Cross-Mediterranean Environment and Health Network

CROME-LIFE

ANNEX 7

Deliverable B.3.3

Findings of the environmental measurement and HBM campaigns

LIFE ENVIRONMENT PROGRAMME

LIFE12 ENV/GR/001040

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Cross-Mediterranean Environment and Health Network

CROME-LIFE

Deliverable B.3.3

Findings of the environmental measurement and HBM campaigns

TASK: 3.2
Environmental and HBM campaigns execution

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LIFE12 ENV/GR/001040

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Contact Person Editing Partner
Name: Prof. Milena Horvat
Phone: +386 15885355
Fax:+386 15885347
e-mail: milena.horvat@ijs.si

Authors Editing Partner
Name: Prof. D. Sarigiannis
Phone: +30 2310994562
Fax:
e-mail: denis@eng.auth.gr

CROME-LIFE web site: http://www.crome-life.eu

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Introduction

Deliverable B.3.3 is a document reporting environmental measurement and HBM campaigns from each demonstration site of the project. With regard to the Cross-Mediterranean study we report here the existing HBM data collected within the PHIME project, involving Slovenia, Croatia, Italy, Greece and within the INMA Project (Environment and Childhood) and data obtained from additional analysis within the CROME. The follow up of the existing cohort of children at 6-8 years of age (14 years in some cases in Spain) in the CROME Cross-Mediterranean study is still in progress, therefore the results of the follow-up are reported only partially in the present document.

In the first step, the existing environmental and HBM data were summarized and the missing gaps were identified. The available environmental pollution data were limited in all demonstration sites of the project. They were enhanced by targeted campaigns in the second step, which focused on the "hot spots" revealed from the residence of the individuals with higher biomarker values. The latter was derived after examination of the existing HBM data. Samples of environmental matrices were collected, stored and analysed with the appropriate analytical techniques, including primarily AAS and ICP-MS (for metals) and GC-MS, HPLC-MS/UPLC-MS (for organic contaminants such as organochlorine compounds and PBDEs). The environmental matrices comprised top soil, surface and groundwater, and ambient air. Some most consumed food items at local areas were also included in the analysis. These analyses will support the derivation of overall exposure patterns of the local population when analysed in combination with the existing environmental and dietary data found in the databases set. In addition, questionnaire-based surveys of behavioural and dietary habits and time-activity diaries of the local population (the individuals for who HBM data exist in the demonstration sites) will be used to derive complete personal exposure profiles for the study participants. Final goal is to assess personal exposure profile as complete as possible.

The obtained environmental monitoring data will be coupled with HBM and epidemiological observations using physiologically-based toxicokinetic (PBTK) and toxicodynamic (PBTD) models. These would allow us to mechanistically associate the observed concentrations of contaminants in environmental media with HBM data already existing within the consortium and collected through targeted field campaigns. Via reverse modelling human exposure to the chemical substances will be reconstructed. These estimates will be used as indices of population exposure and of the environmental health burden due to the anthropogenic pollution in the project demonstration sites. The CROME-LIFE approach will show the feasibility of environment-wide association studies by inter-operably linking environmental, biomonitoring and health status data. Causal associations between the observed health

outcomes and the measured/estimated markers of exposure will be derived by means of advanced statistical models and causal diagrams.

Cross-Mediterranean study

Introduction

Children from the pre-existing Mediterranean cohorts established (1) within the PHIME project, involving Slovenia, Croatia, Italy, Greece and (2) within the INMA Project (Environment and Childhood) are now 6-8 years of age (14 years in some cases in Spain) and are in the progress of resampling and neuro-behavioural testing in the CROME Cross-Mediterranean study.

PHIME was the largest study ever conducted in the general European population on the impact of mercury (Hg) and other trace metals through food consumption. Mother-child pairs were recruited during the pregnancy (Italy, Croatia) or at birth (Slovenia, Greece). Mother's blood and urine have been sampled in the third trimester of pregnancy (Italy, Croatia); mother's hair, cord blood, cord tissue and meconium have been sampled at birth, breast milk and mother's hair 1 month after birth. Hair samples have been analysed for Hg, cord blood and breast milk for Hg, cadmium (Cd), lead (Pb) and arsenic (As), as well as for essential elements (selenium-Se, zinc-Zn, copper-Cu). Children of the PHIME Mediterranean cohort were tested for neurodevelopment (Bayley scales of Infant and Toddler development, Third edition; *Bayley III*) at 18 months of age.

Summary of existing environmental and biomonitoring data – gaps identification

The results of the PHIME Mediterranean cohort have been evaluated within the PHIME project, particularly in relation to methyl Hg exposure. The level of exposure to Hg as indicated by hair and cord blood Hg concentrations is presented in the Figure 1 and are reported by Miklavčič Višnjevec et al (2013).

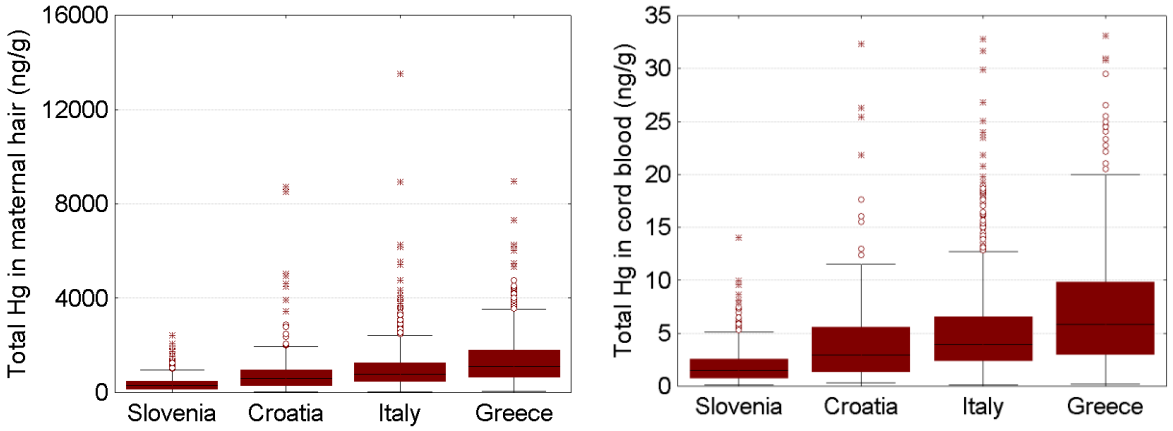


Figure 1: Mercury levels in maternal hair (left) and cord blood (right) by country.

The analysis of results took into account data available from the questionnaires completed by mothers during the recruitment process and later on. The data includes the following parameters:

Environmental data:

- residential area: vicinity of industry, highways and different roads, railway station, airport;
- potential occupational exposure to lead, mercury, gold and other metals, chromium, pigments, PCBs, solvents, radioactive isotopes, amalgams
- general data on occupation, with particular emphasis on: dentistry, jewellery, working with photography, chlor-alkali industry, paints, production of electric appliances, paper industry, pharmaceutical industry, printing industry, traffic, agriculture

Other data available from the questionnaires:

- Smoking behaviour throughout the pregnancy,
- dental visits and new/replaced dental fillings throughout the pregnancy,
- detailed nutritional data: consumption frequency of different types of food including different fish species and origin of a specific food item (locally grown/caught or bought in the supermarket/local market).
- pregnancy history: mother's age at delivery, BMI before pregnancy, weight gain increase, health status and health records, type of delivery and delivery implications if any;
- data on child: sex, birth weight and length, weight and length at 18 months, breastfeeding history (any vs. none), duration and exclusiveness up to 4 months, daycare attendance at 18 months, duration of the intake of fresh and homogenized fish up to 18 months; health implications;
- socio-economic status data (home area <50, 50-100, >100 m^2); home ownership; parental education (the higher of the two); number of children in the family; marital status of the mother at delivery

As presented in Figure 1, the internal exposure was the lowest in Slovenian mother-child pairs (Med 297 ng/g hair and 1.5 ng/g cord blood) and the highest in Greek (Med 1120 ng/g hair and 5.8 ng/g cord blood), and the overall Hg levels were associated significantly with the frequency of fish consumption, while with amalgam fillings only in the Slovenian population. The low level in the Slovenian population comply well with the levels observed in fish from the Slovenian marker reported by Miklavčič et al (2011).

Based on the multiple regression modelling adjusting for all potential confounders and related covariates, Hg in mother´s hair and in cord blood was observed not to predict Bayley scores but a moderate beneficial effect of fish consumption in pregnancy was observed (Barbone et

al, *in preparation*). Other chemical elements were not associated with the outcomes (PHIME 2006).

It is well known that several factors can modulate the neuropsychological outcome. Among the main gaps in knowledge in this field are i) the additive, synergic or competitive effects of different chemicals, which the fetus/newborn/child can be exposed to during different windows of vulnerability; ii) the role of genetic/epigenetic factors that may predispose sunset of individuals to more adverse health effects than those reported in the general population.

As epidemiological studies have demonstrated that the developmental neurotoxicity is associated with prenatal methyl Hg exposure (Grandjean and Landrigan 2006) and that susceptibility to methyl Hg toxicity may be, among other factors, modified by genetics (Grandjean and Julvez 2013), the follow-up study within the CROME aimed at finding those genetic polymorphisms which could modulate the effects of metals, particularly Hg, at such exposure level as was observed in the Mediterranean cohort. In addition, co-exposure to different metals or to metals and organic pollutants is rarely considered in developmental neurotoxicity studies.

In this regard, we focused on gene polymorphisms of glutathione-related genes (GSTM1, GSTT1, GSTP1, GSTM3, GPX1), metal binding protein genes (MT2A, MT1X, SEPP1, APOE), genes involved in scavenging of ROS (CAT, SOD1, SOD2, GSR), and genes implicated in brain development (APOE, PON1, BDNF, PGR) as previous literature has shown that variants in these genes might modulate Hg/methyl Hg developmental neurotoxicity. As possible confounders, other potentially neurotoxic (Pb, Mn) and beneficial elements (Se) were considered in the analysis within the CROME.

