
	Greenhouse and open-field: chloroganics, hydrocarbon derivatives, organotin compounds, nitroorganics, organophosphates, pyrethorids, thio-organics	N.D.	CA		De Ferrari et al.
32/31	Healthy people			Pos (+1.86)	
32/31	Cancer patients			Pos (+1.45)	
28/15	Healthy people	N.D.	SCE	Pos (+1.40)	De Ferrari et al.
14/15	Cancer patients	N.D.	SCE	Pos (+1.50)	De Ferrari et al.
71/75	*Floriculturists and horticulturists:* pesticide mixture; most commonly used pesticides: benzimidazoles, carbamates, chloroorganics, dithiocarbamates, morpholines, nitroorganics, organophosphates, organotins, phthalimides, pyrethroids	2/55	MN	Pos (+1.28)	Bolognesi et al.
27/28	*Floriculturists and horticulturists:* pesticide mixture; most commonly used pesticides: benomyl, captan, deltamethrin, fenvalerate, methomyl, paraquat	>10	SCE	Neg	Carbonell et al.
61/60	*Floriculturists and horticulturists:* pesticide mixture; most commonly used pesticides: amides, carbamates, diazines, organochlorines, organophosphates, pyrethroids, thiocarbamates, triazines	5–29	CA	Pos (+1.39)	Carbonell et al.
67/67			SCE	Neg	Carbonell et al.
29/53	*Floriculturists and horticulturists:* pesticide mixture; most commonly used pesticides: abamectine, acephate, benomyl, buprofecin, captan, chlorpyrifos cypermethrin, cyromazine, deltamethr methomyl, ofurace, paraquat, procymidone in, diquat, endosulfan, fenitrothion, folpet,	N.D.	CA	Pos (+1.55)	Carbonell et al.

Study subjects (exposed/control)	Exposure	Duration (years)	Analysed biomarker	Result	Reference
43/41	*Greenhouse workers: >100 agrochemical* formulations	N.D.	CA	Neg	Scarpato et al.
			SCE	Neg	
			MN	Neg	
34/33	*Greenhouse workers: pesticide mixture; most commonly used pesticides: acephate, azocyclotin, benfuracarb, captan, chlorothalonil, dichlorvos, dimethoate,* dicofol, endosulfan, fenpropathrin, iprodione, mancozeb, metiram, methomyl, procymidone, propineb, toclofos-methyl, trichlorfon, vinclozolin	7/41	MN	Neg	Falck et al.
17/33 pesticide sprayers highly exposed			MN	Pos (+1.22)	Falck et al.
110/29	*Greenhouse workers: pesticide mixture; most commonly used pesticides: amitraz, alfacypermethrin, benomyl, buprofezin, carbendazim, chlorpyrifos, chlorothalonil, chlormequatchlorid, deltamethrin, daminozid, dienochlor, endosulfan,* fenpropathrin, iprodion, pirimicarb, methomyl, praclobutrazol, thiram, vinclozolin	N.D.	CA		Lander et al.
68/29	*Preseason*			Pos (+1.18)	Lander et al.
58/29	*Postseason*			Pos (+1.38)	Lander et al.
30/30	*Greenhouse workers: pesticide* mixture—carbamates, organochlorines, organophosphates	1.5–10	SCE	Pos (+1.77)	Gomez-Arroyo et al.
			MN in buccal mucosa cells	Pos (+2.63)	
104/44	*Greenhouse workers: pesticide mixture—carbamates, organochlorines, organophosphates*	2.5–55.5	SCE	Pos (+1.26)	Shaham et al.
107/61	*Greenhouse and open-field workers:* pesticide mixture—organophosphates, carbamates, benzimidazoles, pyrethroids, tiophthalimides, pyrimidinol compounds, organochlorines, bypirydilics, amides, morpholinics	2–70	MN	Pos (+1.45)	Bolognesi et al.

Table 14.8.-Cytogenetic effects on populations exposed to pesticides – agricultural workers. (Bolognesi, 2003) [a] Chromosomal aberrations, SCE and micronuclei in peripheral blood lymphocytes. [b] The MN frequency was also evaluated in buccal mucosa cells.

Study subjects exposed/(control)	Exposure	Duration (years)	Analysed biomarker	Result	Reference
10/7	Pesticide mixture; most commonly used pesticides: 2,4-D, diquat, MCPA, MCPP, dithiocarbamates	2–29	CA	Neg	Hogstedt et al.
94/76	*Horticulturists: pesticide mixture; most commonly* used pesticides: carbamates, organophosphates, organochlorines, triazines, thiocarbamates, ureics	1–35	SCE	Neg	Gomez Arroyo et al.
71/29	*Open-field and greenhouse workers: pesticide mixture;* most commonly used pesticides: benzimidazoles, carbamates, dithiocarbamates, morpholines, nitroorganics, organochlorines, organophosphates, phthalimides, pyrethroids	2–52	MN	Neg	Bolognesi et al.
30/30	*Potato cultivation: pesticide mixture; most commonly* used pesticides: carbamates, dithiocarbamates, organophosphates	5	CA	Neg	Hoyos et al.
			SCE	Neg	
18/21	*Berry pickers: pesticide mixture; most commonly used* pesticides: captan, carbofuran, diazinon, endosulfan, malathion	1–24	MN	Neg	Davies et al.
20/20	*Banana workers: pesticide mixture—chlorpyrifos,* dibromochloropropene, fenamiphos, gramoxone, imalzabile, terbufos, thiabendazole	N.D.	CA	Pos (+1.26)	Au et al.
23/23	Pesticide mixture; most commonly used pesticides:	0–16	CA	Pos (+3.25)	Antonucci and
	carbamates, organophosphates				Colus [94]
20/16 male	Pesticide mixture; most commonly used pesticides: agrimycin, benlate, cercobin, curzate, dacostar, endosulfan, folidol, folicur, lannate, manzate, methamidophos, microshield, nuvacron, orthene, pyrimicin, recop, roundup, sencor, vertimec	10–40	CA	Neg	D'Arce and Colus

Study subjects exposed/(control)	Exposure	Duration (years)	Analysed biomarker	Result	Reference
64/50	*Greenhouse workers: pesticide mixture; most* commonly used pesticides: abamectine, acrinathrin, cymoxanil, cyromazyne, endosulfan, imidacloprid, malathion, mancozeb, methamidophos, methomyl, oxamyl, piryproxifen, procymidone, tralomethrin	9.82 ± 1.01	MNb	Neg	Lucero et al.
50/66	Pesticide mixture; most commonly used pesticides: cymoxanil, cyromazine, endosulfan, imidacloprid, mancozeb, methomyl, methamidophos, oxamyl, permethrin, procymidone, pyriproxifen, tralomethrin	8.62 ± 1.13	MNb	Neg	Pastor et al.
49/50	Pesticide mixture; most commonly used pesticides: cafenvalerate, carbosulfan, chlorothalonil, deltamethrin, dimethoate, iprodione, lambda-cyhalothrin, methomyl, propanocarb, vinclozolin	16.28 ± 1.10	MNb	Neg	Pastor et al.
39/22	*Greenhouse workers: pesticide mixture; most* commonly used pesticides: abamectine, acrinathrin, cymoxanil, cyromazyne, endosulfan, imidacloprid, malathion, mancozeb, methamidophos, methomyl, oxamyl, piryproxifen, procymidone, tralomethrin	8.31 ± 1.12	MN	Neg	Pastor et al.
84/65	*Greenhouse workers: pesticide mixture; N.D.*	18.75 ± 0.89	MNb	Neg	Pastor et al.

Table 14.9: Cytogenetic effects in human populations exposed to pesticide mixture (summary of the results) (Bolognesi, 2003)

Analysed biomarker		Number of studies (positive/total)	Results (range of effects)
Pesticide sprayers	CA	13/13	1.18–15.8
	MN	2/3	1.20–7.67
	SCE	4/7	1.12–2.36
Floriculturists	CA	5/7	1.02–1.86
	MN	3/4	1.22–1.45
	SCE	4/7	1.18–1.77
Agricultural workers	CA	2/5	1.26–3.25
	MN	0/7	–
	SCE	0/2	–

For references cited in Tables, see Bolognesi, 2003

SINGLE CELL GEL ELECTROPHORESIS (SCGE, COMET ASSAY)

☑ General information

The electrophoresis of individual cells in agar gels (SCGE) (McKelvey-Martin *et al*, 1993; Fairbain *et al*., 1995; Ross *et al*., 1995; Hooghe *et al*., 1995, Collins *et al*., 1997; Delgado *et al*., 2000), more frequently known as the comet assay due to the special morphology of the figures observed that have the form of a comet, has recently become, in this field, an alternative that is full of possibilities.

This test consists of exposing an appropriately pure and concentrated cellular suspension, obtained from animals or cell cultures exposed to the toxin and contained in an agar gel, to an alkaline lysis, to a treatment to uncoil the DNA and to a short electrophoretic migration. The DNA is stained with an appropriate fluorochrome and observed using a fluorescent microscope. Undamaged genetic material grouped in the spherical-shaped nucleoid does not migrate; on the other hand, if fragmentation occurs, the smallest fragments migrate a certain distance that is inversely proportional to their size, producing an image that is similar to the tail of a comet. The analysis of the digitalised images using a computer programme provides a value based on the total fluorescence in the tail and on its length (the *tail moment*) that is correlated with the degree of genotoxicity of the compound under study.

☑ Matrix

- **Invasive**

  SCGE is performed on circulating lymphocytes; a minimal amount of blood (about 8 µl) is needed.

☑ Sampling conditions

As previously described for other biomarkers.

☑ Operational aspects

Blood must be put in ice, and analyzed within a few hours.

☑ Analytical aspects

- **Techniques**

  Cell suspension are embedded in low melting point 0.6% agarose (LMP) prepared in MilliQ water (18 MΩ) and layered on pre-coated slides. The slides are placed in lysing buffer (2.5M NaCl, 100 mM Na_2EDTA, 10mM Tris pH 10, *N*-lauryl-sarcosine 1% (w/v) with 1 % Triton X-100 during 1h at 4ºC. The DNA of the nuclei in the agarose gels is afterwards unwinded for 40 min in electrophoresis buffer (1mM Na_2EDTA and 300 mM NaOH, pH>13). The SCGE slides are then electrophoresed for 30 min at 25V and 300 mA at 4ºC. After neutralization with 400mM Tris buffer (pH 7.5), the slides are dried at room temperature. For image analysis the slides are hydrated and stained with 10 μl 4',6-diamidino-2-phenylindole (DAPI).

- **Sensitivity and specificity**

  Comet Assay is more sensitive and versatile than other tests with similar endpoints, as the Micronucleus Test or the Chromosomic Aberrations Test

- **Units**

  Results are expressed as Tail Moment (migrated DNA x tail length) or as percent of fluorescence in the tail, and compared using the Mann-Whitney "U" test or others statistics.

☑ Data reported in literature

Several studies report SCGE data on pesticide workers (Garaj-Vrhovac et al., 2000; Faust et al., 2004; Shadnia et al., 2005).

We reproduce in Tables 20 to 22 data from Garaj-Vrhovac et al., 2000.

Table 14.10: Demographic characteristics of the study population (Garaj-Vrhovac et al., 2000) a The age (in years) and smoking habits (in cigarettes smoked per day) are reported as mean values ± standard deviations.

Exposed workers				**Control subjects**		
	No.	**Age**	**No. of cigarettes smoked per day**	**No.**	**Age**	**No. of cigarettes smoked per day**
Males	7	34:4 ± 9:3	24.3 ± 14.0	7	32:2 ± 7:7	16.9 ± 3.8
Females	3	44:7 ± 2:5	0	3	29:8 ± 7:9	0
Non-smokers	4	46:0 ± 3:5	0	4	33:0 ± 8:9	0
Smokers	6	33:1 ± 10:5	24.3 ± 14.0	6	29:0 ± 4:3	16.9 ± 3.8
Total	10	39:5 ± 6:0	14.6 ± 7.0	10	31:0 ± 7:7	10.1 ± 1.9

Table 14.11: Mean values of tail length and tail moment, as comet assay parameters, in the exposed workers (after Garaj-Vrhovac et al., 2000) N: non-smoker; S: smoker. b Statistically significant compared to comet

assay endpoints of the same exposed worker after 6 months spent out of the production (P<0:001). c Statistically significant compared to the control (P<0:001).

Exposed worker no.	Comet assay endpoints after 6 months spent in the pesticide production		Comet assay endpoints after 6 months spent out of the pesticide production	
	Mean tail length (mm)	Mean tail moment	Mean tail length (mm)	Mean tail moment
1S	58.5b	73.2b	17.1	13.6
2N	44.8b	56.6b	17.3	13.8
3N	35.4b	28.9b	16.9	13.9
4S	55.7b	71.3b	16.3	13.2
5N	58.8b	77.4b	17.4	13.8
6S	48.0b	62.9b	17.8	13.9
7S	58.8b	75.3b	17.7	14.2
8S	58.3b	73.2b	17.3	13.6
9N	35.0b	43.4b	17.4	14.5
10S	48.0b	61.4b	16.8	13.4
Mean ± S:D.	50:1 ± 9:4c	60:8 ± 18:2c	17:2 ± 0:4c	13:8 ± 0:4c

Table 14.12: Mean values of tail length and tail moment, as comet assay parameters, in the control subjects (after Garaj-Vrhovac et al., 2000) a N: non-smoker; S: smoker.

Control subject no.	Comet assay endpoints	
	Mean tail length (mm)	Mean tail moment
1N	11.8	9.4
2S	15.1	11.5
3N	11.7	9.4
4S	14.7	11.7
5N	11.8	9.6
6S	12.0	9.4
7N	12.2	9.5
8S	13.9	11.3
9S	15.0	12.2
10S	14.3	11.1
Mean ± S:D.	13:3 ± 1:5	10.5 ± 1.1

15.13 CONCLUSIONS

Considerable amounts of data are available on pesticides biomonitoring; however, most of the studies have focused on occupational exposure, so more information on the exposure and effects of environmental pesticides on general population are still needed.

Geographical trends have not been clearly shown within Europe. Data on confounding factors, either, are not conclusive, except, perhaps, in the case of tobacco smoking. Children seem to be the more susceptible group of age.

In general terms, the analysis are carried out in blood; as a consequence, an enhanced development of methods based on non-invasive matrices would be a valuable improvement.

Due to the fact that people are often exposed to complex mixtures of pesticides, indirect or unespecific markers (such as cholinesterase inhibition or genotoxicity tests) are specially suitable for biomonitoring, and also for further integrated risk assessment.

16 PHTHALATES

☑ General information

Phthalates are the dialkyl- or alklarylesters of 1,2-benzenedicarboxlic acid. Table 1 gives an outline of the most commonly used phthalates. Most of these compounds are non-volatile clear liquids with little or no odour.