Additional data collected

Environmental data

In connection to the questionnaire data on consumption of different fish species, fish species that were commonly bought and consumed by the study subjects have been sampled previously in the Slovenian and Italian market. The samples have been analysed for total and methyl Hg as well as other trace elements to link with the exposure as assessed through measurements in blood of study subjects. For the Greek population, selection of most frequently consumed fish species is still missing and the sampling of identified common species followed by trace metal analyses is to be done by the end of CROME. In Spain, previous human biomonitoring studies and questionnaire data were indicating that fish was also the main way of incorporation of mercury into the population, particularly children. However, no comprehensive data on direct measurements of fish species for human consumption was performed. Accordingly, fish of the most commonly eaten species have

been sampled in 2013, 2014 and 2015 and analysed as described in campaign 7th case study of the Spanish contribution.

Biomonitoring data – Slovenia and Croatia

Sampling and analysis protocol

In the first stage, the DNA was extracted from the existing samples of children and their mothers from the Slovenian and Croatian population of the PHIME cohort. Children's DNA was isolated from cord tissue using a QIAamp DNA Mini Kit, QIAGEN, ZDA, following the instructions for DNA purification from tissues provided in the QIAamp DNA Mini and Blood Mini Handbook 04/2010. Maternal DNA was isolated from leukocytes in peripheral blood using the High Pure PCR Template Preparation Kit (Roche), following the manufacturer's instructions. Samples were diluted (1:20) with ultrapure water (UltraPure DNase/RNase-Free Distilled Water, Sigma-Aldrich). The isolated DNA was then genotyped for apolipoprotein E (*Apoe*), genotyping was performed using TaqMan® pre-designed SNP genotyping assay small scale with C_3084793_20 for rs429358 and C_904973_10 for rs7412 (Applied biosystems, Foster City, Ca, ZDA). Apolipoprotein E genotyping was carried out using the Roche LightCycler® 480 II (Trdin, 2015).

Within the statistical analysis, multiple linear regression models were used to evaluate the association between the Hg levels and outcome (Bayley score), taking into account the genotype and other possible confounders, including questionnaire data and the levels of Pb and Se in cord blood.

In the second stage of acquiring additional data, the children are being tested at 6-8 years for neuropsychological performance using Wechsler Intelligence Scale for Children (WISC-IV). Examination includes collection of urine, blood and hair samples for analyses of metals and collection of saliva (Oragene DNA self-collection kit) for genotyping. Mothers need to complete a new questionnaire to update information on their and child's life-style and living environment.

The collected samples of blood and urine will be analysed for Hg, Pb, Cd, Mn, As, Se, Cu, Zn and hair for Hg. From saliva samples, child's as well as maternal DNA will be isolated for the genotyping purposes. As a susceptibility markers, we plan to investigate polymorphism in genes for metallothioneins (MT2A, MT1X) and selenoproteins (SEPP1- 2 SNPs) that are important in the metabolism of metals and genes involved in brain development (PON1, BDNF, PGR). Metallothioneins (MT2A, MT1X) and selenoproteins (SEPP1) are already being genotyped on the existing samples. Other polymorphisms that will be included in further analysis of new samples will be the following: MT1A, MT1F, MT3, MT4, MTF1, PON1, AS3MT, ALAD, VDR, TXNRD1, TXNRD2.

Results

Statistical analysis was performed on 361 children (237 of them were from Slovenia and 124 from Croatia) who had the complete data: total Hg in cord blood/hair, Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III) assessment at 18 months of age and *Apoe* genotyping. The results indicated that even low-to-median Hg exposure in children with normal neurodevelopmental outcome can result in lower cognitive and fine motor scores at 18 months of age as assessed by Bayley-III. The Hg-related decrease in cognitive sores was observed in children carrying at least one *Apoe* ε4 allele, while the decrease in fine motor scores was independent of the *Apoe* genotype. Adjusting for selenium (Se) and lead (Pb) levels, a positive influence of Se on the language domain and a negative influence of Pb on motor domain was observed, but not in the subgroup of children carrying the ε4 allele.

Biomonitoring data – Italy

Within the PHIME project, the University of Udine and the Institute for Child Health Burlo Garofolo of Trieste established in 2007 a prospective mother-child cohort in a coastal area of

Sampling and analysis protocol

The new study protocol focused on the follow up at the age of 7 years of about 200 children born within the NAC II PHIME cohort. Child's hair, child's urine and saliva sample of both mother and child (Oragene DNA self-collection kit) have been collected at the time of appointment. Mothers have completed a new questionnaire to update information on life-style factors and subjected to PSI (Parenting stress index) interview, and children (now at the age of 7) were subjected to neuropsychological testing by trained neuropsychologists. The neuropsychological tests administered included WISC-IV (Wechsler Intelligence Scale for Children), Nepsy-II for assessment of sensorimotor, attention, learning capabilities, MT for assessment of reading skills, BVSCO2 for complete assessment of writing skills, CBCL - Child Behaviour Check List to identify behavioural and emotional problems in children.

Statistics

Data were analysed by Pearson correlations between pairs of exposure and outcome variables and multiple regression analyses by using the SPSS software.

Chemical analyses

Chemical analyses of biological samples collected following neuropsychological assessment consisted in measurements of the concentration of five metals reported as developmental neurotoxicants (mercury, lead, manganese, cadmium, arsenic) in either hair or urine.

Hair: Manganese (Mn) and Mercury (Hg) were analysed on 200 samples of human hair, collected in plastic bags and stored in a dryer until analysis. Before the analysis hair samples,

put in glass bakers conveniently decontamined in HNO3, were treated as follows: (i) three cycles of washes under continuous stirring for 30 min each in a mixture of 3:1 (v/v) ethyl ether-acetone (Sigma-Aldrich, St.Louis, MS, USA) to remove the sebaceous film from the hair, after each cycle hair samples were washed by high purity deionised water (EASY-pure UV, PBI, Milan, Italy) under stirring; (ii) a wash under stirring for 1 hr in 5% sodium ethylenediamine tetracetic acid (EDTA) (Sigma-Aldrich, USA) to bind the chemical elements present on hair surface and, (iii), repeated rinsing by high purity deionised water (EASY-pure UV, PBI, Milan, Italy). After drying in stove for 16h at 85 °C, approximately 0.25 g of hair from each sample was then weighed. A mineralization cycle in ModBlock plate (ModBlock CPI International, Santa Rosa, CA, USA) with 4 ml of HNO3 at 60-70 °C and 1 ml of H2O2 at about half an hour from the end was added. The Hg quantification was performed using both iCAP Qs ICP-MS (Thermo-Fisher, Bremen, Germany). The instrument configuration and operation parameters are shown in the Table 1. The iCAP Qs ICP-MS used in this study was equipped with a PFA ST MicroFlow nebulizer (ESI, Omaha, NB, USA), a Peltier cooled quartz spray chamber (operating at 3 °C), a 2.0 mm ID sapphire injector and a demountable quartz torch with interface Ni sampler and skimmer, and extraction lens system plasma.

Table 1: Instrument configuration

RF power	1550 w
Argon gas flow (L/min)	Plasma 14, Auxiliary 0.8, Nebulizer 1.08
Collision cell gas	He at 4.2 mL/min
KED barrier	2 V
Analytical parameters	Dwell time 80 ms per peak, 50 sweeps, 3 replicates
Analytical masses	55Mn, 202Hg
Internal standard	115In

Urine: Urine samples (ca.100mL) were collected in polyethylene containers and stored at-20 °C until analysis. The containers were previously decontaminated with diluted (10%v/v) ultrapure HNO3 (Normatom, Leuven, Belgium) and rinsed with high-purity deionized water (Barnstead EASY-Pure II, Dubuque, IA, USA). Urine were analysed by the method validated and accreditated by the Italian accreditation body (ACCREDIA). Urine samples were diluted 1:5 (v/v) with high-purity deionized water before metal quantification. Metals were measured by sector field Inductively coupled plasma mass spectrometry (SF-ICP-MS, Thermo Fischer, Bremen, Germany) working at low resolution mode (LR, m/Δm = 300) for not interfered metals: 114Cd, 202Hg, 208Pb; at medium resolution mode (MR, m/Δm = 4000) for interfered metal: 55Mn; at high resolution mode (HR, m/Δm = 10,000) for the interfered

metals: 75As and 82 Se. The instrument was equipped with sampler and skimmer cones in Ni, a Meinhard nebulizer, a water cooled spray chamber (Scott-type) and a guard electrode device. The matrix-matched calibration combined with the internal standardization by 115In and 69Ga (1 ng/mL in the analytical solutions) provides a satisfactory method for compensating for any residual matrix effects and to minimize instrumental drifts. To test the accuracy of the analytical procedure two certified reference materials of lyophilized human urine were used: Seronorm (Sero AS, Billingstadt, Norway) and ClinCheck (Recipe, Munich, Germany).

Results

Data concerning concentration of the metals in study in both urine and hair of the children are reported in Table 2. As for Hg and Pb values are within the reference values established by the PROBE survey for adolescents aged 13-15 in blood (see below).

More in detail, when considering the chemical exposure measured at the different ages, we found a significant correlation between Hg in cord blood and Hg in mother's hair during pregnancy; both are correlated to Hg in hair at 7 years that in turn is correlated with Hg in urine at the same age. On the whole, these results suggest that the maternal life style is among the factors influencing Hg levels in 7 year-old children. This is also supported by the significant correlation found between fish consumption of the mother during pregnancy and breastfeeding and total Hg levels in urine and hair at 7 years ($r=0.26$; $p < 0.001$).

The link between consumption of all kinds of fish by the child and levels of metals in urine/hair is supported by the finding that Hg levels (both urine and hair) are related to consumption of canned fish (mainly tuna fish). Mn levels in urine seem to be related to fresh fish and fried crustacean consumption ($r=0.23$, $p=0.002$; $r=0.33$, $p=0.001$); finally Pb levels are related to consumption of fried fish, crustacean or molluscan.