Table 15.1: List of main phthalates (adapted from http://www.phthalates.com/ accessed on 07/08/2006, and Lorz et al. 2002)

Group	Chemical denomination	Code 1* DIN 7723
C1	Dimethyl phthalate	DMP
C2	Diethyl phthalate	DEP
C3	Dipropyl phthalate	DPP
C4	Diisobutyl phthalate	DIBP
	Butyl benzyl phthalate	BBzP
C5	Diisopentyl phthalate	DIPP
C6	Diisohexyl phthalate	DIHP
C7	Diisoheptyl phthalate	DIHP
C8	Di-2-ethylhexyl phthalate	DOP(DEHP)
	Diisooctyl phthalate	DIOP
	Di-*n*-octyl phthalate	DNOP
C6/C8/C10	Di(hexyl-octyl-decyl) phthalate	HXODP(610P)
C8/C10	Di(octyl-nonyl-decyl) phthalate	ONDP(810P)
C7/C9	Di(heptyl-nonyl) phthalate	HNP(79P)
C7/C9/C11	Di(heptyl-nonyl-undecyl) phthalate	HNUP(711P)
C9/C11	Di(nonyl-decyl-undecyl) phthalate	NDUP(911P)
C9	Diisononyl phthalate	DINP
C9	Diisononyl phthalate	DINP
C9	Di(3,5,5-trimethylhexyl) phthalate	DINP
C9	Diisononyl phthalate	DINP
C10	Diisodecyl phthalate	DIDP
C11	Diundecyl phthalate	DUP
	Diisoundecyl phthalate	DIUP
C13	Diisotridecyl phthalate	DTDP/DITP
	Di(methoxyethyl) phthalate	(DMEP)
	Di(butoxyethyl) phthalate	(DBEP)

C1 & C2 phthalates are used generally in non-PVC applications

C3-C7 phthalates are used in rapid setting and stain resistance applications (e.g. foamed fabrics and flooring)
C8-C10 are the most widely-used general purpose plasticisers – wall-coverings, flooring and medical applications
C11-C13 phthalates are used in high temperature stability applications

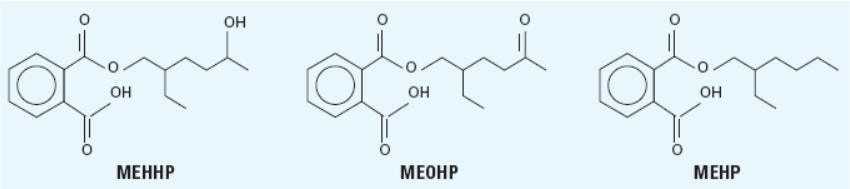
DEHP is the produced in the highest volumes and therefore research tends to focus on this chemical. According to the ATDSR (2002), more than 2Mt of DEHP are produced annually worldwide. As shown in table 1 most phthalates, including DEHP, are used as plasticisers. They are not covalently bonded in the products into which they have been incorporated and therefore are relatively freely leached from them during manufacturing, use and after their disposal (Nuti et al. 2005).

The most common use of DEHP is as a plasticiser in polyvinyl chloride (PVC) products, to which it adds flexibility; these PVC materials may contain as much as 40% DEHP (ATDSR 2002). Other properties phthalates impart on plastics are colour-fastness and durability. Other than plastics, phthalates are also commonly used in many other domestic applications including cosmetics (as plasticisers and fixatives), personal care products (as plasticisers or propellants), adhesives, paints, medical equipment, safety glass, lubricants etc.

The use of phthalates in laboratory equipment can cause widespread contamination of samples and analytes, demonstrated by high blank values and subsequent data quality problems in assessment of exposure to ambient phthalate levels. It is for this reason that exposure to phthalates is better estimated by means of biomonitoring using metabolites of phthalates (Koch et al. 2002).

The most appropriate biomarker for human exposure to phthalates is the urinary concentration of their primary monoesters, since phthalates themselves undergo rapid metabolism upon entering the human body. It should be noted that the measurement of even these metabolites is susceptible to preanalytical data quality problems since these primary esters may be formed from processes in the environment outside of human metabolic pathways including hydrolysis by chemical, enzymatic, microbiological or photolytic means (Koch et al. 2002). One means of taking preanalytical contamination into account has been presented by Koch et al. (2003), whereby measurements are made not only of di(2-ethylhexyl)phthalate (DEHP), but also of its three secondary metabolites mono(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP) and mono(2-ethyl-5-oxo-hexyl)phthalate (5oxo-MEHP). The structures of these secondary metabolites is presented in Figure 1.

Figure 15.1: DEHP metabolites used as markers of human exposure to DEHP (Kato et al. 2004).



☑ Matrix

Several matrices are considered in the sampling of biomarkers of phthalates. These are blood, urine and saliva. Other less common biomarkers include amniotic fluid and breast milk. Toxicokinetic relationships are, however, easier to analyse from blood measurements (whole blood, plasma, serum etc.) (Kato et al. 2004).

'Because of their widespread use, phthalates have a high potential for human exposure as demonstrated by the frequent detection of their metabolites in the general population, both in the United States and abroad (Becker et al. 2004; Blount et al. 2000; CDC 2003; Koch et al. 2003, 2004b, 2005b; Preuss et al. 2005; Silva et al. 2003, 2004a). Previously, we measured phthalate monoester metabolites in urine (Barr et al. 2003; Blount et al. 2000; Kato et al. 2004; Silva et al. 2003, 2004a), in serum (Kato et al. 2003, 2004), in breast milk (Calafat et al. 2004), and in amniotic fluid (Silva et al. 2004b).' (Silva et al. 2005)

- **Invasive**

  Blood: In spite of the invasiveness and associated costs of sampling blood, this method of biomonitoring for phthalates is particularly useful in comparison with urine sampling in as much as the concentrations of analytes in the blood provide better estimations of the concentrations actually circulating in the body (Silva et al. 2005)

  Kato et al. (2003) have shown that blood measurements of phthalate monoesters are susceptible to contamination from the parent phthalates, which are hydrolyzed to their respective monoesters by serum enzymes. If this contamination is not eliminated effectively, measured concentrations of phthalate monoesters (but not oxidative metabolites) in serum may be artificially elevated (Kato et al. 2004).

- **Non-invasive**
  - *Urine:* Urine is used for the biomonitoring of phthalates since urinary levels of metabolites tend to be higher than levels in blood or other matrices. While urine sampling is non-invasive and low cost, the concentrations of excreted substances are dependent on water intake and results must be adjusted for creatinine or expressed in terms of grams of creatinine. In addition, measurement of urine concentrations only reflects concentrations of analytes since the last bladder void (Silva et al. 2005).
  - *Saliva:* Saliva offers many advantages over blood as a fluid for biomonitoring human exposure to phthalates, not least its simplicity of collection and resultant low cost. Saliva can be collected by spitting, swabbing the oral cavity, or making use of special collection equipment (Hold et al. 1995; Silva et al. 2005).

    Since salivary glands have a high blood flow, chemicals and their metabolites can be excreted in saliva by different mechanisms, including passive diffusion, active transport, and ultrafiltration (Hold et al. 1995).

    It should be noted that since saliva has a very low protein concentration (less than 1% of that in plasma), protein-bound molecules will not partition into saliva and salivary levels subsequently reflect the free fraction of the compound in blood (Silva et al. 2005). In addition, it has been shown that saliva contains enzymes that hydrolyse phthalate diesters. Without taking this aspect of enzymatic metabolism into account, concentrations of phthalate metabolites in saliva could be overestimated.

    The similar levels in serum and saliva suggest that saliva could be used as a surrogate matrix for measuring the bioavailable dose of phthalates in biomonitoring studies. However, urine is preferred for exposure assessment to nonpersistent chemicals such as phthalates, because the levels of these chemicals or their metabolites are generally higher in urine than in blood. Because of the relatively small number of saliva samples analyzed and the limited demographic information available, further research is necessary to establish the partitioning of phthalates in serum, urine, and saliva.' (Silva et al. 2005)
- **Comparison of different methods available**

  Levels of phthalate metabolites in serum and saliva suggest that saliva could be used as a means of estimating the bioavailable amount (i.e. that portion unbound by

proteins) of phthalates. Urine is currently more popular than either of the other methods since sampling is cheap, samples themselves are high volume, and levels of phthalates are generally higher than in blood. Little is yet known about the partitioning of phthalates between serum, urine and saliva, however, and this subject represents an area of current research.

☑ Kinetics

- **Uptake**

  Phthalates and, specifically, DEHP, are absorbed in humans by means of ingestion of foods into which phthalates have migrated from packaging, inhalation (of phthalate in gas or particulate-bound form) and dermal absorption (ATSDR 2002) or resorption from e.g. cosmetics, as well as through direct diffusion into blood for patients undergoing dialysis, transfusions or apheresis, or other medical procedures using PVC medical devices (Kato et al. 2004; Koch et al. 2002; Koch et al. 2003; Silva et al. 2003; Duty et al. 2003).

  Uptake is determined in part by the type of phthalate concerned. Human subjects are most likely to be exposed to phthalates with long alkyl chains (such as DEHP) through ingestion, whereas compounds with shorter carbon chains are more likely to be percutaneously absorbed or, depending on volatility, inhaled (Koch et al. 2002; Koo et al. 2002).

  The majority of available information on phthalate exposure and metabolism concentrates on exposure through ingestion. Considerable information is also available on exposure of patients undergoing dialysis or on intravenous lines. Much less information is available on the uptake and metabolism of phthalates through inhalation or dermal exposure (Koo et al. 2002) and these pathways are not nearly so well understood (Adibi et al. 2003) and routes of exposure are generally not adequately characterised (Duty et al. 2003).

- *Ingestion:* In general the primary route of human exposure to phthalates (and DEHP) is through ingestion, particularly ingestion of phthalate-containing foods (Koo et al. 2002). Their presence in foods is not only because of their movement up the food-chain, but also due to their migration from plastic containers, wrapping and other packaging into lipid-rich foodstuffs (Adibi et al. 2003).

  Children may be particularly exposed to phthalates through their ingestion in breast milk and infant formula (Main et al. 2006), but also through swallowing of saliva containing phthalates that migrate out of mouthing and teething toys and drinking bottle teats.

  Exposure through ingesting phthalate-contaminated food is regarded as the most important source of DEHP exposure with an estimated intake of 10 μg per kg body weight and day (Meek and Chan 1994). A German study of ingestion of DEHP-containing house dusts suggests that exposure for a child could be in the order of 10 μg/kg body mass per day (Becker et al. 2004).
- *Inhalation:* Phthalates released by off-gassing from various PVC products in and around the home (e.g. vinyl flooring, wallpaper, furniture etc.) can contribute significantly to ambient air levels (Adibi et al. 2003). Some oven-setting modelling clays have been found to contain very high levels of both non-volatile and volatile phthalates as plasticisers. These plasticisers can potentially be inhaled, both during modelling and curing of the clays, as well as dermally absorbed (see section 4.1.3).
- *Dermal:* The dermal absorption route for phthalates is supported by a limited number of studies. Uptake via this route is most likely to take place through passive diffusion regulated by the stratum corneum; phthalates may also dissolve into lipids between keratin filaments in the skin (Duty et al. 2003). Human dermal dosing experiments have shown and quantified the rates of absorption of several phthalates.

  Many cosmetic and personal care products contain phthalates. Phthalates tend to display considerable lipophilicity and are readily absorbed through the skin (Adibi et al. 2003). While volatile phthalate compounds in personal care products are inhaled, lower molecular weight phthalates are also absorbed percutaneously, particularly in products applied to the skin directly (Koo et al. 2002).

The mouthing of soft PVC and other soft plastic toys, teethers, mouthing rings and bottle teats etc. in young children may present a considerable source of exposure through dermal absorption in the mouth (Koch et al. 2002). The dermal absorption of phthalates during childhood (i.e. a period of developmental and physiological immaturity) is seen to be of particular concern with respect to the development of adverse health outcomes (Silva et al. 2006). This concern is in part reflected by European and US moratoria on the use of certain phthalates in children's toys. Certain modelling clays used by children may well contribute considerably. The percentage contribution of phthalates from personal care products to the total body burden has not yet been sufficiently studied (Duty et al. 2003).

- *Blood treatment:* Products and equipment used in blood transfusion, apheresis and dialysis frequently contains very high levels of phthalates in order to increase their plasticity or other properties. A number of studies have shown that phthalates present in these plastics migrates directly into blood or other blood components by diffusion or adsorption and enters patient blood and tissue. Studies have shown that levels of phthalates may be particularly high in premature babies and those in intensive care continually exposed to phthalates through their blood.

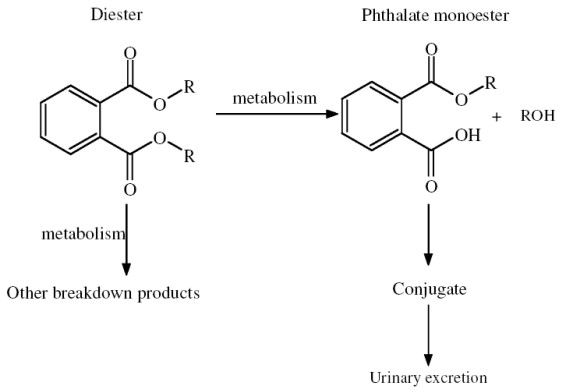
- **Metabolism**
  - *Metabolism of phthalates:* Metabolic pathways of phthalates are specific to differences between different phthalate species and the uptake route. It is therefore outside the scope of this report to go into details on the metabolism of a number of phthalates. It is sufficient to say that low molecular weight phthalates are mostly metabolised to their hydrolytic monoesters (ATSDR, 1995, 2001) and that phthalates with higher molecular weights (including DEHP, see below) are metabolised to hydrolytic monoesters which are then transformed to oxidative products (ATSDR, 1997, 2002). Figure 2 shows a simplified generalised scheme for phthalate diester metabolism and its route to urinary excretion.

    Since phthalates are lipid soluble, it could be anticipated that they would accumulate in fats in the human body. Koo et al. (2002) found, however, that deposition of phthalates into fat does not occur until several hours or months after dosing. Since higher molecular weight phthalates such as DEHP are rapidly

converted to polar metabolites, which may then be rapidly cleared from the body, they are not stored in body fat. The lipophilicity, metabolic pathway and distribution in different fluids and tissues of any particular phthalate are effectively governed by the length of its aliphatic ester side chain (Lottrupp et al. 2006).

Since phthalates are relatively rapidly and completely metabolised by hydrolytic cleavage of at least one ester group, biomonitoring of the resultant metabolites in urine – particularly primary monoesters – is considered appropriate as a means of measuring phthalate exposure (Koch et al. 2002).

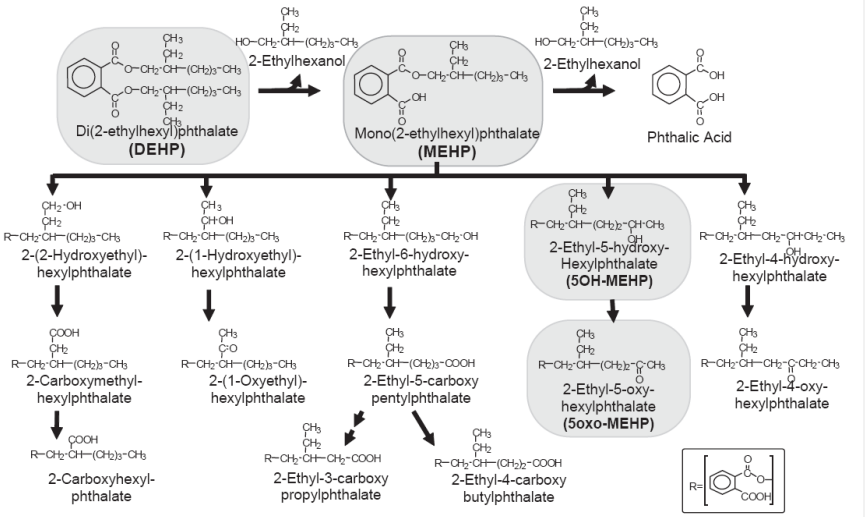
Figure 15.2: Metabolic excretion of phthalates (in Anderson et al. 2001).



- *Metabolism of DEHP:* 'Phthalates are rapidly metabolized to their respective monoesters and further to oxidative products of their lipophilic aliphatic sidechain. Dependingon the phthalate, these metabolites are partially glucuronidated and excreted through the urine and feces. They do not appear to bioaccumulate (Schmid and Schlatter, 1985).' (Koch et al. 2003)

  DEHP is relatively rapidly metabolised first to its respective monoesters and then to oxidative products of its lipophilic aliphatic sidechain. These monoester metabolites are then glucuronidated and excreted through the urine and faeces (Koch et al. 2003) or undergo further hydroxylation in the liver or kidney (Lottrupp et al. 2006).

Figure 15.3: Metabolism of DEHP (in Koch et al. 2003)



Long ester side chain species of phthalate such as DEHP undergo further oxidation and/or hydroxylation to a number of other oxidative metabolites, as shown in Figure 3. DEHP is first metabolised to the primary metabolite mono(2-ethylhexyl) phthalate (MEHP), which is converted by o-1-oxidation of the monoester alkyl chain to a number of secondary metabolites. The two found to be predominant in urine are mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono(2-ethyl-5-oxylhexyl) phthalate (MEOHP) (Lottrupp et al. 2006), both of which are present in higher concentrations than MEHP. Kato et al. (2004) found that MEHHP and MEOHP are excreted in the urine predominantly as glucuronide conjugates and at higher concentrations than MEHP. Given that MEOHP and MEHHP were found in 10-fold greater concentrations than MEHP in urine, and given that detection of these secondary metabolites was also greater, it is considered they may be more sensitive urinary markers of DEHP exposure than MEHP measurements alone.

There are drawbacks of using hydrolytic monoesters as biomarkers of DEHP exposure e.g. some amount of the phthalate monoesters detected may not originate from DEHP metabolism, but exposure to other lower molecular weight phthalates, thus overestimating exposure to DEHP. Equally, many other phthalate monoesters may be present from exposure to mixtures of other high molecular weight phthalate species, but these remain undetected. This results in potential underestimation of high isomeric molecular weight phthalates (Calafat et al. 2006).

- **Biological half-life**

  The biological half-lives of diester and monoester phthalates are relatively short owing to rapid metabolism through oxidative processes as described in section 4.2. Typical half-lives for these phthalates and their metabolites is in the range 6-12 hours (Duty et al. 2003).

☑ Sampling conditions

- **Blood**

  Blood sampling methodologies used for DEHP biomarkers:; Venous non-fasting blood samples were collected the same day as a paired breast milk sample was delivered to the hospital. After clotting, blood samples were centrifuged, and the sera were separated and stored at -20°C until analysis. (M*ain et al. 2006)*

- **Urine**

  Urine sampling methodologies used for DEHP biomarkers:

  - Spot urine sample made and delivered immediately to laboratory. Sample frozen at -70°C. Within 5 months sample sent for analysis. Analysis done for various biomarkers. Urine samples matched to personal air samples. (Adibi et al. 2003)
  - First morning urine samples taken from 550 children. Questionnaires used to collect information on exposure conditions, i.e. food selection, housing condition and quality of the residential environment. Samples were taken by parents or the children themselves in polypropylene or polyethylene containers. Samples were stored and frozen (-20ºC) in polypropylene tubes. None of the materials used contained detectable levels of DEHP. Samples were coded and analysed in a randomised sequence to avoid observer bias. (Becker et al. 2004)

- Single spot urine sample taken on the same day as a single spot semen sample. Urine sample collected in sterile specimen cup. (Duty et al. 2003)
- Single spot urine sample was collected from each participant in a sterile plastic specimen cup. Specimen cup was pre-screened for phthalates. (Duty et al. 2005)
- First morning urine was collected from 85 subjects. All subjects were not occupationally exposed to phthalates. An extensive questionnaire was designed to collect data on each subject including sex, age, bodyweight or occupation, possible routes of exposure to phthalates (use of vinyl gloves or close contact with PVC products), extent of application of bodycare products (perfumes, deodorants or hairspray), eating and drinking habits, smoking habits and pesticide use. (Koch et al. 2002)
- Women provided two consecutive first morning void urine samples on the second and third days of their menstrual cycles. Samples were shipped to the storage laboratory by overnight mail in a specially designed styrofoam kit that included a frozen cold pack. Samples were aliquoted and frozen at –20°C for future analyses. Urine samples were collected and stored in plastic containers. These containers were screened for phthalate monoesters before the pilot study began, and none contained detectable levels. This analysis was restricted to urine samples collected on Tuesdays through Fridays, to minimize variation associated with potential differential exposures on weekends as compared to weekdays. First morning voids were used because these were expected to vary less than other types of urine samples and because they are generally more concentrated. (Hoppin et al. 2002)
- Single spot urine samples collected in polypropylene centrifuge tubes and stored at -20°C until analysis. Travel blanks were prepared and treated similarly. Participants completed a brief, self-administered questionnaire (recording height, body weight, age and sex in order to calculate the personal daily excretion rate of urinary creatinine). Sample containers and questionnaires were anonymous. An aliquot of each urine sample was shipped to the lab for measurement of creatinine by an enzymatic method. (Itoh et al. 2005)
- Urine grab samples were collected in plastic cups and stored in polyethylene tubes until analysis. (Jonsson et al. 2005)

- Urine samples were taken from 176 subjects from a population with no documented exposure to DEHP. 127 subjects also provided serum samples. The samples were collected at different times throughout the day, and the urine samples were not necessarily first-morning voids. Serum and urine samples were collected on the same day. (Kato et al. 2004)
- First morning void urine samples were collected from 85 subjects. Sample collection containers were prescreened to verify absence of phthalates and phthalate monoesters. The least interfering sample container type was selected following pre-screening of various types. All laboratory equipment suspected of containing phthalates was either excluded or screened. PVC material was generally avoided. (Koch et al. 2003)
- Urine specimens for analyses, including phthalate metabolites and creatinine concentrations, were collected from each participant ≥ 6 years of age during one of three daily examination periods (0830–1200 hr, 1230–1600 hr, 1630–2000 hr). (Silva et al. 2004)

- **Saliva**

  Unstimulated saliva samples were obtained from 39 subjects directly into prescreened glass test tubes. Saliva samples were frozen at -40°C in the original vials after collection. Saliva was thawed, spiked with phosphoric acid, vortex mixed, and spiked with a solution of isotope-labelled internal standard analytes, 4-methylumbelliferone glucuronide, and b-glucuronidase. Sample incubated at 37°C for 90 min to allow for the deglucuronidation of phthalate metabolites. Ammonium acetate buffer was added to each saliva sample after deconjugation. (*Silva et al. 2005)*

- **Breast milk**

  Breast milk samples from a joint prospective, longitudinal cohort study. Sample consisted of many small aliquots collected over successive infant feedings over several weeks. Storage of the breast milk sample was carried out in Pyrex glass bottles with Teflon-coated caps. Mothers instructed orally and in writing to feed the baby first and then to sample milk aliquots (hind milk), starting from 1 month after birth. Mothers instructed to collect samples into glass containers or porcelain cup, avoiding the use of mechanical breast pumps. Breast milk frozen consecutively in

household freezers in a single glass bottle as additive aliquots. Delivered frozen to hospital at 3-month examination. At the hospital, samples stored at -20°C until analysis. (Main et al. 2006)

☑ Analytical aspects

- **Techniques**
  - *Blood:* Blood samples analyzed as duplicates and blinded for the technician at one laboratory. Determination of phthalate monoesters was accomplished by high-pressure liquid chromatography with a Betasil Phenyl column (100 × 2.1 mm × 3 μm). The instrument was run in negative mode using the electro spray source (ESI). Detection limits were in the range of 0.01 to 0.5 μg/L. Recoveries at two different levels ranging from 2 to 120 μg/L were included using different milk samples and the CV (percent) was calculated from measurements of real duplicate determinations during the project period. (Main et al. 2006)
  - *Urine*

- Analysed according to Blount et al. 2000a. Urine first treated with glucuronidase enzyme in order to deconjugate monoester metabolites. Samples then extracted twice with Oasis HLB solid-phase extraction cartridges (Waters, Milford, MA, USA) and resuspended in the mobile phase. Samples analysed using high-performance liquid chromatography using tandem MS. 4-Methylumbelliferone used as a quality control measure for the deconjugation step. Urinary creatinine was measured with an ASTRA analyzer (Beckman Coulter, Palo Alto, CA, USA) based on the Jaffe rate reaction. Creatinine (reported in micrograms per deciliter) was used to adjust for variability in urine dilution. Creatinine excretion changes during pregnancy may have an effect on the validity of creatinine adjustment in the data analysis. However, in this data set, the results were essentially the same whether the data were analyzed with or without creatinine adjustment. Measures were taken to eliminate phthalate contamination from the laboratory environment, including substitution of certain materials that were shown to contain phthalates. Method blanks were used to monitor contamination. If a blank had > 5 ppb of any phthalate analyte, all the analytical results for that

day were rejected until the source of contamination was identified. (Adibi et al. 2003)

- Urine samples were analysed using multidimensional liquid chromatography tandem mass spectrometry (LC/LC-MS/MS). Internal quality control performed by analysing a low and high concentration control urine (MEHP: 5.8 µg/l and 16.8 µg/l, 5OH-MEHP: 14.1 µg/l and 78.8 µg/l, 5oxo-MEHP: 11.0 µg/l and 55.3 µg/l). Variation was less than 8% except for the low concentration of MEHP with a variation of 15%. LOQ (limit of quantification) was 0.5 µg/l. The dust samples were extracted with toluene and analysed using both GC/MS and LC/MS. As the results showed no difference (0.53 t (95%)) averages were used for further calculations. Entirely extracted pool samples were spiked and analysed for internal quality control. The variation found for these samples was less than 10% and recovery was between 81 and 125%. The LOQ was 20 mg/kg. (Becker et al. 2004)
- The analysis carried out according to Blount et al. 2000a. Urinary phthalate metabolite determination involved enzymatic deconjugation of metabolites from the glucuronidated form, solid-phase extraction, separation with high-performance liquid chromatography, and detection by tandem mass spectrometry. Detection limits were in the low nanogram per milliliter range. Reagent blanks and 13C4-labeled internal standards were used along with conjugated internal standards to increase precision of measurements. One method blank, two quality control samples (human urine spiked with phthalates), and two standards were analyzed along with every 10 unknown urine samples (Blount et al. 2000a). Analysts were blind to all information concerning subjects.(Duty et al. 2003)
- Analytical approach used was Blount et al. 2000 adapted to enable the detection of additional monoesters and improve efficiency of the analysis. Measurement of monoester metabolites, namely, MEP, MBP, mono(2-ethylhexyl) phthalate (MEHP), MBzP, and monomethyl phthalate (MMP), entailed enzymatic deconjugation of the phthalates from their glucuronidated form, solid-phase extraction, HPLC separation, and tandem mass spectrometry detection. Limits of detection (LODs) were approximately 1 ng/mL. One method blank, two quality control samples (human urine spiked with phthalate monoesters), and two sets of

standards were analyzed along with every 21 unknown urine samples. Analysts at the Centers for Disease Control and Prevention (CDC) were blind to all information concerning subjects. To control for urinary dilution, urinary concentrations were adjusted according to specific gravity. Specific gravity was measured using a handheld refractometer (National Instrument Company Inc., Baltimore, MD). Specific gravity–adjusted monoester phthalate levels were used as continuous outcome variables in statistical models. (Duty et al. 2005)

- Urine samples were analysed applying a newly developed multidimensional liquid chromatography tandem mass spectrometry (LC/LC-MS/MS) method according to Koch et al. (2003). Analytes were recorded in multiple reaction monitoring mode and quantified by isotope dilution. Metabolites determined were the secondary metabolites of DEHP, mono(2-ethyl-5-hydroxyhexyl)-phthalate (5OH-MEHP) and mono-(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP) next to the monoester metabolites monoethylhexylphthalate (MEHP), monoethylphthalate (MEP), mono-n-butylphthalate (MnBP), monobenzylphthalate (MBzP) and mono-n-octylphthalate (MnOP). Urinary metabolite excretion values were determined for the 85 subjects of the collective. (Koch et al. 2002)
- One 1.2 mL aliquot of each day's urine was analyzed. Both urine samples from each individual in the same laboratory batch was included to eliminate interset variation. All samples were shipped frozen to the analytic laboratory and remained frozen until chemical analysis. We analyzed urine samples for seven phthalate monoesters using methods described previously. The phthalate monoesters analyzed were monobenzyl phthalate (mBzP), monobutyl phthalate (mBP), monocyclohexyl phthalate (mCHP), monoethyl phthalate (mEP), monoethylhexyl phthalate (mEHP), monoisononyl phthalate (mINP), and monooctyl phthalate (mOP). Samples were spiked with 13C4-labeled phthalate monoesters and 4-methylumbelliferone glucuronide. The samples were then treated with β-glucuronidase to release the phthalate monoesters from their conjugated forms. Deconjugated urine samples were extracted twice with Oasis hydrophobic lipophilic balance (Waters Corp, Milford, MA) solid-phase extraction and resuspended in mobile phase. Chromatographic separation by high-pressure liquid chromatography was followed with tandem mass

spectrometry on a triple quadrupole instrument using atmospheric pressure chemical ionization. We monitored levels of 4-methylumbelliferone as quality control for the deconjugation step. Samples that failed to meet the laboratory quality assurance criteria were not included the analysis; as a result, MEP measurements on 11 subjects and MINP and creatinine in one sample were unavailable. (Hoppin et al. 2002)