When considering the correlation between levels of metals in urine and hair at 7 years and the different neuropsychological scores obtained in the selected test batteries, no major effects of total Hg (or other metals) levels in urine on the different behavioural functions scored (scale MT, CBCL, NEPSY-II, WISC) was found. At variance, Hg in hair is positively related with cognitive scores in WISC-III (the higher the Hg levels the better the performance) and with CBCL scores, with children with higher Hg levels showing the worse behaviour (more anxiety and retreat).

Mn as measured in child's hair produced adverse effects ($r=0.16$, $p <0.04$) on the five WISC-III score, with statistically significant decrement of the general IQ and of verbal comprehension.

As expected we confirmed a positive association between maternal IQ and IQ of the child assessed in the WISC-III test, as well as a positive correlation between IQ assessed at 18 and 40 months and that assessed at 7 years, and a positive effect of higher socioeconomic status on the child's IQ.

Altogether it is worth mentioning that, though Hg levels did not adversely impact on neuropsychological performances at either 18 months, 40 months or 7 years, when we calculated the variation in cognitive scores assessed in the Bayley scales from 18 to 40 months of age, children with the higher level of exposure to Hg showed a significantly lower increase in performance with age. Such trend is evident also in the language and motor components of the Bayley scales. A more thorough analysis of behavioural data, including integrated comparison of similar neuropsychological domains is warranted to confirm this trend on a longer time span.

These analyses confirm the results of other large epidemiological studies on the neurobehavioral toxicity of prenatal/neonatal mercury exposure at low doses. In absence of significant impairments of behavioural functions at different ages, the results highlight the complex interaction between environmental exposures, life style, and time of outcome assessment and support the usefulness of the integrated CROME-LIFE approach we applied under Action B5 in health impact assessment.

No updated indications concerning the levels of metals in food items in the areas where the population under study lives are available. A report on the national levels of some metals in fish and seafood product in Italy has been published by Pastorelli et al (2012).

Table 2:NAC-II cohort follow-up at 7 years (n=200): mean concentration of the five neurotoxic metals in urine (A) and hair (B).

(A)	As	Cd	Hg	Mn	Pb	Se
mean	35,8	0,47	0,48	0,20	0,717	38,9
SD	97,8	0,38	0,29	0,30	0,448	20,9
rSD%	274	79,6	60,4	152	62,6	53,8
min	1,20	0,07	0,23	0,06	0,145	6,53
max	920	3,10	1,89	3,45	3,230	129

(B)	**Hg**	**Mn**
mean	0,76	0,21
SD	0,66	0,17
rSD%	86,9	80,4

min	0,06	0,04
max	4,09	1,09

Conclusions

The study indicates that even low-to-moderate Hg exposure in children with normal neurodevelopmental outcome can be associated with lower cognitive and fine motor scores at 18 months of age. Despite the relatively low number of subjects carrying the Apoe ε4 allele, the study provided firm evidence of Hg-associated decrease in cognitive performance among ε4 carriers, while the decrease in fine motor scores was independent of the genotype. Gene-environment interaction was indicated for the cognitive score. We demonstrated that accounting for beneficial factors like Se and other potentially neurotoxic substances like Pb is crucial in assessing such associations. Re-evaluation of the obtained results is needed with higher predictive values of neuropsychological test used at later age and with consideration of other polymorphisms identified that can influence neurodevelopmental performance. In addition, the results do not make clear what is the contribution of inorganic Hg exposure to the overall negative association between Hg and neurodevelopment. Speciation analysis in biological samples would help reveal this and is planned for the follow-up.

Re-analysis of up of a sample of 200 children aged 7 years and originally enrolled in the Italian Northern Adriatic Birth Cohort II (NAC II) revealed significant association between metals/metalloids' exposure and neuropsychological scores at school age in childrenAt 7 years if age, some children presented very high levels of As. Hg levels in the hair were significantly related to higher scores in CBCL, indicating that both externalizing (anxiety) and internalizing (depression) behaviours were increased in children. Manganese levels (related to fish consumption as well) in the hair of the children were significantly associated to lower levels of the general IQ and verbal comprehension with the higher levels of Hg.

Moreover, life style factors exerted a significant effect on neuropsychological scores, which are positively influenced by 1) fish consumption, 2) maternal IQ and educational level of the parents.

A significant role in neuropsychological functioning is also attributable to perinatal exposure. The reanalysis of the data on metals' concentrations in the different matrices highlights the importance of all the exposures occurred during postconceptional development, and suggest that together with neurotoxic metals, essential elements such as Zn, Cu and Se concur to modulate neurobehavioural development.

Spain

The INMA study

The INMA – INfancia y Medio Ambiente [Environment and Childhood] Project is a network of research groups in Spain that established a project aiming to study the role of the most important environmental pollutants in air, water, and diet during pregnancy and early childhood in regards to child growth and development. The Project was based on experience acquired by 3 birth cohorts: the cohort of Ribera d'Ebre (n=102), which assessed neurological development in an area with high levels of OCs and mercury originating from the emission of a chlor-alkali plant; the cohort of Menorca (n=482), which studied the relationship of early life exposure to airborne irritants and allergens with allergy and asthma; and the cohort of Granada (n=668), which studied the incidence of infant reproductive health disorders at birth in relation to environmental endocrine disruptors. Based on knowledge from these cohorts, a common protocol was developed by setting up 4 new birth cohorts: Valencia (n=855), Sabadell (n=657), Asturias (n=494), and Gipuzkoa (n=638) cohorts, and unifying all 7 cohorts.

The INMA Project is a prospective population-based cohort study concerned with the effects of pre- and postnatal environmental exposures on growth, health, and development from early foetal life until adolescence.

The general aims of the project are:

1. To describe the degree of individual prenatal exposure to environmental pollutants and the internal dose of these chemicals during pregnancy, at birth, and during childhood in Spain;
2. To evaluate the impact of the exposures to different contaminants on foetal and infant growth, health, and development;
3. To evaluate the interaction between pollutants, nutrients, and genetic variants on foetal and infant growth, health, and development.

The studied population involved pregnant women of general population who live in each study area and their child. The inclusion criteria of the mothers were: (a) to belong to the study area (specific in each cohort), (b) to be at least 16 years old, (c) to have a singleton pregnancy, (d) to not have followed any programme of assisted reproduction, (e) to wish to deliver in the reference hospital, and (f) to have no communication problems.

Each cohort had different period of recruitment and there were some differences on it. In Ribera d'Ebre and Granada cohorts, mothers were recruited during hospital admission for delivery. The recruitment took place between March 1997 and December 1999 in Ribera d'Ebre cohort and between October 2000 and July 2002 in Granada cohort. In Menorca cohort, all eligible pregnant women presenting for prenatal care at all general practices of the island (in public or private health centres) were invited to participate to the study over 12 months starting in mid-1997. In the 4 new cohorts, the recruitment took place during the first

prenatal visit (10-13 weeks of gestation) in the main public hospital or health centre of each study area. The recruitment period in Valencia was from November 2003 to June 2005. In Sabadell it took place from July 2004 to July 2006. Recruitment in Asturias was carried out from May 2004 to July 2007, and in Gipuzkoa from April 2006 to January 2008. A common characteristic of these areas is that the vast majority of the population attends the public health sector. From 45% to 98% of eligible pregnant women agreed to participate (96% in Ribera d'Ebre, 98% in Menorca, 54% in Valencia, 60% in Sabadell, 45% in Asturias, and 68% in Gipuzkoa; information not available for Granada). In Sabadell, the educational achievement of women who refused was lower than that of participants although no differences in age. In Valencia, older women and a high proportion of working women were included. No differences in age between participants and non-participants in Asturias, whereas a high proportion of working women were included in Gipuzkoa.

The CROME-LIFE study focussed on the cohorts closer to the Mediterranean: Menorca, Valencia, Ribera d'Ebre and Sabadell.

Introduction

The study has been centered in assessing the impact of metals and organochlorine compounds in the above mentioned cohorts. Therefore, biomonitoring considered the body burden of these compounds assessed from the study of their concentrations in blood serum and urine. Ribera d'Ebre, Menorca, Valencia and Sabadell were chosen as test cases for evaluation of this exposure. Specifically, metals were assessed in the cohorts of Sabadell and Menorca and the organochlorine compounds in the cohorts of Ribera d'Ebre, Menorca and Valencia.

Environmental assessment was concerned with the exposure to pollutants by inhalation (atmosphere) and ingestion (diet). The former were studied in the cohort of Ribera d'Ebre and the second in the cohort of Menorca. This second study was extended to the whole Balearic Islands.

Summary of existing environmental and biomonitoring data – gaps identification

Assessment of exposure to metals.

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

compounds by maternal transmission. Thus, analysis of metals in urine collected at different pregnancy periods may provide useful information for assessment of the efficacy of the use of urine samples as exposure markers.

Therefore, we aimed: i) to develop an analytical method using acid digestion prior to analysis by inductively coupled plasma quadruple mass spectrometry (Q-ICP-MS) for the simultaneous analysis of 12 metals in maternal urine and ii) to determine the concentrations of 12 metals, namely cobalt (Co), nickel (Ni), Cu, Zn, selenium (Se), As, molybdenum (Mo), Cd, antimonium (Sb), cesium (Cs), Th and Pb, in urine samples of pregnant women living in a highgly industrialized urban town (Sabadell), which were collected during their first and third trimester of pregnancy. This approach afforded an assessment of the steadiness of the concentrations of these metals in urine during the pregnancy period and their usefulness for epidemiological studies, maternal and prenatal exposure estimates.

The urine samples from 489 pregnant women of the Sabadell cohort were collected in 100 mL polypropylene containers in the first and third trimester of pregnancy. The samples were stored in polyethylene tubes at -20ºC until further processing. Prior to Q-ICP-MS analysis, the samples were digested and diluted to oxidize and remove organic matter and to minimize the concentrations of inorganic solids (Castillo et al. 2008; Krachler 1996). Q-ICP-MS analysis was performed by an X-SERIES II (Thermo Fisher Scientific) instrument. Specific isotope ions for Co and Ni were selected in order to avoid potential calcium interferences from the sample matrix. Cl atoms may also potentially interfere in the determination of As and Se. In these cases the collision/reaction cell technique should be added to the instrumental methods but no interferences were observed in the present samples and these cells were not used. Instrumental limit of detection (LOD) for all metals was 0.2 ng/mL attending to the most reliable lowest calibration point. The two samples corresponding to the first and third trimesters of each subject were digested and analyzed at the same time. One MilliQ water blank was processed in each batch of samples to control for possible contamination. If there was any contamination, thorough cleaning of all material was performed and digestion was repeated. Field samples were also obtained by analysis of Milli Q water which was previously stored in the containers used for maternal urine bottles and transported together with the samples.

The concentrations of trace metals in urine collected in the first and third trimesters of pregnancy were significantly correlated in all metals studied, with high statistical significance in most cases. These significant correlations likely reflect an absence of major changes in metal exposure during pregnancy and the differences between both trimesters observed for most metals may reflect metabolic changes during this period. Accordingly, the findings from this studied cohort indicate that the measurements of these trace metals at any stage of pregnancy provide a representative estimate of the exposure to these compounds during the

whole period. Sampling in specific stages may be chosen according to logistics or specific study purposes and the observed concentrations must always be interpreted in reference to this selected phase.

Assessment of mercury exposure.

The body burden of mercury in four year-old children was estimated in the Menorca cohort by collection and analysis of hair samples. Significant associations between the concentrations of this metal and socio-demographic factors such as sex, residence site and other maternal and paternal characteristics have been investigated. The influence of fish and seafood consumption in the observed mercury concentrations was also assessed. The study also encompassed an assessment of the effects of this contamination in neuro-development. The McCarthy Scales on Children's Abilities (MSCA) was used for this purpose. 302 children provided complete outcome data at 4 years and hair for mercury measurements. More female children were included due to the easier availability of hair samples. The subset with both THg measurements and MSCA neuropsychological test included 298 children (99%) and no differences between the subsets were found.

Assessment of organochlorine body burden

A total of 102 singleton children born in the main hospital of the Ribera d'Ebre between 1997 and 1999 were included in the Ribera d'Ebre birth cohort study. Organochlorine compounds were measured at three different follow-ups: at birth (cord blood, N=73) and at the age of 4 years (serum, N=58, years 2001-2003).

Between 1997 and 1998 the Menorca birth cohort study recruited all women presenting for antenatal care. In total, 482 children were enrolled and OCs were measured at three different follow-ups: at birth (cord blood, N=405, years 1997-1998) and at the age of 4 years of children (serum, N=285, years 2001-2002).

Additional data collected

Environmental data

Fish

The concentrations of mercury in 515 fish specimens were determined.0.5 g of fish muscle or other food items were treated with HNO3 (Baker Instra) and H2O2 (Merck Suprapur) in a Teflon vessel (90ºC overnight). The digested sample was diluted with deionized water (Purelab Ultra). One procedural blank was included in each sample batch for possible contamination control. THg determination was performed using inductively coupled plasma mass spectrometry (Agilent 7500 CE) operating under standard conditions and using rhodium as internal standard. All samples were above detection limit.

Diet

Food samples were acquired in local markets and department stores from the main two cities: Maó and Ciutadella (December 2014, n = 46). The selection was based on Food Frequency Questionnaires (FFQs) from 4 year old children from Menorca cohort (see next subsection below) and interviews with local experts. They included fish species of several trophic levels and fat contents and shellfish, all them captured in the island surroundings. The samples also included meat, e.g. beef, chicken and lamb, fruits and vegetables, cheese and chicken eggs, all them produced locally. After collection, each food item was dissected in two composite samples for organochlorine compound and Hg analyses. The former were wrapped in aluminium foil and the second were sealed in plastic bags. The samples were then frozen at -23ºC until further analysis in the laboratory. Only the edible parts of each food item were analysed.

Air

Air sampling for the control of organochlorine solvents and synthetic by-products in the City of Flix and surroundings was implemented in order to identify possible risk situations generated by the inhabitants of Flix (n = 100). Initially, sampling was performed by staff from IDAEA-CSIC and later by personnel of the City Hall of Flix.

The decision criterion for sample collection was based on the detection of odors in the village. Therefore, the samples taken likely correspond to maximum levels of contamination impact, not average levels.

Estimate of the risks associated to these concentrations observed in episodes of strong odors were performed. These estimates should be interpreted as a limit and not as representing the real situation, which probably corresponds to lower values.

Biomonitoring data

Additionally, in the cohort of Menorca, schoolmates of children of the original cohort were invited to participate in the studyat the age of 14 years (serum, N=36, year 2012). In the cohort of Ribera d'Ebre children were invited to participate again at the age of 14 years. The participation was N=43.

Conclusions

The results suggest that environmental contaminants that are common in soil, water, and food are present in tissues of mothers and children. Several compounds have been found in placentas, cord blood, mother's and child's blood, mother's and child's urine, child's hair, and mother's milk. Their ubiquity supports the plausibility of embryo-foetal exposure during pregnancy. Moreover, concentrations of environmental chemical pollutants were also measured in tap water, swimming pool water, indoor and outdoor air, and personal samples.

The concentrations of all metals in the first and third trimesters were significantly correlated which reflect the absence of major changes of metal inputs in the studied women during pregnancy. The significant concentration differences between these two sampling periods may respond to metabolic pregnancy changes. Accordingly, the measurements of the studied trace metals in urine provide representative estimates of exposure during the whole pregnancy period. Prenatal exposure of several of these environmental contaminants has been related with an alteration of maternal and child thyroid hormones levels, an increased risk for congenital anomalies, an impairment of the foetal and child growth, a delay in child cognitive and behavioural development and an increased risk of child respiratory symptoms and atopy. However, long-term breastfeeding and some maternal diet factors during pregnancy, such as fish consumption or high adherence to Mediterranean diet score may counteract some of these pollutants' negative effects on child health. Moreover, genetic differences have shown to lead to different effects of environmental factors on child health outcomes.

National targeted environmental problems – Greece

1st case study – Asopos Basin

Introduction

A major local environmental issue in Greece is related to the presence of hexavalent chromium Cr(VI) in drinking water of the Oinofyta municipality, within the wider area of Asopos basin and the related cancer mortality. The Oinofyta municipality (***Figure 2***) is situated 50 km North of Athens, Greece, and it includes four villages that were initially rural but transformed into industrial areas in the early 1970s. In 1969, a ministerial decision gave permission for depositing processed industrial waste in the Asopos river, which runs through Oinofyta. This decision, furthered by a presidential decree in 1979, permitted free disposal of processed liquid industrial waste into the river. According to the Technical Chamber of Greece (TCOG 2009), in the 80s there were about 700 industries operating in the Oinofyta area, of which 500 generated liquid industrial waste. After protests from citizens who complained about the discoloration and turbidity of their drinking water in 2007 the Ministry of Environment, Regional Planning and Public Works of Greece imposed fines on 20 industries for disposing industrial waste with high levels of hexavalent chromium into the Asopos river. Official limits on total chromium have been set by both the United States Environmental Protection Agency (US-EPA), equal to 100 μg/l, and the European Union (Council directive 98/83/EC), equal to 50 μg/l. However, as of yet, there are no limits set by any international body for Cr(VI). In 2009, the California Environmental Protection Agency proposed a public health goal level of 0.06 μg/l for Cr(VI) in drinking water (OEHHA 2009).

Figure 2: Asopos Basin and Oinofyta municipality

Because areas characterized by high Cr(VI) concentrations in drinking water are relatively uncommon, human epidemiologic studies are scant; the study carried out by Zhang and Li (1987) is one of the most cited and controversial studies analyzing the effects of oral exposure to Cr(VI) on population cancer mortality rates conducted near a chromium smelting plant in the Liaoning Province, China.

In order to examine the potential effects of elevated oral exposure to hexavalent chromium, an ecological mortality study (Linos et al., 2011) was performed in an industrial area of Greece where the water consumed by the population was contaminated with hexavalent chromium (maximum levels ranging between 41 and 156 μg/l in 2007-2009, and presumed exposure for at least 20 years). The goal of the study was to examine the cancer mortality in an area of Greece, historically satisfying its potable needs with a Cr(VI)-contaminated aquifer. According to the study, a total of 474 deaths were observed, of which 118 were cancer related. These figures (i.e. one in four deaths being cancer related) are in accordance to the general Greek, EU15 and EU27 averages. The all cause standardized mortality ratio (SMR) for the Oinofyta municipality was similar to that of the prefecture of Voiotia (SMR = 98, 95% CI 89-107). The SMR for all cancer deaths over all the years was slightly increased but not statistically significantly (SMR = 114, 95% CI 94-136). There were eight observed deaths of the hepatobiliary system, and more specifically: six primary liver cancers, one bile duct, and one gallbladder. For primary liver cancer, the observed deaths were eleven fold higher than the expected number of deaths (SMR 1104, 95% CI 405-2403, $p < 0.001$); statistically significant SMRs for primary liver cancer were observed among both males and females. Observed deaths associated with kidney and other genitourinary organ cancers (five deaths with ICD-9 code 189, and one death with ICD-9 code 184) were more than threefold higher than expected in women (SMR 368, 95% CI 119-858, $p = 0.025$). The SMR for lung cancer was also statistically significantly elevated (SMR 145, 95% CI 101-203, $p = 0.047$). Furthermore, elevated SMRs were noted for several other cancer sites, including cancers of lip, oral cavity and pharynx, stomach, female colon, female breast, prostate, and leukaemia, but did not reach statistical significance. Tests for linear trend performed after grouping the period specific SMRs into 3 time intervals, i.e. 1999-2002, 2003-2006, 2007-2009, did not reveal any significant evidence of a linear trend. However, there was a statistically significant SMR of 193 (95% CI 114-304, $p = 0.015$) for all cancer deaths that was found for the year 2009.