- Measurement of the phthalate monoesters was performed using a tandem quadrupole mass spectrometer connected to a liquid chromatograph with a C18 column. 1ml of acetic acid in 1-l water (solvent A) and 1ml of acetic acid in 1-l acetonitrile (solvent B) was used as a mobile phase with a constant flow rate (1.2 ml/min). Five microliters of the sample was injected into a HPLC column using an auto sampler. The percentage of solvent B was elevated as follows: 10% (0 min), 86% (7 min), 100% (9–10 min), and 10% (11–31 min). The temperature of the column was kept constant at a 40˚C. After HPLC separation, the line was forked. Only one part of the post column flow (approx. 0.2 ml/ min) flowed to the electrospray ion source. Multiple reaction monitoring using a combination of the precursor and product ion listed in Table 1 was employed. In a series of instrumental analyses, a standard solution and blank were also measured in parallel, to monitor the variation of sensitivity of the instrument and sample carryover. (Itoh et al. 2005)
- Aliquots of 2 mL of urine were combined with 500 ng of tetra-deuterium labeled phthalic acid, used as an internal standard for all analyzed compounds plus 2.5 L of glucoronidas/ arylsulfatase (helix pomatia) from Boehringer Mannheim (Mannheim, Germany) and 0.5 mL of 1 M ammonium acetate (pH 6.5). The samples were incubated at 37°C overnight, then 0.5 mL of 6 M NaCl and 0.1 mL of 6 M HCl were added and the samples were extracted by 1 portion of 6 mL of ethyl acetate. The organic phase was evaporated and the residue dissolved in 0.2 mL of water containing 0.5% acetic acid. We analyzed samples by LC-MS-MS using an autosampler coupled to an LCMS-MS. Analyses were performed in the negative ion multiple reaction monitoring mode and the mass fragments used were for phthalic acid. Standards were prepared by addition of different amounts of MEP, MBP, MBzP, MEHP, and phthalic acid to normal urine. The urine sampling procedure was tested by use of purified water, and no measurable

contamination of the phthalate metabolites was found during this procedure. The detection limits were 15, 30, 15, 7, and 15 ng/mL for phthalic acid, MEP, MBP, MBzP, and MEHP, respectively. (Jonsson et al. 2005)

- The analytical methods for measuring phthalate metabolites in urine and serum were adapted from previously developed methods and modified to meet our requirements for the analysis of MEOHP and MEHHP (Blount et al. 2000a; Kato et al. 2003; Silva et al. 2003a). For the free metabolite (nonglucuronidated) analysis, the β-glucuronidase enzyme treatment for both unknown samples and QC materials was eliminated. The urine and serum samples (1 mL) were spiked with 13C4-labeled internal standards and MeUmb-glu (to evaluate the completion of the hydrolysis), and the phthalate metabolites were extracted from the matrix by solid-phase extraction. The final eluate was concentrated and resuspended in water. The analytes were chromatographically separated (HP 1100, Agilent Technologies, Wilmington, DE, USA) on a Keystone phenyl Betasil column (Keystone Scientific, Bellefonte, PA, USA) using a nonlinear water:acetonitrile gradient and analyzed by tandem mass spectrometry on an API 3000 (Applied Biosystems, Foster City, CA, USA) using electrospray ionization. The limits of detection (LODs) were calculated as 3S0, where S0 is the standard deviation value as the concentration approaches zero. S0 was determined from the replicate analysis of low-level standards. The relative standard deviations ranged from 8% to 13%. The LODs in urine were 1.2 ng/mL (MEOHP), 1.6 ng/mL (MEHHP), and 0.9 ng/mL (MEHP) (Silva et al. 2003a); the LODs in serum were 1.3 ng/mL (MEHP) (Kato et al. 2003), 1.4 ng/mL (MEOHP), and 1.9 ng/mL (MEHHP). All of the samples, blanks, standards, and QC materials were processed identically. (Kato et al. 2004)
- Urine samples were analyzed, applying a newly developed LC/LC-MS/MS method (Koch et al., 2003a). Metabolites determined were the secondary metabolites of DEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP) and mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP) next to the monoester metabolites monoethylhexylphthalate (MEHP), monoethylphthalate (MEP), mono-n-butylphthalate (MnBuP), monobenzylphthalate (MBzP), and mono-noctylphthalate (MnOP). Mono-iso-butylphthalate (MiBuP) was registered but not determined quantitatively, since no isotope labeled internal standard was

available. The synthesis of the secondary metabolites 5OHMEHP and 5oxo-MEHP and their isotopically marked analogs (D4) was described by Gilsinget al. (2002). All samples were spiked with the D4-ring labelled phthalate metabolites prior to analysis. The samples were then treated with a special b-glucuronidase to release the monoester metabolites from their conjugated form. The applied b-glucuronidase had no nonspecific lipase activity to minimize the generation of monoesters out of the ubiquitously present diesters. For the analysis of the secondary metabolites of DEHP no special precautions had to be taken. After hydrolysis each sample was vortex mixed, sonicated and frozen, thawed again, centrifuged to separate cryophilic proteins, and the supernatant was transferred into a glass screw-cap vial for subsequent on-line LC-MS analysis. There, HPLC online enrichment and column-switching techniques were combined with state-of-the-art MS/MS detection. The phthalate metabolites were stripped from urinary matrix by on-line extraction on a restricted access material (LiChrospher ADS-8, Merck, Darmstadt) precolumn, transferred in backflush mode. (Koch et al. 2003)

- After collection, urine specimens were aliquoted and then stored cold (2–4°C) or frozen until they were shipped. Samples were analyzed for creatinine using an AS/ASTRA clinical analyzer at the University of Minnesota Medical Center. Samples collected for phthalate metabolite measurements were shipped on dry ice to the CDC's National Center for Environmental Health. Urine samples were stored frozen at –20°C until analyzed. The samples were analyzed by isotope dilution high-performance liquid chromatography coupled with tandem mass spectrometry. Phthalate urinary concentrations are reported both in micrograms per litre of urine and in micrograms per gram of urinary creatinine. Creatinine adjustment was used to correct for urine dilution. (Silva et al. 2004)

- *Saliva:* The phthalate metabolites were extracted from saliva using an automated solid phase extractor, separated from other extracted compounds by reversed phase HPLC on a liquid chromatograph, and detected on a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer by negative ion electrospray ionization-MS/MS. Quality control (QC) materials were made using pooled human urine spiked with phthalate metabolites. Reagent blanks, QCs, and analytical standards were analyzed along with unknown saliva samples. The

esterase activity of saliva was evaluated by spiking the saliva with D4-DBP (800 ng/mL), D4-DEP (200 ng/mL), and D4-DEHP (300 ng/mL) and estimating the concentration of D4-MBP, D4-MEP, and D4- MEHP, respectively, produced by hydrolysis of the labelled phthalate diesters. The labeled, DEP, DBP, and DEHP were used to eliminate potential contributions from ubiquitous DBP and DEHP contaminants. (Silva et al. 2005)

- *Breast milk:*Blood samples were analyzed as duplicates and blinded for the technician at one laboratory. Each run contained blood samples of both cryptorchid and healthy boys from both Finland and Denmark to minimize any effect of interassay variation. For determination of phthalate monoesters, breast milk samples were thawed and placed in a water bath at 37°C to get a homogeneous sample without a separate fat layer. An aliquot of 3 mL was removed for liquid extraction using a mixture of ethyl acetate and cyclohexane (95:5) followed by a two-step solid phase extraction as described in detail previously. Determination of phthalate monoesters was accomplished by high-pressure liquid chromatography with a Betasil Phenyl column (100 × 2.1 mm × 3 μm). The instrument was run in negative mode using the electro spray source (ESI). Detection limits were in the range of 0.01 to 0.5 μg/L. Recoveries at two different levels ranging from 2 to 120 μg/L were included using different milk samples and the CV (percent) was calculated from measurements of real duplicate determinations during the project period. (Main et al. 2006)

- **Sensitivity and specificity (detection range)**
  See above.
- **Units**
  See above.

☑ Performance characteristics

- ***Analytical reproducibility***
  See above.
- ***Inter- and intralaboratory variability***
  N/A

☑ Validation

- *Scientific Committee for Toxicity, Ecotoxicity and the Environment (CSTEE)*
  CSTEE published carried out risk assessments for DEHP and other phthalates in the late 1990s, including the publishing of tolerable daily intake (TDI) figures and no observed effect levels (NOAEL). These are given in Table 10.

  Table 15.2: Estimated TDI and NOAEL based on urinary metabolite excretion (figures in mg/kg body-weight/day)

	Metabolite	Diester	TDI	NOAEL
Secondary metabolites	5oH-MEHP	DEHP	37	3.7
	5oxo-MEHP	DEHP	37	3.7
	DEHP metabolites	DEHP	37	3.7
Monoesters	MEHP	DEHP	37	3.7
	MnBuP	DnBP	100	52
	MBzP	BBzP	200	20
	MEP	DEP	8000	800
	MOP	DOP	370	37

☑ Confounding factors

Oxidative metabolism of phthalate esters is significantly greater in children than in adults (Koch et al. 2004).

☑ Concentrations reported in literature

- **Reference value per age group/sex...**
  N/A
- **Critical values**
  N/A
- **Normal range in populations (including power calculations)**
  Only a small body of data is available for normal ranges of phthalates (or DEHP) in populations. Work that has been done seems to focus on typical doses, as opposed to concentrations. Tables 11-13 are derived from http://www.reason.org/peg2.html (accessed 14 August 2006).

Table 15.3 : airborne DEHP concentrations – inhalation exposure

Source of exposure	Dose in mg/kg of body weight per day
Air (worst case)	0.100
Air (indoor, PVC paved room)	0.014-0.086
Air (in cars at 60° C)	.030
Air (in cars at 25° C)	<.0001
Air (outdoor, urban)	0.000006-0.0000225
Air (outdoor, non-urban)	~0

Table 15.4: ingested DEHP concentrations

Source of exposure	Dose in mg/kg of body weight per day
Food (hypothetical worst case)	0.485
Food (typical situation)	0.025
Mouthing toys	0.00024-0.00166
Drinking water	<0.001

Table 15.5: Injected / Infused DEHP Exposures and Potential Doses

Source of exposure	Dose in mg/kg of body weight per day
DEHP (long term)	
• Hemodialysis, one session	0.01-7.2
• Peritoneal dialysis	0.800
• Clotting factors in hemophiliacs	0.030
DEHP (short term, < 10 days)	
• Extracorporeal oxygenation in infants	42-140
• Adult blood transfusion	0.2-8.5
• Newborn blood transfusion	0.5-4.2
• Platelet concentrates in adults	0.4-2.5
• Cardiopulmonary bypass	0.3-2.4
• Platelet concentrates in newborns	1.9

☑ Dose –response/effect relationships

N/A

☑ Time trend, geographical variation, susceptible groups

The use of phthalates in medical devices (particularly di-(2-ethylhexyl) phthalate) represents a significant source of exposure particularly for neonatal patients and patients

undergoing blood exchange transfusions, extracorporeal membrane oxygenation (ECMO), and cardiovascular surgery (Rais et al. 2004). Extreme DEHP exposure levels have been documented for such patients (Lottrupp et al. 2006).

Children's toys made of polymers are a source of oral exposure, particularly in young children, who are prone to suck and chew on these items. The European Union (EU) has banned certain such items containing DEHP, DBP, BBP, DnOP and DiDP. The baking of polymer modelling clays in ovens may cause short-term, high-level exposure through inhalation to phthalates of higher molecular weights (Shettler 2005). The fact that children use these clays raises concern about their potentially high exposure levels to phthalates through this route (Miller et al. 2002).

Biomonitoring studies carried out so far have provided the first evidence that urinary metabolites of some phthalates such as DBP, di-2-ethylhexyl phthalate (DEHP) and benzyl butyl phthalate (BBP) are consistently higher in children than in adults. This may indicate a higher average exposure for children (Koch et al., 2004, 2005; Silva et al., 2004; Lottrupp et al. 2006).

17 POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

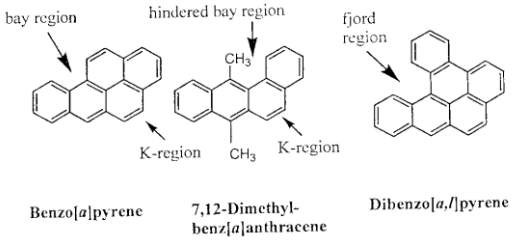
☑ General information

The most commonly used biomarkers of PAH exposure are metabolites of PAHs, particularly 1-hydroxypyrene, and PAH-DNA or protein adducts.

PAHs are products of the incomplete combustion or pyrolysis of organic material. They are ubiquitus in the in the environment, leading to measurable background levels of exposure in the general population. Pollution of air by PAHs is mainly due to the incomplete combustion of wood or fuel used for residential heating and industrial or motor vehicle exhaust (IARC 1983; Peluso et al. 1998).

Eight compounds, including benzo[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-c,d]pyrene, are considered by several agencies as human carcinogens. The most common compound used as a reference substance for carcinogenic PAHs is benzo[a]pyrene (B[a]P).

Fig. 16.1: Structural features of PAHs that contribute to carcinogenicity.



B[a]P, a known carcinogen, contains a bay region. The diol epoxide metabolite of B[a]P, which binds to DNA, contains an epoxide group next to the bay region. Methyl groups in DMBA hinder this structural feature further, as does the extra benzene ring in DB[a,l]P. The compounds are arranged in order of their increasing carcinogenic potency, with DB[a,l]P being the most potent. Adapted from Baird and Ralston, 1997.

☑ Matrix

- **Invasive**

  DNA adducts and protein adducts are determined in blood (WBC, lymphocytes) or placenta samples.

- **Non-invasive**

  1-hydroxypyrene is determined in urine.

☑ Kinetics

- **Uptake**

  The main sources of human exposure to PAHs are occupation, passive and active smoking, food and water, and air pollution (Jongeneelen 1997). The total intake of carcinogenic PAHs in the general population has been estimated to be 3 µg/day (Mumtaz et al. 1996). In smokers, B[a]P levels range from 0.5 to 7.8 µg/100 cigarettes when exposure is from mainstream smoke and from 2.5 to 19.9 µg/100 cigarettes when it comes from side-stream smoke. Levels from passive smoking are lower, ranging from 0.0028 to 0.76 µg/ m^3 of B[a]P (IARC 1983). (Beside occupational exposure, dietary intake seems the most important source of PAHs in non-smokers; in the USA, dietary intake of total PAHs has been estimated to be almost 2 µg/kg food (IARC 1983), with high levels of B[a]P found especially in charcoaled meat (8 µg/kg), while in a variety of other food products B[a]P levels from 0.09 to 30 µg/kg (IARC 1983; Scherer et al. 2000) have been reported. Levels in water may range from 0 to 13 µg/l B[a]P. There is a high variation in atmospheric PAHs levels across geographical areas with B[a]P concentrations ranging from 0.01 to 100 ng/m^3 B[a]P) (Vyskocil et al. 1997).