Summary of existing environmental and biomonitoring data – gaps identification

Existing environmental data

To the best of our knowledge, there are no systematic measurements of Cr(VI) before 2007. Since 2007, three independent sets of hexavalent chromium measurements are available for the Oinofyta area. These include:

- a study of the Institute of Geology and Mineral Exploration (IGME 2008) during the period November 2007 to February 2008, which detected 35 samples (out of 87) taken from different wells in the same area, where levels above 10 μg/l with a maximum value 156 μg/l were detected;
- a study conducted by the faculty of the Geology and Geo-environment department of the University of Athens (Vasilatos et al. 2008) during the period September 2008 to December 2008, in which Cr(VI) levels ranged from 41 up to 53 μg/l in three samples taken from the public drinking water supply of Oinofyta; and
- repeated measurements by the Oinofyta municipality in the public drinking water supply during the period July 2007 to July 2010, in which there are 13 measurements with levels above 10 μg/l and with a maximum value of 51 μg/l

According to official Oinofyta municipality authorities, in early 2009 the main drinking water supply of Oinofyta was diverted to receive water from Mornos lake (reservoir) which is part of the drinking water supply network of the city of Athens. Therefore, more recent measurements made by the Oinofyta municipality (June 2009- July 2010) record relatively lower levels of Cr(VI) (<0.01-1.53 μg/l).

Furthermore, in the study carried out by Economou-Eliopoulos et al. (2011), groundwater samples from the Asopos aquifer showed a wide spatial variability, ranging from <2 to 180 ppb Cr total content [almost same to the Cr(VI)-values] despite their spatial association. The presence of Cr(VI)-contaminated ground water at depths >200m is attributed to a direct injection of Cr(VI)-rich industrial wastes at depth rather than that Cr(VI) is derived from the Asopos river or by the interaction between water and Cr-bearing rocks.

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

Additional data collected

Biomonitoring data

Sampling and analysis protocol

Study design

50 individuals age stratified (4 to 65 years old) where recruited so as to evaluate current as well as past and cumulative exposures to Cr. For this purpose, both urine and hair samples were taken. Population covered the wider basin of Asopos area, reflecting the variability of exposures related to the different levels of Cr sampled in the environmental campains mentioned above. Hair samples were collected from the occipital region of the head. The samples of hair were obtained using stainless steel scissors from the nape of the neck. The hair samples were cut into approximately 0.5-cm pieces in length and mixed to make a representative hair sample.

Analysis

The analytical method was based on the described by Afridi et al (2006) where biological samples were collected from a total of 56 long-term exposed steel production workers (PW), 35 quality control workers (QCW) and 75 unexposed normal controls (all male, age range 25 55 years). Hence, the working solution of Cr was prepared from certified standard solutions of all analytes under study in 2M nitric acid. All solutions were stored at 4^{o}C. In the case of each person, hair strands were washed with diethyl ether-acetone (3+1) mixture, non-ionic detergent solution (distilled water) and ultrapure water, respectively. After washing, the hair samples were dried at 80^{o}C for 6 h. Hair samples for each participant were placed in separate plastic envelopes, which indicated the identification (ID) number of the participant. Duplicate 0.5 mL of each certified urine samples, while 0.2 g of human hair samples BCR 397, were placed into 50-mL Pyrex flasks. A 5-mL volume of a freshly prepared mixture of concentrated HNO_3 -H_2O_2 (2:1, v/v) is added to each flask, and the solutions will be heated on an electric hot plate at 80^{o}C for 2-3 h, until the clear transparent digests is obtained. Final solutions was made up to 10 mL with 2M HNO_3. The final solutions were collected in polyethylene flask for determinations of Cr by ETAAS. Blank digestions were carried out. Duplicate samples of QCW, PW and normal controls were treated as described above. The calibration was periodically verified by analyzing the standard at the frequency of 10 readings. A microwave-assisted digestion procedure was carried out in order to achieve a shorter digestion time. For digestion of biological samples, duplicate samples of dried scalp hair (200 mg) three replicate samples of CRM 397, were weighed into Teflon PFA digestion vessels directly, to which 2 mL of HNO_3 and 1 mL of 30% H_2O_2 were added and left to stand for 10 min, then the vessels were sealed and placed in a PTFE reactor. This was then heated following a one-stage digestion programme (250 W, 15 min for hair samples). After cooling the digestion vessels in an ice bath for 20 min before opening, the resulting solution was evaporated almost to dryness to remove excess acid, and then diluted to 10.0 mL in volumetric flasks with 2M HNO_3. Blank extractions (without sample) were carried through

the complete procedure of both methods. The concentrations were obtained directly from calibration graphs after correction of the absorbance for the signal from an appropriate reagent blank.

The methodology to identify Cr in urine samples was based on the one proposed by Scheepers et al. (2008). Spot urine samples were stored at 4 ^{0}C in the dark and transferred to a laboratory for further storage at −18 ^{0}C. The analysis of Cr is performed according to Lewalter et al. (1985). Urine was diluted with a solution of magnesium nitrate with Triton-X and sulphuric acid (matrix modifier). Cr levels were determined at 357.9nm using electro thermal atomic absorption spectrometry, AAS (Solaar M, Thermo Analytical) with Zeeman background correction. The LOQ was 0.05 μg/L of urine and the coefficient of variation was 5.4% at 0.31μg/L.

Results

The results of the biosamples analysis for the various individuals (for both urine and hair) are presented in Figure 3, while various statistic metrics per age group and matrice are presented in Figure 4.

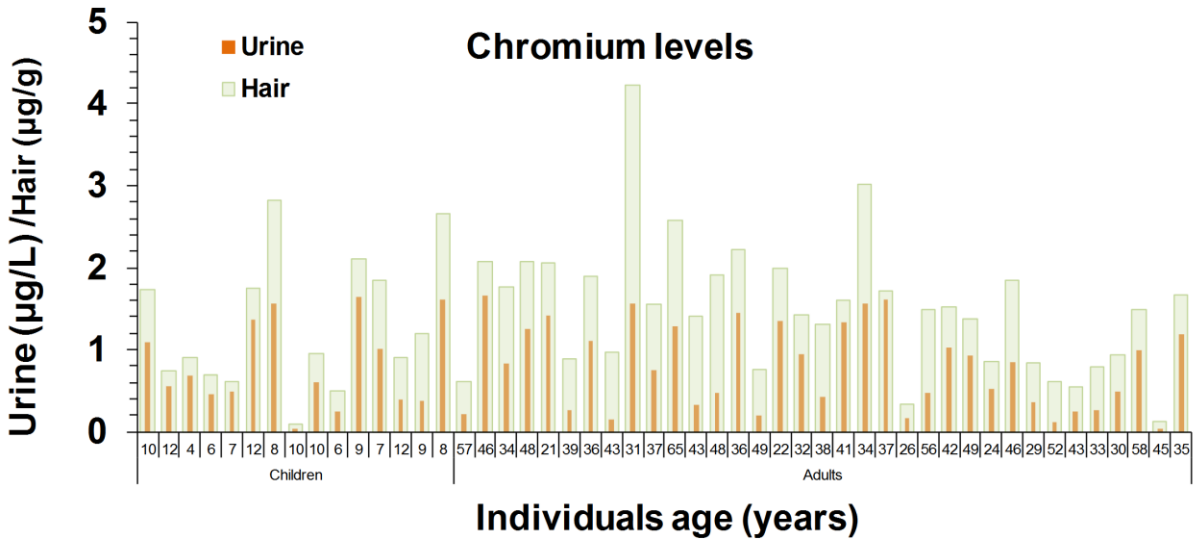


Figure 3. Urine and hair Cr levels for the 50 individuals in the Asopos Basin

Urinary levels range between 0.1 and 1.7 (mean value of 0.7) μg/L, while hair levels vary between 0.1 and 4.2 (mean value of 1.3) μg/g. Slightly higher values were observed in adults, potentially related to higher amounts of tap water consumption. Cr levels in hair showed higher variability that the respective urinary levels, however, this is somehow expected. The lack of highly sensitive methods and the limited weight of the material used (50-200 mg, instead of 2-5 ml for biological fluids such as urine and blood, or even 10 ml, which is the case for breast milk) act as limiting factors for the widespread use of this matrix. In addition,

parameters such as external deposition of chemicals, or hair pigmentation have been amended as potential confounders.

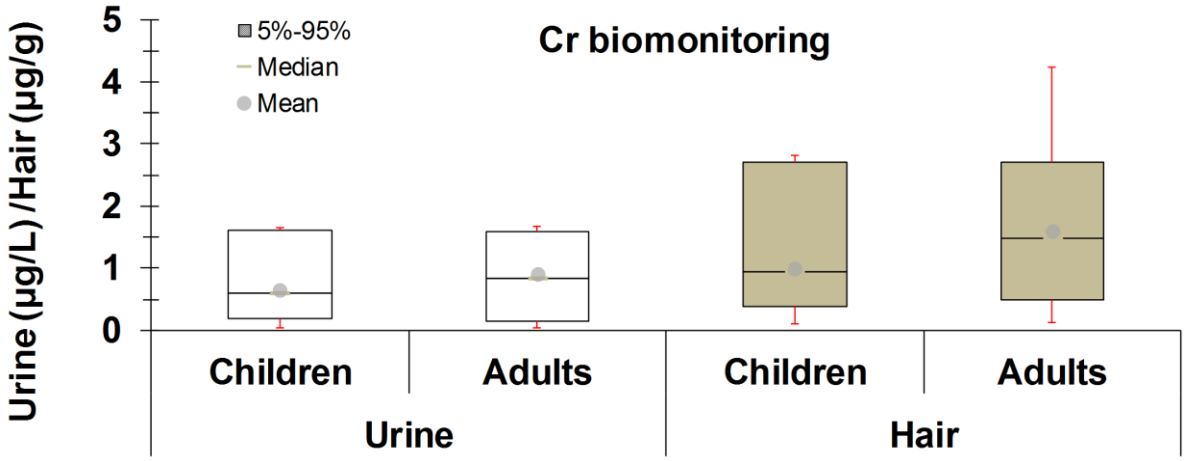


Figure 4. Urine and hair Cr levels for adults and children in the Asopos Basin

The correlation between urinary and hair Cr levels is quite good (Figure 5), indicating that both matrices seem able to capture the long term exposure related to exposure to multiple sources of Cr.