  In a US study exposure to PAHs from food were commonly 70% in the homes of non-smokers (Lioy and Greenberg 1990). Tobacco smoke adds around 2-5 µg/day for a 1 pack/day smoker. Data from the Czech Republic indicate that more than 70% of exposure is by air pollution (Benes et al. 1999).

- **Metabolism**

  Inhaled PAHs are absorbed mainly through the bronchial epithelium. Absorption can be influenced by the size of the particles, with a slower clearance of those adsorbed on particles compared to PAHs in pure crystals (Creasia et al. 1976;

Sun et al. 1982). After absorption, PAHs are distributed to tissues where they are biotransformed by phase I metabolic enzymes to chemically reactive intermediates, that may bind covalently to DNA (DNA adducts), give rise to mutation and, eventually, tumour initiation. Furthermore, PAH metabolites are mostly conjugated with glucuronic acid by phase II enzymes and excreted as hydroxylated metabolites or in a small proportion as sulphate or even unconjugated. PAHs are excreted mainly through the faeces; only about 10% are excreted in the urine.

Specific cytochrome P450 isozymes and epoxide hydrolase can form reactive diol epoxides that comprise ultimate carcinogenic metabolites. The major cytochrome P450s that are involved in the formation of diol epoxides are CYP1A1, CYP1A2 and CYP1B1. The major phase II ezymes include glutathione S-transferases, which are involved in the conjugation of PAH metabolites (GSTM1, GSTP1, and GSTT1).

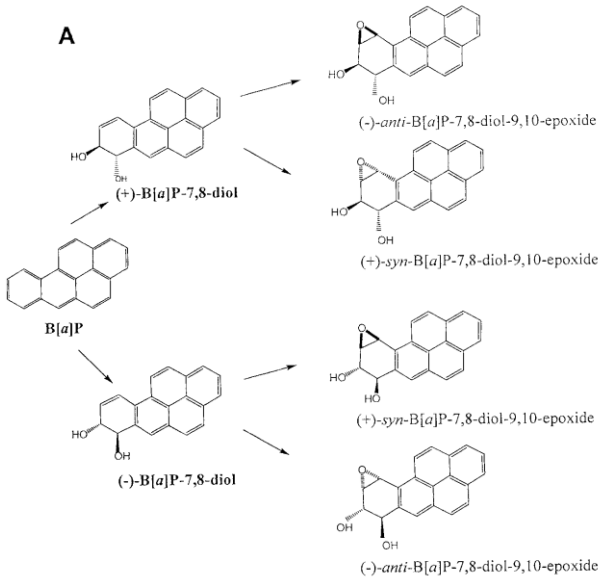
- **Biological half-life**

  The half-life of airborne PAHs is of the order of days but can be longer when they are bound to small particles (Mumtaz et al. 1996).

  Following inhalation, the half-life of 1-OHP is on average about 18–20 hours (Jongeneelen and Bos 1990; Buckley and Lioy and Greenberg 1992; Van Schooten et al. 1995).

  The half-life of DNA adducts in lymphocytes is in the order of months, while for protein adducts it depends on the type of protein where the chemical is bound. In the case of albumin adducts, the half-life is around 20 days, while for haemoglobin adducts it is around 120 days.

Fig. 16.2: A: Metabolism of B[a]P to diol epoxides. The BPDE isomers containing the epoxide oxygen and 7-hydroxyl group on the same face of the molecule are called *syn;* opposite face, *anti.* The *syn* and *anti* pairs are enantiomers. The (+)-*anti*-B[a]P-7R,8S,10R-epoxide is the most tumorigenic. The (+)-*syn* metabolite also binds to DNA. B: Opening od the epoxide to adduct to DNA. The bay region epoxide may open in a *cis* or *trans* fashion to adduct to DNA bases (Baird and Ralston 1997).



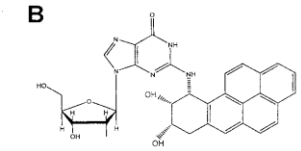
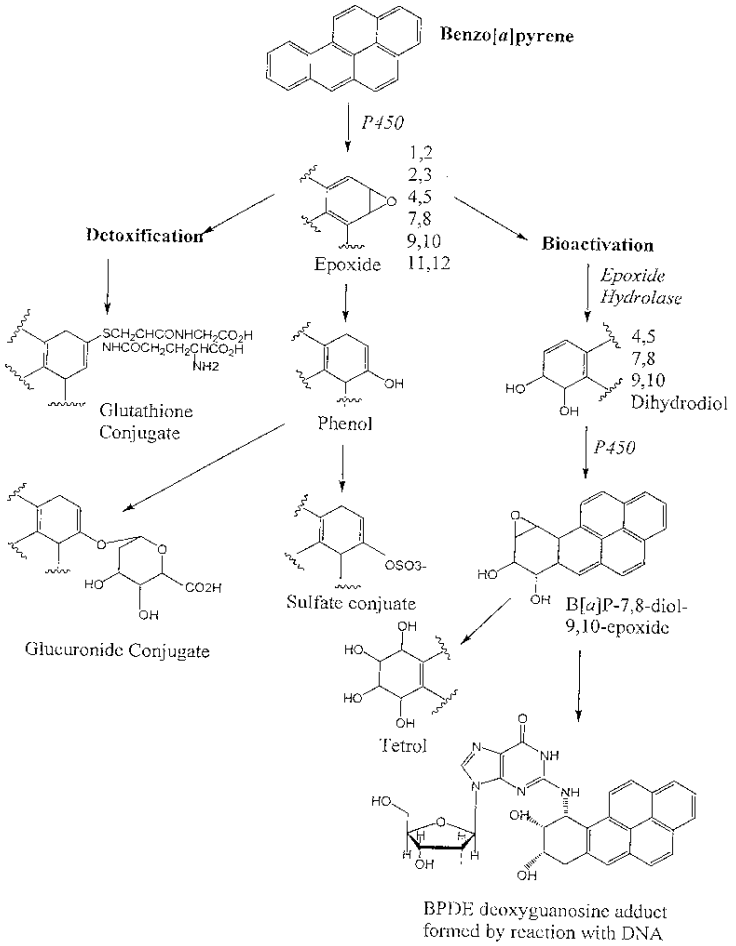


Fig. 16.3: Activation of PAHs to DNA-binding species. CYPs add an epoxide group to hydrophobic PAHs. This species may be conjugated and detoxified or further bioactivated by epoxide hydrolase and additional epoxidation by CYPs. Bay of fjord region diol epoxides have the potential to bind to DNA (Baird et al. 2005).



☑ Sampling conditions

- **Blood**

  10 ml of of human vein blood collected in heparinized vacutainers is used for lymphocyte isolation by using Ficoll 400 gradient centrifugation. Alternatively total WBC cells might be isolated. Cell pellets may be stored for several months in -80^{o}C until DNA isolation.

- **Placenta**

  Tissue samples taken from the middle of placenta, opposite to the umbilical cord, should be frozen shortly after delivery and may be stored at -80^{o}C for several years.

- **Urine**

  Urine samples should be frozen shortly after delivery and may be stored at -80^{o}C for several years.

☑ Analytical aspects

Airborne PAHs are usually analyzed by gas chromatography/mass spectrometry (Poirier et al. 1998; Mumford et al. 1993) or high performance liquid chromatography (Peluso et al. 1998; Lewtas et al. 1997; Binkova et al. 1996; Kang et al. 1995), mostly from particles collected in a filter after extraction with organic solvents.

Hydroxypyrene (1-OHP) is the principal product of pyrene metabolism, representing 90% of its metabolites (Brzeznicki et al. 1997). Pyrene is the only known precursor of 1-OHP (Jacob et al. 1982); it forms a consistent proportion of higher molecular weight PAHs in the environment (Butler and Crossley 1979). The two main analytical methods employed to measure 1-OHP are high performance liquid chromatography combined with fluorescence detection (HPLC/FD), and gas chromatography with mass spectrometry (GC/MS). For HPLC/FD, the detection limit is 0.05 µg/l, with a recovery rate of 85% (Jongeneelen et al. 1987) and for GC/MS it is around 0.1 µg/l with a higher recovery rate that reaches more than 93% (Bouchard et al. 2001).

Procedures for DNA adducts monitoring exposure to c-PAHs include ^{32}P-postlabeling, immunoassay using antibodies to carcinogen modified DNA, and mass spectrometry (Farmer 2004). PAH adducts have been mainly employed as a measure

of PAHs linked to DNA in target tissues (biological effective dose), although protein adducts have also occasionally been determined. ELISA is used to measure specific DNA adducts, and ^{32}P-postlabelling is used for the determination of bulky DNA adducts, also called aromatic adducts. Protein adducts are measured mostly in blood proteins using CG/MS, HPLC, or ELISA.

☑ Validation

- **Standardization**

  Standardization of ^{32}P-postlabelling has been achieved by means of an inter-laboratory trial organized by IARC (Phillips and Castegnaro 1999).

- **Analytical reproducibility**

  ^{32}P-postlabelling requires precision and several variations of the assay can be performed, which have been shown to produce very different results. These assay variations can explain some of the differences in DNA adduct levels measured by ^{32}P-postlabelling. Higher adduct levels resulted from ELISA compared with ^{32}P-postlabelling, although it is not clear if this was due to higher exposure to PAHs levels in studies using ELISA, or to higher sensitivity of the method. However, absolute comparisons between the two assays should only be made after calibration in a laboratory.

- **Inter- and intralaboratory variability**

  Despite the inter- and intralaboratory variability should be checked by using positive control samples which are included in each postlabelling experiment.

☑ Confounding factors

- **Diet**

  Higher intake of vitamin C affected level of DNA adducts in placenta (Sram et al. 1999).

- **Ethnicity**

  According to Perera et al. (2005) comparing exposure to c-PAHs in New york and Cracow, African Americans may be more sensitive than Europeans (comparing DNA adduct levels vs. exposure to c-PAHs).

☑ Dose –response/effect relationships

Biomarkers of exposure measure internal dose as metabolites in blood or urine or biologically effective dose, as DNA adducts, protein adducts or DNA breaks by Comet assay. Studies on environmental exposure to mutagens and carcinogens in many cases lack data on exposure. For example, personal exposures to PAHs are often below or near detection limits for environmental exposure. It is also sometimes difficult to relate the effects observed on biomarkers only to pollution, if information on ambient exposure and life style are not fully presented for each individual.

Regardless, toll booth workers, traffic police, bus drivers or postal workers have been generally used as model groups for air pollution in big cities. Binkova et al. (1995) studied the effect of personal exposure to carcinogenic PAHs (c-PAHs) on DNA adducts in women working outdoors as postal workers or gardeners in the city of Teplice (polluted region). There was a significant effect on personal exposure to c-PAHs and DNA adducts in the group of nonsmokers. In the repeated follow up study, Binkova et al. (1996) compared women and control (Prachatice) districts. A significant correlation was found between individual personal exposures to c-PAHs and DNA adducts.

The effect of environmental pollution has been measured in several groups of traffic police in Italy. Peluso et al. (1998) conducted the study in Genoa, determining B[a]P exposure using personal monitoring and analyzing the formation of DNA adducts in WBC using a ^{32}P-postlabeling technique. A higher level of B[a]P and DNA adducts in the police officers was observed during the summer (no effect was observed in the winter). Similar results for workers exposed to traffic-related pollution in Florence and Prague have been reported elsewhere (Palli et al. 2001; Binkova et al. 2002). The effect of environmental exposure to airborne-particulate-bound PAHs on DNA adduct levels was studied in male police officers spending > 8 hours outdoors and matched controls spending > 90% of daily time indoors in Prague (Binkova et al. 2002). The total DNA adduct levels did not significantly differ between exposed and control groups, whereas the level of B[a]P-derived DNA adducts was significantly higher in exposed group.

A large study in Denmark evaluated several endpoints and exposure in bus drivers and postal workers using 1-OH-pyrene in urine, and exposure dose by DNA and protein adducts (Hansen et al. 1998; Autrup et al. 1999; Loft et al. 1999).

Significantly higher levels of bulky-aromatic DNA adducts were observed in bus drivers working in the central part of Copenhagen. The biomarker levels in postal workers were similar to the levels in suburban bus drivers. In contrast, significantly higher levels of malondialdehyde (marker of lipid peroxidation) in plasma and PAH-albumin adducts were observed in a suburban group, which was used as a control group because of lower exposure to ambient air pollutants. Autrup et al. (1999) recommended analyzing oxidative damage to DNA as pollution by diesel exhaust particles may induce just oxidative damage. Oxidative damage on DNA in bus drivers was determined by urinary excretion of 8-oxo7,8-dihydro-2'-deoxyguanosine (8-oxodG) (Hansen et al. 1998). Comparing drivers in the centre and the rural/suburban areas of Copenhagen, there was a significant difference in the 8-oxodG excretion and suggests that exposure to ambient air pollution causes oxidative damage to DNA (Loft et al. 1999).

Exposure to c-PAHs in environmental pollution on exogenous and oxidative DNA damage was specifically studied by the EC project EXPAH (Farmer et al. 2003) in Prague, Kosice and Sofia (city policemen, bus drivers, controls). Lymphocyte bulky DNA adducts (by ^{32}P-postlabeling method) were significantly correlated with exposure to c-PAHs when subjects were classified either by personal exposure measurements or by job description (Taioli et al. 2006). Also plasma levels of p53 and $p21^{WAF21}$ correlated in the same groups with c-PAHs exposure (Rossner Jr et al. 2006). When the oxidative DNA damage was determined (8-oxodG) by liquid chromatography-tandem mass spectrometry and cyclic pyrimidopurinone N-1,N^2 malondialdehyde-2´-deoxyguanosine (M_1dG) by the immunoslot blot assay from lymphocyte DNA, it seems, that the environmental air pollution may alter the endogenous oxidative DNA damage levels in humans but the effect appears to be related to the country where the individuals reside (Singh et al. 2006). Using Comet assay with the X-rays challenge, Cebulska-Wasilewska et al. (2005) proved the impact of c-PAHs exposure to decrease the cellular DNA repair processes.

In the Greek study (AULIS project) of non-smoking students from urban and rural areas (Georgiadis et al. 2000, Kyrtopoulos et al. 2001), a positive correlation between DNA adducts and personal exposures to chrysene and benzo[a]pyrene was found only in the group from rural area. The authors observed a significant effect of gender on DNA adduct levels (males having higher levels) and no effects for other dietary and

exposure reflecting factors. The AULIS project resulted in the conclusion that moderate-to-low exposures to airborne particulate-bound PAHs do not exhibit a simple correlation with biomarkers of genotoxicity. The authors suggested that additional factors could contribute to the overall genotoxic burden (Kyrtopoulos et al. 2001). Palli et al. (2001) demonstrated the effect of XPD-repair polymorphisms on DNA adduct levels of traffic workers and general population exposed to high levels of genotoxic agents related to vehicle emissions. The study of Godschalk et al. (2001) provided the evidence for combined effects of genetic polymorphisms in GSTM1, GSTT1, NAT1 and NAT2 on DNA adduct formation in smoking individuals and indicated that simultaneous assessment of multiple genotypes may identify individuals at higher cancer risk. Another study of Palli et al. (2003) confirmed that biomarkers of dietary intake of antioxidants as well as genetic susceptibility markers (GSTM1 genotype) modulate DNA adduct levels in healthy adults.

Overall, results of various studies indicate that different biomarkers are worthwhile under different types of pollution scenarios. Due to the lower sensitivity, the classic 1-OH-pyrene seems to be an inconvenient biomarker for environmental exposure. DNA adducts measured by a ^{32}P-postlabeling method have become the most popular of biomarkers of exposure and are probably the biomarker of choice for evaluating PAH exposure. Contemporary study indicate that the biomarkers of exposure should be used obligatory with the personal and stationary exposure monitoring and biomarkers of susceptibility, determining metabolic and DNA repair genotypes and antioxidant levels.

For oxidative damage, 8-oxodG and Comet assay seem to be convenient biomarkers. Nonetheless, both of them need reliable standardization and international validation for human biomonitoring in the future.

Lewtas et al. (1997) postulated for air pollution exposure-DNA adducts dosimetry in humans the non-linearity at high doses (e.g. comparing environmental vs. occupational exposure).

☑ Time trend, geographical variation, and identification of susceptible groups

- **Time trend**

  In the cities where residential heating is the main source of PAHs, air pollutant PAH levels during winter are frequently higher than in summer (Kure et al. 1997; Perera et al. 1992; Motykiewicz et al. 1998). Nevertheless, some studies that found very low levels of B[a]P (range 0.03–3.3 ng/m^3) did not show a seasonal variation (Peluso et al. 1998).

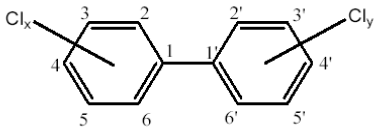
- **Susceptible groups**

  The finding of higher DNA adduct levels in the infant compared to the mother suggests an increased susceptibility of the developing fetus to DNA damage (Perera et al. 1999). This finding is consistent with the idea of a primary role for c-PAHs in fetal growth modulation (Ridgon and Renneis 1964; MacKenzie and Angevine 1981; Guyda 1991; Zhang et al. 1995). In addition, it appears to be an interaction between exposure to PAH and genotype to produce DNA-adducts (Whyatt et al. 2001).

18 POLYCHLORINATED BIPHENYLS

☑ General information

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



Because of their insulating and non-flammable properties, PCBs have been widely used as coolants and lubricants in transformers, capacitors, and other electrical equipment.

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

Table 17.1: WHO-TEF values for dioxin-like PCBs

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

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

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

169) (TEF=0.03) and a single TEF value (0.00003) for all relevant mono-*ortho* substituted PCBs. Additivity, an important prerequisite of the TEF concept was again confirmed by results from recent *in vivo* mixture studies (Van den Berg et al, 2006).

☑ Matrix

- **Invasive**

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

- **Non-invasive**

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

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☑ Kinetics

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

- **Uptake (by different routes)**

  The lipid solubility of PCBs increases with chlorination and promotes absorption across lipophilic cell membranes of the skin, the lung and the gastrointestinal tract (Van den Berg et al, 1998; Van Birgelen and Van den Berg, 2005).

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

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

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

- **Metabolism**

  For the purpose of this project an excellent source of information on PCB metabolism can be found in several published reviews treating this topic (WHO, 2000; Lotti, 2003; WHO, 2003). The rate limiting step in the elimination of PCBs is metabolism, which primarily occurs via the hepatic cytochrome P-450-dependent monooxygenase system, and which varies depending on the

chlorination pattern of the congener. Hydroxylated products are major metabolites, with hydroxylation occurring primarily at the *para* or *meta* positions if these sites are unsubstituted. Arene oxides occur as intermediate metabolites in the oxidation of some PCBs. They are reactive and can be converted both spontaneously and enzymatically to detoxified products (phenols, dihydrodiols, glutathione conjugates), which are excreted. Alternatively, they can form other potentially toxic (cytotoxic, mutagenic, carcinogenic), covalently-bound substrate macromolecular adducts. Besides hydroxylation and subsequent conjugation, sulfur-containing metabolites (e.g. methyl sulfones) and partially dechlorinated metabolites have also been identified. The methyl sulfonyl and hydroxylated PCB metabolites have been detected in human milk and plasma as well as in other biological samples from the environment. Both types of PCB metabolites can preferentially accumulate in specific tissues such as the lung and the fetus. The rate of metabolism of PCB and the resultant metabolite pattern vary between different species. The excretion of PCB congeners is, to a large extent, dependent on their rate of metabolism to more polar compounds. Most congeners show biphasic elimination, where the initial half-life is relatively short for all congeners, but the later half-life is much longer and clearly structure-dependent. There is a large variation in half-lives between different PCB congeners depending on the number and position of the chlorine atoms; the range is from a few days to 450 days depending on the congener.

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

Most of the toxic coplanar and mono-*ortho* coplanar PCB is not readily metabolised. Lower chlorinated PCBs and those congeners that have two adjacent unsubstituted carbon atoms and an unsubstituted *para*-position are thought to form

arene oxide intermediates by cytochrome P450 isoenzymes and subsequently to rapidly produce more polar metabolites. As with polychlorinated dibenzofurans and polychlorinated dibenzodioxins, the cytochrome P4501A isoenzymes seem to play an important role in the metabolism of those PCB congeners that are more or less isosteric with 2,3,7,8-tetrachlorodibenzodioxin. Whereas several studies have investigated the metabolism of PCB in different species and shown the involvement of various P450s, much less is known about the isoenzymes involved in their metabolism in humans. Metabolism of PCBs in humans results in the formation of the hydroxylated metabolites. Whereas many hydroxylated PCBs are further converted to either glucuronic acid or sulfate conjugates by phase II enzymes, which facilitates their excretion, some OH-PCBs persist in the body. Glucuronidation of OH-PCBs seems to be correlated with surface area and surface volume of the molecule, thus explaining, in part, why some OH-PCBs may persist in the body. The dominating OH-PCBs retained in human plasma, accounting for up to 20% of total plasma PCBs, are 4-OH-PCBs derived by 1-*ortho*-PCBs through a 1,2-shift of a chlorine in the *para* position (Lotti, 2003).

- **Biological half-life**

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

☑ Sampling conditions

- **Operational aspects**

  The sampling procedures vary greatly according to the type of sample matrix. Adipose tissue should be frozen after sampling; then the sample can be freeze-dried or homogenised using liquid N_2 and ground with anhydrous sodium sulfate.

  Breast milk (or other milk samples) can also be frozen and, after sampling and transporting to the laboratory, freeze-dried or chemically extracted. If the milk samples are not frozen immediately after sampling they should be treated by potassium dichromate.

  Foodstuffs represent a very broad matrix category. Meat and fish samples should be frozen right after the sampling and the handling with samples after transport is similar to adipose tissue. Butter, fats and oils are generally assumed to be homogenous and usually do not require sample pre-treatment before clean-up procedure (no more than drying with anhydrous sodium sulfate).

  Finally, dried samples should be stored at $-20^{o}C$ until they are treated for analyses, preferably in glass containers.

  Blood should be clotted and centrifuged to isolate serum. The serum samples should be frozen and stored at $-20^{o}C$ until analytical procedure.

☑ Analytical aspects

- **Techniques**
  - *Chemical-analytical:* Many of the analytical methods used for biological and environmental samples are the methods approved by various agencies and organizations such as EPA and the National Institute for Occupational Safety and Health (NIOSH), Association of Official Analytical Chemists (AOAC) and the American Public Health Association (APHA). Additionally, analytical methods were developed that modify previously used methods to obtain lower detection limits, and/or to improve accuracy and precision (Turci et al., 2004).

    Methodology for PCB analysis includes several steps: sample collection and storage, extraction, cleanup, and determination (Hess et al., 1995; EPA, 1995c; EPA, 1999k). The trend is toward congener-specific analysis by high-resolution gas chromatography (HRGC).

PCBs are routinely analysed by one-dimensional capillary gas chromatography equipped with electron capture detector (ECD) or micro-ECD in biological and food samples (EN-1528-1, -2, -3, -4, 1996). The analysis of non-ortho, mono-ortho and some non-dioxin like PCB congeners requires pre-separation column liquid chromatography and the use of high-resolution mass spectrometry (HRMS) is inevitable. Recent advances include analytical methods that are able to quantify individual PCB congeners to enable TEQ calculations (Patterson et al., 1994; EPA, 1999k; Frame, 1999). EPA Method 1668 (Revision A) is the most efficient and precise current methodology used to measure individual PCB congeners in water, soil, sediment, and tissue by HRGC/ HRMS (EPA, 1999k).

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

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

In a sample containing PCBs, there are often several dozens of different congeners. For practical reasons, all of them are not always measured, but the most important congeners are used as indicators. Σ7PCB, a selection of PCB congeners denotes the sum of the seven marker PCBs. In Belgian chicken incident, only seven abundant congeners were usually measured: congeners with IUPAC numbers 28, 52, 101, 118, 138, 153, and 180 (2,4,4'-TriCB, 2,2',5,5'-TCB, 2,2',4,5,5'-PeCB, 2,3',4,4',5-PeCB, 2,2',3,4,4',5'-HxCB, 2,2',4,4',5,5'-HxCB, 2,2',3,4,4',5,5'-HpCB, respectively). The seven congeners are estimated to constitute about one third of all PCBs in the contaminated feed.

2,2´,4,4´,5,5´-hexachlorobiphenyl (CB-153**)** is also frequently used as a biomarker for POP exposure, because it correlates very well with both total PCB concentration in plasma and serum (Grimvall et al,. 1997; Glynn et al,. 2000) and with the PCB derived dioxin-like effect as well as the total POP derived dioxin-like effect (Gladen et al,. 1999).

- *Bio-analytical:* U.S. Environmental Protection Agency Method 4425 (US EPA http://www.epa.gov/epaoswer/hazwaste/test/pdfs/4425.pdf) utilizes a reporter gene system (RGS) based on cytochrome P450 to screen samples for a range of organic compounds including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), coplanar polychlorinated biphenyl congeners (PCBs), and high molecular weight polynuclear aromatic hydrocarbons (PAHs). The method is a screening procedure that will detect the total amount of planar compounds in solvent extracts of environmental samples of soil, sediment, tissue, and water. Method 4425 serves as both, a biomarker simulating the response of an organism (with CYP1A) exposed to inducing compounds, and as a bioanalytical technique measuring the levels of these chemicals in the samples. Methods based on similar principles are commercially available (CALUX , CAFLUX assay). For more references see http://www.biodetectionsystems.com/lit_ldr.html. The ELISA (Enzyme Linked Immunosorbent Assay) is commonly used for the analysis of environmental samples and have recently started to be used for food samples as well (Kočan, 2004). The enzyme immunoassay method uses an immobilized antibody to facilitate the separation of targeted analytes (antibody-bound components) from non-target substances using a washing step and an enzyme conjugate to generate the signal used for the interpretation of results. While GC-ECD and/or GC/MS is able to quantify individual PCB congeners of different reactivity and toxicity, the data reported indicate that immunoassay offers a rapid and inexpensive alternative method for estimating "total" PCBs (Zajicek et al., 2000; Fillmann et al., 2002).

- **Sensitivity and specificity**

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

- **Units**

  Results on polychlorinated biphenyls are expressed in several ways. The most common is to express concentrations of the analyte as weight per wet matrix weight

basis either as ppb or microgramm per g. Since polychlorinated biphenyls are highly lipophilic, the concentrations of individual congeners are generally expressed as a weight unit per gram of lipid (usually ng/g lipid).

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

☑ Performance characteristics

- **Analytical reproducibility**

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

- **Inter- and intra-laboratory variability**

  Inter-laboratory comparison studies in PCB analysis in different matrices are organised by many accredited/certified institutions worldwide. Inter-laboratory assessment studies on PCDDs/Fs and PCBs in human milk and blood serum has been organized by WHO/EURO in 1989, 1992, and 1997. Laboratories have the opportunity to assess their quality in inter-laboratory quality assessment organised by G-EQUAS (German External Quality Assessment Scheme) – Intercomparison programme for toxicological analyses in biological materials - occupational and environmental medical field (blood serum), yearly. Variability of analytical results is reported by Youden-pairs and if the results of the laboratory on two concentration

levels (in two samples) of each compound lie within statistically appointed intervals (mean of results ±3 SD, standard deviations), the laboratory gains the certification. The variation coefficients for PCBs ranged from 7 to 14% in Intercomparison programme 35 in 2005. Another proficiency testing or inter-calibration studies are organized by Food Analysis Performance Assessment Scheme (FAPAS), Ministry of Agriculture, Fisheries and Food, United Kingdom, by Norwegian Institute of Public Health, Oslo (Dioxins in Food, dioxin-like PCBs analysed), and by University Consortium Environmental Chemistry, Marghera.

☑ Validation

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

☑ Confounding factors

- **Age**

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

- **Gender**

  Complex role of gender should be taken into consideration when PCB exposure in human population is being assessed. Results do not show clear trend in exposure between genders among studies. Higher PCB levels in men, if compared to women, were often reported (Falk et al., 1999; Hanrahan et al., 1999; Petrik et al., 2006), but

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other authors did not confirm this association (Apostoli et al., 2005; de Saeger et al., 2005; Dirtu et al., 2006).

- **Diet**

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

- **Socio-economic status**

Borell et al. (2005) reported higher PCB serum concentrations associated with family income, but not with maternal education in Afro-American women. The authors concluded that maternal socioeconomic indicators may influence the effects of exposure to PCBs among African-American pregnant women. Vartiainen et al. (1998) and Chao et al. (2004) found positive association between PCB levels in breast milk and maternal education. Race differences in exposure to several PCB congeners were reported with higher exposure in nonwhite participants (James et al., 2002).

- **Body Mass Index (BMI)**

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

- **Parity and lactation**

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

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☑ Concentrations reported in literature

Table 17.2: Median PCB-153 congener levels in human milk reported in the 4th WHO-Coordinated Survey of Human Milk for Persistent Organic Pollutants - Report of status and results as available on August 11, 2006 by Dr. Reiner Malisch.

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

Table 17.3: PCB levels in human milk reported in the 3rd round of the WHO-coordinated exposure study 2001 – 2002 (Van Leeuwen and Malisch, 2002).