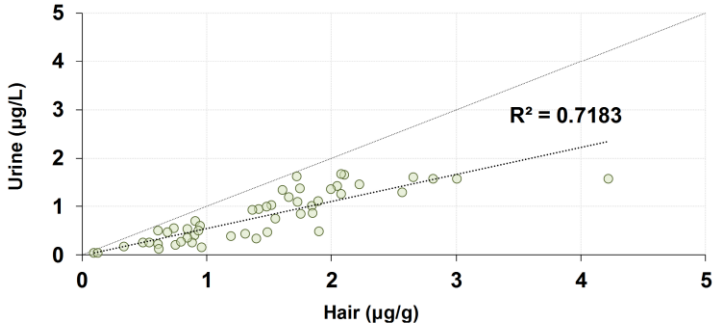


Figure 5. Correlation between Cr levels in urine and hair

Conclusions

Exposure to Cr(VI) in the Asopos Basin seems to be lower than in the previous years, as a result of the counter measures actions that relate to tap water. This was also verified by the low levels of Cr identified in the various biological matrices sampled of the relevant population. However, these results will be further analysed with internal dosimetry models for better associating external exposure levels and the respective biomonitored data.

2nd case study – PAHs emitted from biomass burning from space heating

Introduction

Over the last couple of years, the use of biomass as heating source was allowed in Greece as a CO_2-neutral means of space heating in the large metropolitan areas of Athens and Thessaloniki affecting more than half of the country's population. At the same time the use of light heating diesel was heavily taxed. In the same period Greece faces a financial crisis with significant repercussions on the average household income. That combination of parameters resulted to increased use of biomass for residential heating in year 2012, followed by a significant increase of ambient air, indoor air and exposure to PM_{10} and $PM_{2.5}$. In this case study, we aim to quantify the health effects related to that shift from light heating diesel to biomass burning, as well as to evaluate alternative scenarios of residential heating energy share.

Preliminary data for the verification of the case have been delivered by Sarigiannis et. al (2013). Interesting conclusions are given by the comparative study of daily values for the same dates between the years 2011 and 2012.

During the warm period of early October-early November 2011 at the traffic station, PM_{10} concentrations were 59.8 and $PM_{2.5}$ 47.0 μg/m^3, while during the cold period, amounted to 82.9 and 68.3 μg/m^3 respectively. During the warm period of 2012, the respective concentrations remained lower, being 53.1 and 29.5 μg/m^3 for PM_{10} and $PM_{2.5}$, while a significant increase occured during the cold period (76.5 and 59.7 μg/m^3 for PM_{10} and $PM_{2.5}$ respectively). Since average wind speed was similar in both the seasons, the reduced concentrations observed in 2012 at the traffic station are attributed to reduced traffic emissions, which in turn are due to the reduced traffic load by 30% in 2012, as evidenced by *in situ* traffic measurements carried out.

Moreover, while in 2011 the ratio of $PM_{2.5}/PM_{10}$ remained almost constant (~ 0.8) in the two periods (warm-cold), in 2012 it increased significantly during the cold period (from ~ 0.55 up to 0.78); the latter indicates that additional contribution beyond traffic sources was becoming important. Instead, at the background station, whereas concentrations of PM_{10} and $PM_{2.5}$ were higher during the warm period of 2011 (41.4 and 31.1 μg/m^3), with respect to the ones measured in 2012 (30.6 and 19.4 μg/m^3 respectively), during the coldest period of 2012 PM concentrations were significantly higher (73.1 and 62.7 μg/m^3 for PM_{10} and $PM_{2.5}$ respectively) than the ones in 2011 (53.1 and 43.5 respectively).

The increase of concentrations in year 2012 was accompanied by a sharp increase in the ratio $PM_{2.5}/PM_{10}$ (from 0.63 to 0.86), in contrast to 2011, where the corresponding change was smaller (from 0.75 to 0.82). Between the two years, there was a significant variation among

the emission patterns, where the traffic component appears to be reduced in 2012, while during the colder period the component associated with the biomass heating was dramatically increasing. Given that temperatures during the cold season of 2012 are close to the ones of 2011 (daily average 11.1 ^{0}C for both years), these differences cannot be attributed to increased need for domestic heating. During the last winter (2013-2014), the pattern of traffic and biomass burning emissions seems to be similar to the previous year, thus, the problem of biomass burning still remains.

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

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

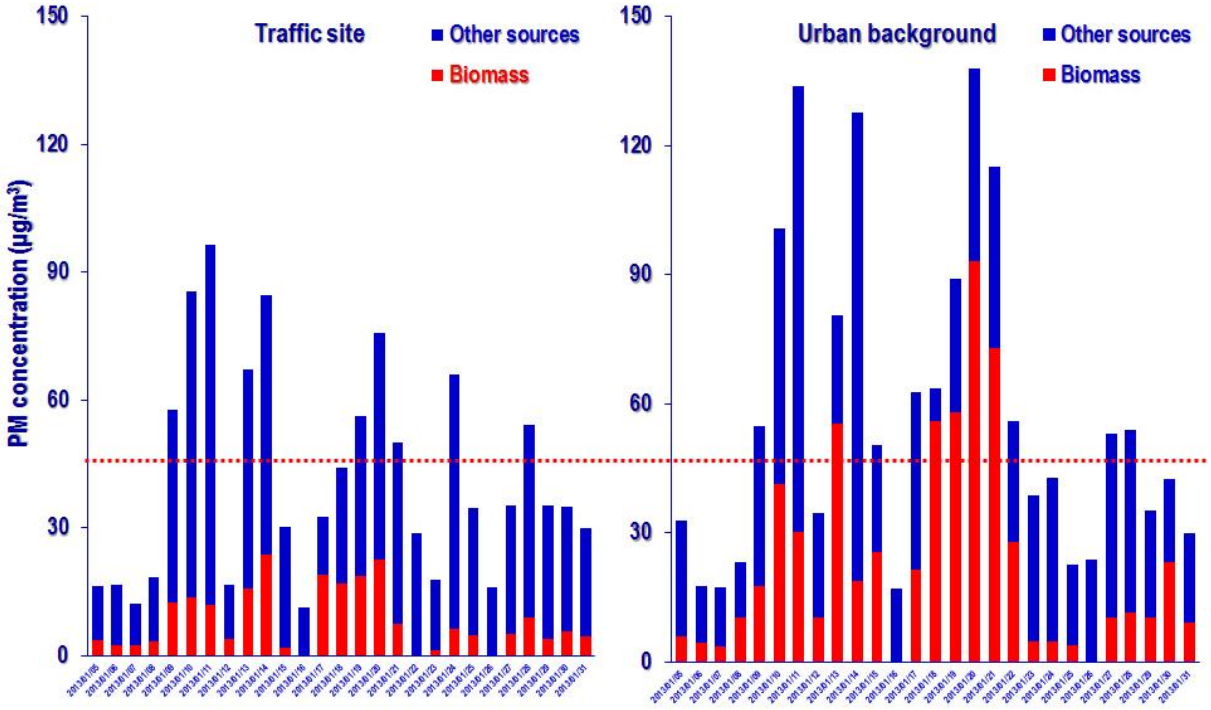


Figure 6: Biomass burning contribution in two distinct sites (traffic and urban background) in the city of Thessaloniki

Increased biomass emissions, are linked to elevated exposure to PM, as well to toxic compounds adsorbed on them, PAHs (IARC, 2010), being the ones of highest concern among them. It is also interesting that higher concentrations of PM due to biomass burning, where

accompanied by equally high concentrations of PAHs; the latter shows that toxic potency of biomass emitted PM is at least equally toxic to traffic emitted PM (***Figure*** 7).

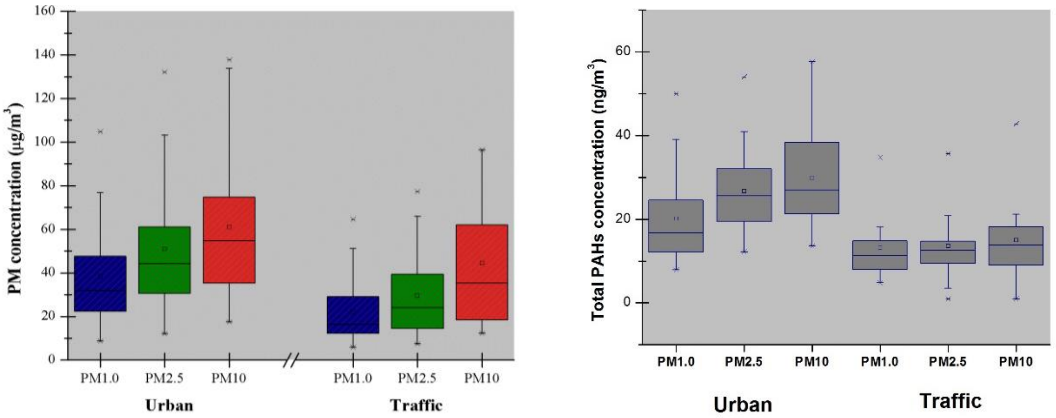


Figure 7: PAHs concentration between urban and traffic site for the different PM fractions

Summary of existing environmental and biomonitoring data – gaps identification

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

In order to fill the biomonitoring gaps, a biomonitoring sampling campaign took place in winter of 2014-2015 in Thessaloniki, aiming to capture the effect of biomass burning in PM and PAHs exposure. Overall 50 of non-smoker individuals aged 8 to 64 will be sampled, covering a wide spatial distribution of the city. This will be necessary, since differences in exposure are expected to be found based on the levels of the performed activities, the ventilation conditions, as well as the respective distribution of PM and PAHs levels in the city.

Additional data collected

Environmental data

Sampling and analysis protocol

Considering that there are a lot of data regarding the mean daily exposure to PM and PAHs in the area of Thessaloniki, it is of particular interest to identify the diournal variability of ambient air and indoor air to PM and to associate this to specific activities. This will allow us

to further refine exposure estimates for the various groups and eventually the lung cancer risk estimates associated to PAHs exposure.