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

Country	N° of pools	PCBs (WHO-TEQ pg/g fat, median)		Σ PCBs (ng/g fat, median)	
		median	range	median	range
Norway	2	8.08	6.56 – 9.61	119	106 – 132
Philippines	2	2.38	2.22 – 2.54	26	26 – 26
Romania	3	8.06	8.05 – 8.11	173	165 – 198
Russia	7	13.45	12.92 – 22.95	126	84 – 311
Slovak Republic	4	12.60	10.72 – 19.49	443	331 - 621
Spain	6	9.42	6.93 – 17.94	241	162 – 467
Sweden	1	9.71	-	146	-
The Netherlands	3	11.57	10.90 – 13.08	192	178 – 210
Ukraine	3	19.95	14.10 – 22.00	136	103 – 148
USA	2	4.61	3.69 – 5.52	54	43 - 64

Table 17.4: Median PCB-153 congener levels and other PCB levels in human milk reported in the 2nd round of the WHO-coordinated exposure study 1992 - 1993 (WHO European Centre for Environment and Health. Levels of PCBs, PCDDs and PCDFs in human milk. Environmental Health in Europe No.3)

Country	Town	N° of individual samples in pool	PCB-153 (ng/g fat, median)	dl PCBs (WHO-TEQ pg/g fat, median)	Σ marker PCBs (ng/g fat, median)
Albania	Tirana	10	32.2	2.3	63.4
	Librazhd	10	22.2	1.7	42.5
Austria	Vienna	13	146.0	11.7	380.6
	Tulln	21	119.0	12.4	302.5
	Brixlegg	13	174.0	19.0	449.4
Belgium	Brabant Wallou	8	128.6	7.4	275.5
	Liege	20	142.6	4.7	306.2
	Brussels	6	122.1	7.8	260.5
Canada	Maritimes 92	20	37.7	4.1	86.1
	Quebec 92	20	59.5	6.8	137.3
	Ontario 92	20	49.6	7.7	128.0
	Prairies 92	20	21.6	3.2	57.9

Country	Town	N° of individual samples in pool	PCB-153 (ng/g fat, median)	dl PCBs (WHO-TEQ pg/g fat, median)	Σ marker PCBs (ng/g fat, median)
	British Columbia 92	20	25.5	3.5	70.2
	All provinces 92	100	44.5	5.3	111.7
	Gaspe	12	94.7	12.7	219.7
	Canada all provinces 81	200	87.0	12.1	212.1
	Basse Cote-Nord	4	283.9	25.4	558.6
	Ungave Bay	4	282.8	14.1	576.0
	Hudson Bay	5	716.7	21.3	1361.4
Croatia	Krk	10	103.0	6.1	218.4
	Zagreb	13	99.8	8.0	219.5
Czech Rep.	Kladno	11	215.0	6.0	531.6
	Uherske Hradiste	11	424.8	9.8	1068.5
Denmark	7 different cities	48	99.6	4.5	209.4
Finland	Helsinki	10	88.8	4.6	188.9
	Kuopio	24	65.1	2.4	133.5
Germany	Berlin	10	165.0	11.7	375.0
Hungary	Budapest	20	31.6	1.7	61.3
	Scentes	10	23.2	1.4	45.0
Lithuania	Palanga	12	163.5	20.4	362.0
	Anykshchiai	12	127.4	20.7	287.0
	Vilnius city	12	143.3	20.5	321.7
Netherlands	mean of 17 individual samples	17	113.3	11.0	253.1
Norway	Tromso	10	127.5	19.5	272.6
	Hamar	10	127.9	10.4	264.5
	Skien/Porsgrunn	10	140.8	9.5	301.5
Pakistan	Lahore	14	5.7	2.3	18.9
Russian Fed.	Arkhankelsk	1	94.4	8.6	196.8
	Karhopol	1	50.5	4.9	102.3
Slovak Rep.	Michalovce	10	434.9	13.3	1015.1
	Nitra	10	207.7	6.1	489.0
Spain	Bizkaia	19	185.7	10.6	461.3
	Gipuzkoa	10	187.6	8.2	451.7
Ukraine	Kiev nr.1	5	122.7	15.0	263.5

Country	Town	N° of individual samples in pool	PCB-153 (ng/g fat, median)	dl PCBs (WHO-TEQ pg/g fat, median)	Σ marker PCBs (ng/g fat, median)
	Kiev nr.2	5	91.2	11.5	191.3
United Kingdom	Birmingham	20	56.9	4.3	129.5
	Glasgow	23	57.8	4.0	130.8

Table 17.5: Median PCB-153 congener levels in plasma from humans reported by Lotta Hofvander (Polychlorinated biphenyls and their metabolites in human blood, Method development, identification and quantification, Doctoral thesis in environmental chemistry, Faculty of Science, Stockholm University 2006).

Country	District	Sampling year	sex/matrix	N° of samples	PCB-153 (ng/g fat, median)
Slovakia	Michalovce	2001	female/male	175	240
	Svidnik/Stropkov	2001	female/male	122	570
Netherlands		1998/2000	maternal	51	100
		1998/2000	cord plasma	51	115
		2001/2002	maternal	90	63
		2001/2002	cord plasma	9	62
Faroe Islands		1994/1995	maternal	57	430
		2000/2001	children	42	310
Sweden		1995	female	16	50
		1995	female	16	290
		1991	male	20	220
		1991	male	12	450
		2000/2001	maternal	15	56
		2000/2001	cord plasma	15	44
Latvia		1993	male	19	160
		1993	male	26	920
Nicaragua		2002	female	4	14
		2002	female	4	100
Canada Quebec	Nunavik	1993/1996	cord plasma	10	131
	Lower N.Shore	1993/1996	cord plasma	10	215
	Southern Quebec	1993/1996	cord plasma	10	52

Table 17.6: Median PCB-153 congener levels in blood serum presented by Dr. Kočan at UC Davis, CA, July 10-13, 2006

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

Table 17.7: Median PCB-153 congener levels and sum of PCBs in blood serum reported in literature

Country	Note	N° of samples	PCB-153	Σ PCBs
Germany[1]	Infants breast-fed/bottle-fed (serum)		0.38/0.10 ng/ml serum	1.19/0.29 ng/ml serum
Japan[2]	Maternal serum	32		61.5 ng/g lipid (0.467 ng/g serum)
	Umbilical cord serum	32		63.8 ng/g lipid (0.136 ng/g serum)
Japan[3]		24	0.171 ng/g whole blood	0.771 ng/g whole blood
Alaska, St. Lawrence Island[4]	Human blood serum	60		1500 ng/g lipid
Mohawk women[5]	Human blood serum	111		1.2 ng/g serum

Lackmann, et al. 2004; Fukata et al., 2005; Hirai et al., 2005; ACAT, 2003; Fitzgerald et al., 2004.

☑ Dose-response/effect relationships

Despite an enormous number of reports on PCBs, both the causal interpretation of epidemiological studies (Wolff et al., 1997; DeVoto et al., 1997) and the risk assessment of human exposures (Safe, 1994; Safe, 1990; Safe, 1998) have so far been hampered by the different toxicological properties of various congeners and isomers, their large

variability in kinetics and dynamics, and the relative paucity of key data regarding humans. In particular, numerous factors including isomer and congener composition, various degrees of contamination with other chemicals (e.g. chlorinated benzofurans), and quantitatively inconsistent data have made it difficult, up to now, to interpret PCB levels in human blood.

Chlorines at the *ortho* positions introduce constraints on the rotational freedom of the phenyl rings. Thus, PCBs without *ortho*-substitution are referred to as coplanar PCBs and all others

as non-coplanar PCBs. Non-*ortho*-substituted coplanar PCB congeners have 'dioxin-like' activity, based on their ability to interact with and activate the aryl hydrocarbon receptor (AhR). The AhR is one chemical/ligand-dependent intracellular receptor that can stimulate gene transcription in response to xenobiotics. Only a small portion of the total mass of PCB mixtures are coplanar non-*ortho* congeners. *Ortho*-substituted non-planar PCB congeners do not interact with the AhR and could elicit 'non-dioxin-like' effects.

☑ Health effects

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

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

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

☑ Introduction to the complex issue of biomarkers of PCB exposure, effects and susceptibility

Because of their ubiquitous occurrence in all kinds of environmental media, persistent organic pollutants (POPs) have become some of the most important global environmental contaminants (Jones and Voogt, 1999). Laboratory and field observations of animals, clinical and epidemiological studies and studies with cell cultures demonstrated that exposure to certain POPs may be associated with a wide range of biological effects including biochemical deviations, carcinogenesis, neurological changes, reproductive and behavioral abnormalities and dysfunctions of the immunological network (Ritter et al. 1995). Among POPs, the polychlorinated biphenyls (PCBs) represent one of the most important groups of chemicals. Aroclor-1254 is one of the many technical mixtures of PCBs that were widely used in industrial insulation materials, plasticizers and heat transfer fluids. Like DDT various PCBs have been detected in human tissues, especially in fat tissues including breast milk (Kodama and Ota, 1980; Mersch-Sundermann et al., 1996). The use and production of PCBs have been banned for decades, but PCBs are still an environmental problem. The concentrations in food have declined since the pioneering reports by Sören Jensen and others in the 1970s and 80s. However, it was recently reported that in fish from some parts of the Baltic Sea, the decline has not continued during the 90s (Atuma et al.1996; Bignert 2002; Kiviranta 2003).

- **General frame format for use of biomarkers**

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

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

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

  The aquatic environment is particularly sensitive to the toxic effects of contaminants since a considerable amount of the chemicals, PCBs included, used in industry, urbanization, and in agriculture enter marine and other aquatic environments. The aquatic environment is a sink for endocrine disrupting chemicals (EDCs) and other organic chemicals; therefore it is not surprising that there exist many examples of endocrine disruption in fish (Kime, 2001). Molecular and cellular biomarkers measured in aquatic organisms respond rapidly to the stress caused by environmental contaminants, and can be used to assess the health status of organisms and to obtain early-warning signals before irreversible damage occurs at a higher level of

biological organization (Huggett et al. 1992). This property makes aquatic biomarkers indispensable for human environmental health. In a multi-pollution context, it is now recognized that the use of a series of biomarkers is necessary to provide a good understanding of the actual impact of contaminants on organisms. A critical aspect of the multi-biomarker approach is the selection of complementary biomarkers in order to obtain the most complete and reliable information (Cajaravillem et al., 2000; De Lafontaine et al., 2000). The choice of biomarkers to be assessed is most often determined a priori, by considering their physiological role and the (eco)toxicological significance of their responses as characterized in information concerning the numerous potential interactions between contaminants involved in the mechanisms responsible for biochemical, cellular or physiological responses. Providing evidence of the mechanisms involved for each chemical element alone constitutes the first step in understanding how they can interfere when they are present in mixtures (Ait-Aissa et al., 2003). As sentinel aquatic organisms the following species occur in available literature: blue mussels *Mytilus sp.* (Devier et al., 2005), zebra mussels *Dreissena Polymorpha* (Minier et al., 2006), smallmouth bass *Micropterus dolomieu* (Anderson et al., 2003), demersal fish *Solea ovata* (Au and Wu, 2001) and others.

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

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

  exposure as a result of their induction in wildlife (fish, birds, mammals) that inhabit industrialized areas (Arinç et al., 2000; Široká and Drastichová, 2004). In particular carps from the Kalamazoo River Superfund Site contaminated with polychlorinated biphenyls (PCBs) were responding to PCB exposure via upregulation of CYP1A independent of activation of the oxidative stress response genes normally thought to be co-regulated with CYP1A (Fisher et al. 2006).

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

  The review published by Whyte et al. (2000) compiles and evaluates existing scientific information on the use, limitations, and procedural considerations for EROD activity (a catalytic measurement of cytochrome P4501A induction) as a

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

Exposure of rainbow trout to 3,3‘,4,4‘-tetrachlorobiphenyl (PCB77) (1 mg kg^{-1}) for 21 days strongly induced EROD activity (Ait-Aissa et al., 2003). In newly fertilised rainbow trout eggs the order of EROD induction potential was: total extract > polycyclic aromatic compounds

-fraction > dicyclic aromatic compounds-fraction including PCBs > aliphatic and monocyclic aromatic compounds fraction in all matrices (Sundberg et al. 2005b). The lack of a clear relationship between toxicopathic effects and EROD induction in the same test system underlines the need for a battery of biomarkers for estimating environmental risk (Sundberg et al., 2005a; Sundberg et al., 2006).

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

  Heat-shock proteins (HSPs) are a family of diverse proteins, of molecular size ranging from 10 to 150 kDa, involved in the transport, folding and assembly of newly synthesised proteins. These proteins have been found in organisms as diverse

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

- **Expression of multixenobiotic resistance (MXR) proteins as biomarker of human exposure to PCB**

  Multixenobiotic resistance (MXR) phenotype is derived from the expression of membrane-bound proteins (MXR-proteins or P-glycoproteins) that can actively transport toxic compounds out of the cell, thus decreasing their toxicity (Endicott and Ling, 1989). These proteins belong to a superfamily of transporters termed ABC-proteins or traffic-ATPases (Ames et al., 1990; Higgins, 1992). Members of this family have been identified in nearly every organism in which they have been looked for: these include micro-organisms, yeast, plants and animals (Higgins, 1992). The importance of this system for aquatic organisms has been shown by its potential to protect them from nuclear damage (Waldmann et al., 1995) and deleterious effects on cell division during embryonic development (Toomey and Epel, 1993). The expression of MXR proteins (or P-glycoproteins, Pgp) is inducible by exposure to toxic compounds (Minier and Moore, 1996) and the amount of Pgp varies significantly between differentially polluted sites (Minier et al. 1993).

Accordingly, the use of the MXR phenotype could be an index of exposure to toxic compounds (reviewed by Minier et al., (1999)). In zebra mussels (*Dreissena polymorpha*) from Seine estuary with PCB toxic equivalent quantities varying between 20 to 40 pg dioxin equivalents/g dry weight high levels of multixenobiotic resistance proteins were detected (Minier et al., 2006).

- **Antioxidant defence biomarkers in relation to PCB exposure**

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

- **Oxidative damage as biomarker of PCB effect**

  2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and PCBs have been shown to elicit oxidative stress as indicated by decreased antioxidant levels and increased superoxide anion production, lipid peroxidation, GSSG:GSH ratio, and DNA damage in mammals and fish (Hassan et al., 1983, 1985a,b; Stohs et al., 1983; Wahba et al., 1989; Pohjanvirta et al., 1990; Stohs et al., 1990; Alsharif et al., 1994; Palace et al., 1996; Hori et al., 1997; Hassoun et al., 1997; 2000; Shertzer et al., 1998).

  After incubation of calf thymus DNA with either higher- or lower-chlorinated PCB congeners, no significantly increased levels of oxidative DNA damage above background levels, measured as 8-oxo-7,8-dihydro-2′deoxyguanosine.could be detected (Schilderman et al., 2000).