For this reason, time dynamic measurements of PM (in parallel to the existing equipment for gravimetric PM sampling and chemical analysis) was continuously monitored during the cold period of the years 2014-2015 and 2015-2016 using the Mini Laser Aerosol Spectrometer (Mini-LAS) 11-R (Figure 8).

Figure 8. Mini Laser Aerosol Spectrometer (Mini-LAS) 11-R

The 11-R captures every single particle ranging from 0.25 to 32 μm and classifies them into 31 size channels. The sample air enters the measuring chamber from the top in such a way, so that only one particle at a time is measured (this is the difference to any Nephelometer method). The outlet after the pump is closed prior each start, so that there is only internal filtered air circulation and therefore the count is "zero" (self-test).

The use of this instrument, allowed us to capture (a) the time dynamics of ambient air PM and (b) the contribution of specific sources such as open fireplaces in overall exposure to the various fractions of PM.

Results

The use of the the aerosol spectrometer allowed us to capture the time dynamics of PM air pollution in the city of Thessaloniki. The results of the average hourly PM concentrations (for the three main fractions PM10, PM2.5 and PM1) during the winter period of 2015-2015 for the urban background station are illustrated in Figure 9.

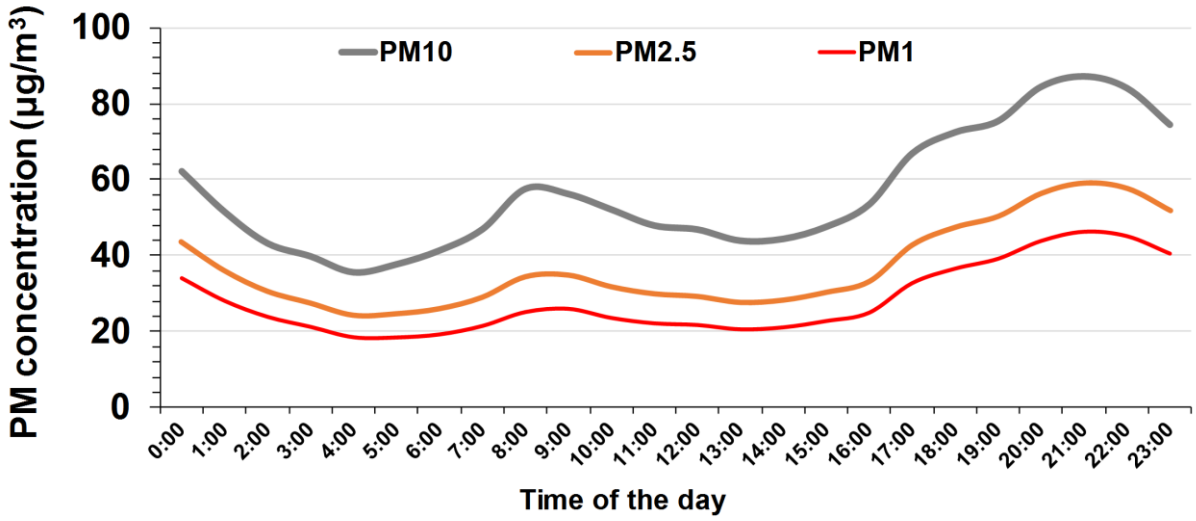


Figure 9. Ambient air PM diournal variability in the urban background station (average for the cold period)

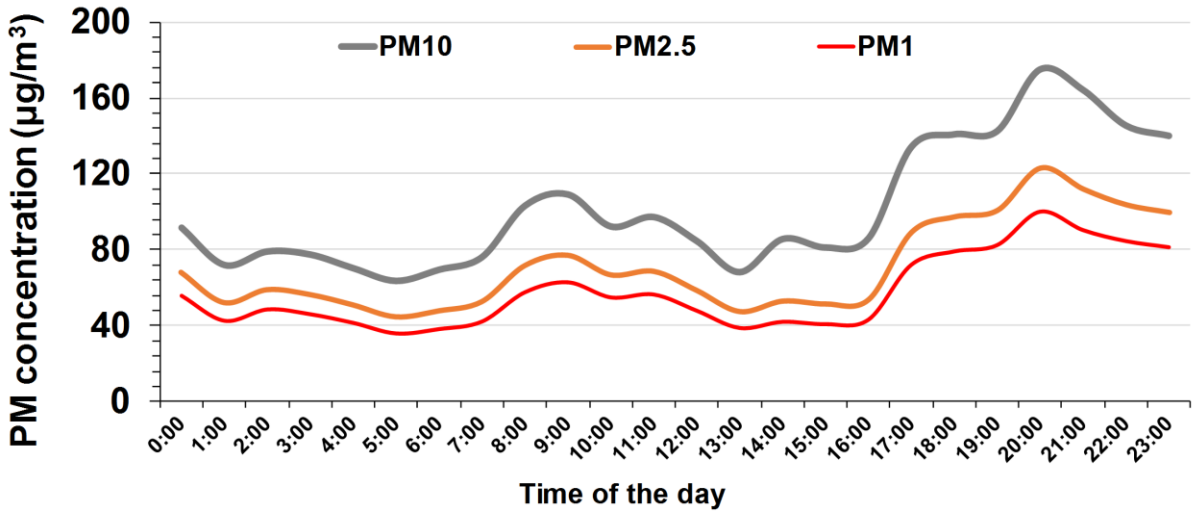


Figure 10. Diournal variability of PM levels for a characteristic winter day (27-12-2015)

From the results it is indicated that concentration peaks occur during the night at about 9:00 – 11:00 pm. These levels are attributed to the use of space heating (and especially the use of biomass for space heating), since it is well known from previous studies that traffic emissions do not have any peak at this hour. This behavior is further highlighted, by selecting characteristic days where PM concentrations were very high such as on 27/12/2015 (Figure 10).

An additional capability provided by the time dynamic measurements was the contribution of open fireplaces in the indoor pollution levels. It was found that operation of open fireplaces contributed significantly on the measured indoor concentrations, although this contribution

was highly variable, depending on the type of woods used, the position of the woods in the fireplace and ambient weather conditions (Figure 11).

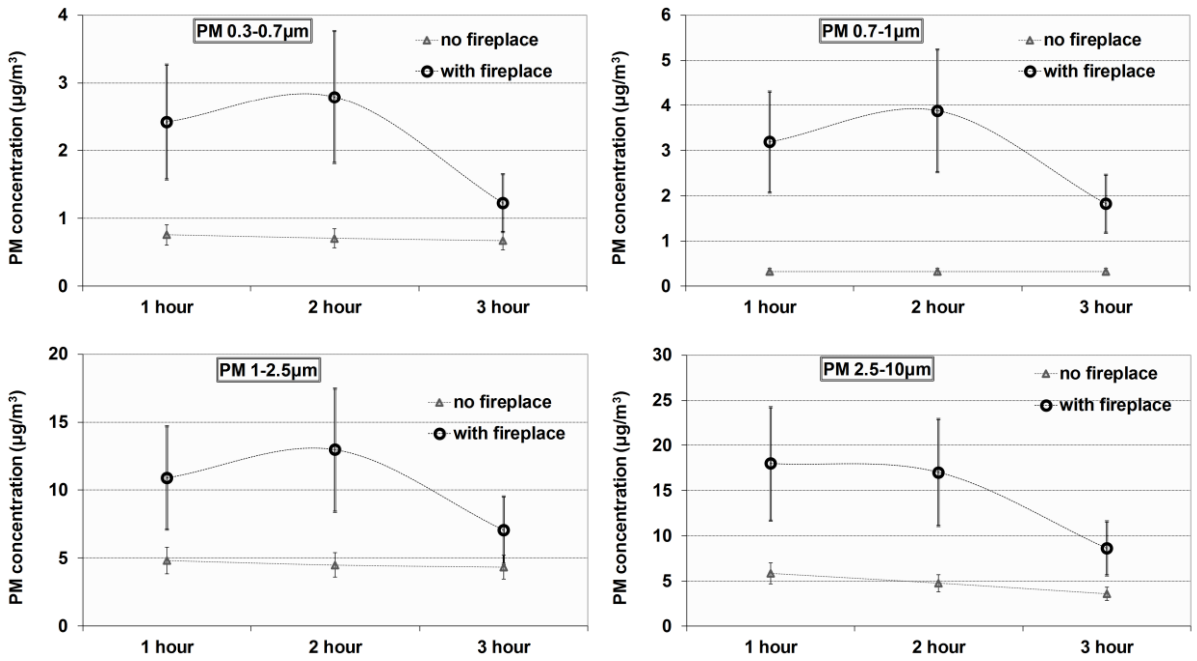


Figure 11. Increase of PM of various aerodynamic diameters during fireplace operation

From these measurements it was indicated tat the PM emitted from biomass burning are mostly of lower aerodynamic diameter, although in most of the cases, fireplace operation resulted up to 3 times higher level of exposure across the whole range of PM size spectrum.

Biomonitoring data

Sampling and analysis protocol

Sampling

Within the CROME-LIFE project, 1-OH pyrene in urine was used as a metric of internal exposure to PM, which is a well validated (Bouchard and Viau 1999; Miao et al. 2014) major metabolite traced after exposure to PAHs. The straightforward HPLC method is a good basis for the analysis of 1-OHP in human urine. It is generally sensitive, specific and can be made very reproducible with some minor modifications (Bouchard and Viau 1999). Urine samples will be collected in standard polyethylene tubes and of a small amount of thymol will be added so as to prevent bacterial growth. Tests conducted have further shown that storing of

urine samples (collected over thymol) at room temperature, 4 °C or -20 °C causes no loss of the analyte for a period of several weeks.

Figure 12. Location of the 50 residential sites

Urine samples

Preservation

- At least 10 ml of urine were collected in polyethylene tubes and were kept in the dark.
- Label for storage was put on the polyethylene tubes.
- The bottles were stored immediately at -80 °C until analysis; 1-OHP is stable for at least 3 days even at 4 °C in urine adjusted to pH 5 and buffered, while urine samples are stable for at least 6 months at -20 °C (Boos et al. 1992).