- **Hormones thyroxin, testosterone and gonadotropins as biomarkers of PCB exposure**

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

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

  There was a significant linear association between CB-153 and total amount of PSA (slope [b]522.5, 95% confidence interval [CI] 24.0, 20.9; P 5 0.02). With age, abstinence time and smoking included in the model the association became non-significant (b521.4, 95% CI–3.0, 0.1; P 5 0.07) (Rignell-Hydbom et al., 2005a). In the same population group a significantly lower DNA fragmentation index (%DFI), a measure of sperm chromatin structure, was found in the lowest CB-153 quintile (< 113 ng/g lipid) compared with the other quintiles (Rignell-Hydbom et al., 2005b). When CB-153 was categorized into quintiles, the subjects in the quintile with the highest concentration (>328 ng/g lipid), tended to have decreased sperm motility compared with the subjects in the lowest quintile (<113 ng/g lipid) (Rignell-Hydbom et al., 2004).

- **Biomarkers of DNA damage, mutagenesis and carcinogenesis**

  Among the adverse effects caused by POPs, DNA damage, mutagenesis and carcinogenesis are crucial. For example, POPs such as chlordane, toxaphene, DDT, PCBs, heptachlor, hexachlorobenzene and benzofuran have been classified as probable carcinogens. Numerous toxicological studies have been published, which showed negative results for PCBs in the Salmonella assay in the absence and presence of an exogenous metabolizing system (S9) (Wyndham et al., 1976). Aroclor-1254 was not able to induce micronuclei in vivo using polychromatic erythrocytes obtained from mammalian bone marrow (Heddle et al., 1983), supplying evidence that the PCB-mixture does not act as a direct genotoxicant. Also no treatment-related effects were seen on bone marrow micronuclei following subchronic exposure at the minimum risk level to a complex mixture of persistent contaminants, including polychlorinated biphenyls [PCBs] (Wade et al., 2002) in male rats. Administration of Aroclor 1254 was per se devoid of any influence on

cytotoxic and cytogenetic effects in bone marrow polychromatic erythrocytes and pulmonary alveolar macrophages but tended to attenuate these changes produced by cigarette smoke or ethanol in both types of cell (Balansky et al., 1993). Both PCB 126 and non dioxin-like PCB 153 were not genotoxic to the porcine follicles independently of the stage of their development. (Gregoraszczuk et al., 2004). The teachers in a school polluted by polychlorinated biphenyls did not manifest an increased level of micronucleated cells or sister chromatid exchanges (Wiesner et al., 2000). Several other studies are in agreement with these data (van Pelt et al., 1991). Concerning PCB metabolites five methylsulphonyl polychlorinated biphenyl congeners failed to induce the formation of micronucleated cells at doses of 5.2 to 9.6 ppm, which were about 35,000 times higher than the concentrations in the lungs and adipose tissue of healthy Japanese people (Nagayama et al., 1995). On the other hand significant increase of micronuclei were recorded in lymphocytes from newborns born 12 months after contamination (9.36 +/- 5.60), in comparison to controls (5.53 +/- 3.02) and newborns born 18 months after contamination due to leakage of several tons of polychlorinated biphenyls (PCBs) into the environment and groundwater (6.14 +/- 3.57) (Milosevic-Djordjevic et al., 2005) and also in workers exposed to polychlorinated biphenyls the incidence of micronuclei as well as exchange of sister chromatids were elevated as compared to controls (Joksic and Markovic, 1992). The PCB congener, 3, 4, 5, 3', 4'-pentachlorobiphenyl, was found to be a very potent inducer of micronuclei what can be considered as indicator of biological and genetic damage due to exposure to carcinogens or mutagens, in cultured human lymphocytes (Nagayama et al., 1995).

However, aroclor-1254 is a well-known xenobiotic-metabolizing enzymes (XME) inducer especially of cytochrome-dependent oxigenases, e.g. aryl hydrocarbon hydroxylases. CYP1A1 play a key role in the activation of BaP and other polycyclic aromatic hydrocarbons (PAHs). Therefore, from the mechanistical point of view, aroclor-1254 should be able to enhance the genotoxicity of PAHs, especially of BaP in metabolically complement cells such as HepG2. This supposition was supported by studies (Mendoza-Figueroa, 1985) in which aroclor-1254 enhanced the genotoxicity of BaP in primary liver cells of rats. Additionally, (Mersch-Sundermann et al., 1996) the in vivo treatment of rats with PCB-mixtures enhanced the toxifying potency and the CYP1A content of S9 derived from the livers of the

treated animals. As shown in another study (Wu et al., 2003), aroclor-1254 alone did not affect the MN frequencies in HepG2 cells, but when the cells were pretreated with 23-181 microM aroclor-1254, BaP exposure caused significantly more MN than BaP alone.

- **Genetic susceptibility to environmental PCB – impact for human health**

  Most important chronic diseases of modern industrialised societies, including heart disease and cancer have a multifactorial origin, and a great number of environmental and genetic risk factors have been identified. Gene-environment interactions are of primary importance in the area of individual susceptibility to the development of specific diseases in their response to environmental hazards. Many scientific studies demonstrate that certain genetic polymorphisms can serve as biomarkers of susceptibility to specific environmentally-initiated diseases. Biomarkers of susceptibility, indicates the inter-individual variation in mechanistic processes on the continuum between exposure and effect. An individual's susceptibility to environmentally-mediated disease may arise from genetic causes or from non-genetic factors such as age, gender, disease state, or dietary intake. Genetic polymorphisms may function as biomarkers of susceptibility, but it is important to keep in mind that it is actually the phenotype that is of importance for the final response to the hazardous insult. With regard to the multifaceted biological effects of polychlorinated biphenyls the spectrum of clinical endpoints to which these biomarkers should target is very broad: development of Parkinson's Disease, susceptibility to allergic responses, association between glutathione *S*-transferase pi polymorphism and susceptibility to a variety of diseases including cancers of the lung, oesophagus and stomach, genetic polymorphisms associated with susceptibility to breast cancer, endothelial cell activation and the pathology of atherosclerosis.

- **DNA adducts as biomarkers of PCB exposure**

  Biphenyls are metabolically activated to electrophilic quinoid species capable of binding to DNA. Rodents as well as human hepatic enzymes metabolize lower chlorinated biphenyl congeners to reactive intermediates that form DNA adducts in vitro The para-quinone metabolites of PCBs are, in part, involved in direct DNA adduction (Pereg et al., 2002). In vivo PCB-DNA adducts could not be detected in rats orally treated with a mixture of PCBs (Aroclor 1242) by either the butanol- or by the NP1-enrichment procedure in rat target tissue DNA (Schilderman et al.,

2000). In another quantitative study by HPLC/ESI-MS/MS and (32)P-postlabeling-HPLC the adduct levels were in the range of 3-1200 adducts per 10(8) nucleotides. These results demonstrate that increasing chlorine substitution is associated with lower yields of DNA adduct (Zhao et al., 2004).

19 RETINOL BINDING PROTEIN (RBP)

☑ General information.

Retinol-binding protein (RBP) is 22.2 kD microprotein which is electrophoretically heterogeneous with four pI values between 4.4 and 4.8. It belongs to the lipocalin protein superfamily, consisting mostly of lipophilic carrier proteins. The function of RBP is to transport vitamin A in the form of retinal from the liver to epithelial tissues.

☑ Matrix

Untimed or 24-h urine sample.

☑ Kinetics

- **Production**

  RBP is synthetized in the liver where it binds one molecule of retinol.

- **Distribution**

  RBP is secreted in plasma as a non-covalent complex 1:1 with transthyretin (formally called prealbumin). The size of this bimolecular complex is sufficient to prevent its elimination in urine. Upon recognition by a cell surface receptor in target tissues, RBP undergoes a conformational change abrogating its affinity for transthyretin. The concentrations of total RBP in serum fluctuate in a narrow range around 45 mg/l. Approximately 10-15 % of RBP in plasma is not bound to prealbumin (free RBP).

- **Excretion**

  Free RBP is rapidly eliminated from plasma by glomerular filtration and catabolized by the proximal tubule cells. Its concentration in urine is very similar to that of β_2-m averaging 60-80 µg/g cr.

- **Biological half-life**

  Free RBP in plasma has a half-life of 2-3 hours.

☑ Sampling conditions

- **Sampling conditions**

  RBP is stable in acidic urine and requires no special precautions for the collection of the urine sample. Sampling time is not critical. RBP can be determined on a 24-h

urine sample or on a spot (ideally mid-stream) urine sample. A preservative must be added to the urine (e.g. NaN_3 0.1 %).

- **Transport**

  If possible, the sample should be shipped on dry ice. However, shipment at room temperature should not significantly affect the stability of RBP provided the urine contains a preservative.

- **Storage conditions**

  RBP is stable in urine for several days at room temperature and at least for two weeks at 4 °C. At -20 °C, the protein is stable for years. In practice, if the analysis cannot be performed within 2 or 3 weeks after collection, it is preferable to store the samples frozen. Repeated freezing/thawing cycles should be avoided.

☑ Analytical aspects

- **Techniques**

  Radioimmunoassay (RIA), enzyme immunoassay (EIA), latex immunoassay (LIA).

- **Sensitivity**

  Around 1 µg/l

- **Units**

  µg/l, µg/24 hours and µg/g cr

☑ Performance characteristics

- **Analytical reproducibility**

  5-10 %

- **Inter- and intralaboratory variability**

  5-10%

☑ Validation

Published immunoassays are usually validated by comparison with alternative methods. Commercially available calibrators can be used.

☑ Confounding factors

Age, sex and diuresis are potential confounders of urinary RBP concentration. Severe liver disease can affect the plasma levels of RBP and thus also the urinary excretion of the protein.

☑ Concentrations reported in literature

- **Reference values**

  The urinary excretion of RBP by healthy subjects averages 60 to 80 µg/g cr. The reference limits for urinary RBP reported in the literature for healthy subjects are close to that of β_2-m and also vary between 200 and 300 µg/g cr.

- **Critical value**

  200-300 µg/g c

☑ Dose –response/effect relationships

See cadmium for examples.

☑ Time trend, geographical variation, susceptible groups

No data

20 FURTHER STRATEGY TO INTEGRATE BIOMONITORING IN THE RISK ASSESSMENT PARADIGM (MONTHS 10 - 18)

☑ Step 1: Collection of information on the availability and performance of biomarkers which are currently used in environmental health surveillance and research in different EU countries

The first 12 months of the workprogram have been dedicated to establish a number of reviews on the applicability of human biomonitoring (HBM) to evaluate exposure from specific pollutants with high policy and environmental relevance. The biomarkers have been selected based on the experience of the partners of the project with national and EU programs involving biomonitoring. These reviews list the performance criteria of the biomarkers and provide an overview on the state-of-the-art of the biomarkers, including sampling procedures, confounding factors, analytical procedures and baseline values.

The next steps should focus on guidance on how biomarkers can be used in integrated risk assessment in order to reduce uncertainty.

☑ Step 2: Review the collected information on each biomarker to see whether it provides information on aspects which are essential for risk assessment : hazard, dose and exposure (month 10-12).

Each biomarker review will be independently looked at by two other WP partners . The aim is to critically evaluate the information as to whether it is possible to answer following questions which are considered as being the basic input for further elaborating the use of biomarkers for risk assessment:

1. What is the <u>baseline</u> or background level against which individual biomarker levels should be compared? Are limit values (levels of health concern) or reference values (normal values occurring in the population) for biomarkers available?
2. <u>Linkage to the environment:</u> What is the relationship between levels of chemicals in the environment and human biomarker values ? Is information on uptake by various exposure routes (absorption , PBPK models) available?

3. Linkage to health: How can internal biomarker levels be used to draw conclusions about individual or population health risk? Are health endpoints associated with biomarker concentrations? If biomarker levels of health concern are known, this would allow direct interpretation of the data in terms of health risks. If dose- response relationships are known health risks can be extrapolated from biomarker levels. How solid is this information? Are threshold values known below no health concern is known or expected to exist?
4. Comparability of data at the EU level ? How should data be expressed, which confounders and co-variables should be taken into account ?
    - Geometric means, median,…
    - P_{25}, P_{50}, P_{75}, P_{90}
    - How to deal with non-detects?
5. How was the biomarker used for Health Impact Assessment
    - In the past ? reflecting time trends?
    - Geographic spread of use ?
    - Future availability and development of data ?

☑ Step 3: Relationship of biomarkers with policy areas of SP3 (month 10-12)

One of the further steps in translating the biomarkers into the risk assessment paradigm (developed by Sub Project 1 - Integrated Assessment methods) is to identify which biomarkers are relevant for the different policy areas. We are seeking input from SP3 workpackages, but meanwhile we will follow our own strategy which will be matched with input from SP3 workpackages in a later stage. Based on the available information in the biomarker reviews, the RIVM partner will identify which biomarkers are useful for application in following policy areas:

- Transport
- Housing
- Agricultural land use
- Water
- Household chemicals
- Waste
- Climate

The outcome will be some kind of an elaborate "matrix" format, which might look something like this:

	Water		
	Pollution	Treatment	…
Lead	X	O	
Pesticides	X	O	
Disinfection byproducts	O	X	
…			

☑ Step 4: Case studies from which we can learn how biomarkers and biomonitoring can be used in the risk assessment frame work (month 12-18)

The identification of appropriate indicators is something that should be received from the different work packages 3.1-3.7 of subproject 3 (Policy assessments). From figure 14 from the INTARESE project description (page 62), it is clear that SP3 needs to construct a "systems model to define the sources, agents, pathways, target population, exposures, effects of interest" (upper box).
However, since it is not expected that this contribution of SP3 will be sufficiently complete and readily deliverable, WP 2.2 will also start its own case studies to illustrate the efficiency with which HBM can be incorporated within risk assessment.

It is proposed that WP 2.2 will act beforehand and will already start developing some case studies, illustrating how HBM has contributed in:

- Identifying environmental problems
- Interaction with 'other lines of evidence'
- Illustrated (causal) links between environmental stressors and health problems
- Helping policy makers to adopt appropriate legislation, or indicate how HBM altered policies

We plan to provide at least one case study for each policy area. By providing these case studies, we can illustrate that HBM is a good alternative for the current "black box" approach, linking environmental contamination and health effects. Furthermore, these case studies hopefully will trigger increased communication with other SPs, leading to more pertinent questions and more explicit case studies. These case studies should specifically focus on how HBM contributed to the specific case, how the link was established between HBM and sources and/or effects, and how policy was altered due to these HBM data

The following are potential examples, but specific case studies should be developed either on demand from WP3, or by the different WP-Members after deliberation:

- Lead in gasoline, IQ reduction in children (Transport)
- Smoking, indoor air pollution and PAHs (Housing)
- Effects of pesticides on genotoxicity in farmers (Agricultural land use)
- …

A workshop is planned (beginning of 2007) to digest the information among workpackage members (day 1) and to present it and discuss this with WP leaders from WP2 and WP3 (day 2).

☑ Step 5: cross cutting issues (month 1-18)

Three topics have been identified as areas for progress and will be further elaborated and reviewed

- Biomarkers in Ecotoxicology: a tool for Ecosurveillance
- Biomarkers and their use for evaluating mixtures
- Non invasive biomarkers

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