- Always recorded exact time of urine collection, centrifugation and storage to -80 °C (on a worksheet), and always record any deviation from the procedure.

Materials

- Solvents: HPLC-grade acetonitrile and methanol from Sigma and water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).
- Materials: b-glucuronidase / aryl sulfatase (type H-2: from *Helix pomatia*: b-glucuronidase activity 100 000 units /mL and sulfatase activity, 5000 units / mL) was from Sigma, Waters Sep-Pak C_{18} (Waters, Mil ford, MA, USA)
- Evaporative Concentrators: Rotary evaporator, dry-nitrogen "blow-down" device
- Analytical balance: capable of accurately weighing 0.0001 g. The balance was properly calibrated and maintained.
- Pipettes: Mechanical pipettes with capacities from 10 to 1000 µL were used.

Sample Preparation

Hydroxylated metabolites of PAHs in urine were determined using a slight modification of the method of Feunekes at al. (1997).

1. Prior to performing the procedure, Waters Sep-Pak C_{18} (Waters, Mil ford, MA, USA) cartridges were primed by performing two cycles of filtering 5 ml of HPLC grade methanol followed by 10 ml of Milli-Q water.
2. The frozen samples of urine were thawed in a warm water bath while stirring.
3. Once thawed, 10 ml of the samples were transferred to 50 ml conical tubes and adjusted to pH 5.0 (with ± 0.05 error) using 0.1M HCl. Once properly adjusted for pH, 15 µl of β-glucuronidase/ aryl sulfatase (1655/63 units) and 20 ml of 0.1 M acetate buffer were added to each sample.
4. After the deuterated internal standard (*1-OHP-d_9)* was added the reaction mixtures were placed and incubated at 37ºC for 2 hours.
5. After incubation, primed Sep-Pak cartridges were added to 10 ml syringes with the plungers removed and 60% of the samples were poured in the syringes. The samples were then slowly pushed through the cartridges at a rate of 2.5 ml per minute to allow the 1-OH-P to be collected on the Sep-Pak.
6. Once the syringe was empty, the rest of the samples were added and the step was repeated to finish loading the cartridges. Once loaded, the cartridges were washed with 8 ml of Milli-Q water by slowly pushing it through the Sep-Pak.

7. After all contaminants were removed from the cartridge, 10 ml of HPLC grade methanol was added to the syringe and the 1-OH-P was eluted at a rate of 2.5 ml per minute into 25 ml glass vials.
8. The vials were placed in a warm water bath nitrogen evaporator and placed under a gentle flow of nitrogen until all of the solvent evaporated.
9. The samples were resuspended by adding 2 ml of HPLC grade methanol to each vial.
10. A 3 ml syringe was used to draw up the remaining methanol solution and a 0.45 µm filter was added to each syringe before filtering the sample into 2 ml HPLC vials.

High-Performance Liquid Chromatography with Fluorescence detection

The HPLC system included a RF German 10AXL fluorescence detector and a C-R7A plus integrator. An analytical column was: an RP-Amide column (Discovery RP-Amide C16, 2503 4.6 mm I.D., 5 mm Supelco, Bellefonte, PA, USA) The mobile phases for the RP-Amide column was acetonitrile–phosphate buffers (pH 7.0) (57:43 and 47:53, v/ v, respectively) at a flow-rate of 1 mL/min. The column temperature was set at 40 ^{o}C. The excitation and emission wavelengths are 240 and 387 nm, respectively.

Calibration Standards

Standards were placed every tenth sample to validate the results. A standard calibration curve was determined with solutions containing 1-OHP standard and 1-OHP-d_9 as internal. For the linear regression, r^2 = 0.997 and the detection limit of 1-OHP is 0.1 mg/L (signal-to-noise ratio > 3). The quantification range was from 1 to100 nmol/L.

Recovery

The percentage of 1-OHP recovered had to be determined comparing the amount of detection of the standards to the expected amount with known amounts of 1-OHP. This recovery percentage was used to adjust the samples accordingly.

Results

From the results of the analysis of the urinary samples, it was identified that urinary 1-OH pyrene levels ranged from 0.05 to 0.80 (Figure 13). It has to be noted that higher 1-OH pyrene levels were identified in children. This is explained by the fact that the actual uptake (bodyweight normalised) of PAHs is higher in children. The differences of the distributions of both age groups are further presented in Figure 14.

These differences are explained by the higher inhalation rate (bodyeight normalized) of children, as a result of the higher metabolism, as well as of the the more intensive activities

performed daily by the children. In addition, children commute more often than the adults, thus they are spending more time outdoors, where PM levels are usually higher.

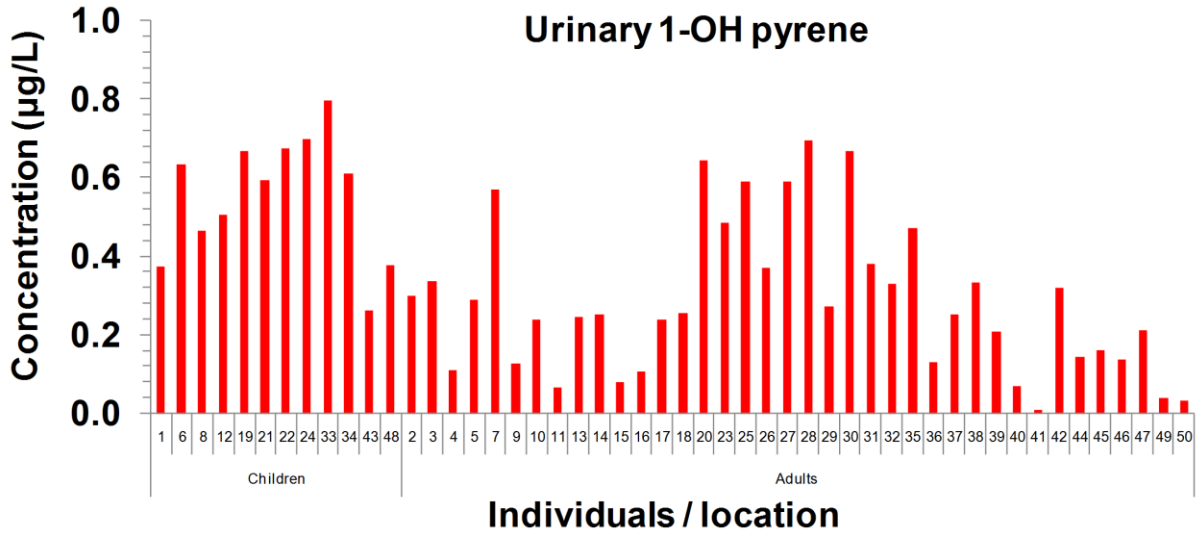


Figure 13. Urinary 1-OH pyrene levels in the population of Thessaloniki

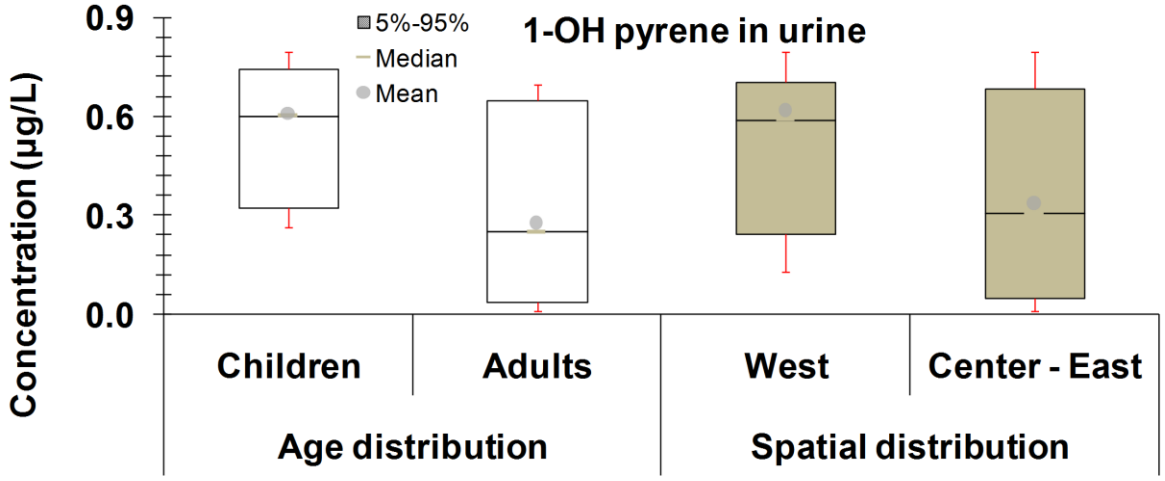


Figure 14. Box-plots of urinary 1-OH pyrene levels in the population of Thessaloniki

It has also to be highlighted, that higher 1-OH pyrene levels were identified in the west area of the city compared to the east-center area. These results also corroborate with the findings of previous analysis of the ambient air PAHs analysis in the metropolitan area of Thessaloniki, where exposure to PAHs TEQ was higher in the western side. It has also to be noted, that higher difference (of almost 3 times) of the actual TEQ had been identified within the two locations, while the difference in 1-OH pyrene levels is lower (almost twice). This is attributed by the fact that TEQ is calculated taking into account the toxicity of the individual PAHs identifyied in the ambient air particles, hence, each compound contributes to the overall

TEQ through a fraction, defined by its TEF. On the contrary, 1-OH pyrene is a metabolite indicating the overall burden of exposure to PAHs, irrespective of their toxicity.

Conclusions

The additional environmental and biomonitoring data obtained in the frame of CROME, provide additional information towards the association between exposure and effects, for a population heavily exposed to biomass burning for space heating particulates and to the main carcinogenic compounds adsorbed, namely PAHs.

The additional measurements of ambient air PM diurnal variability, provide us the ground for improving exposure estimates, by deriving data related to the peaks of exposure and how these peaks correspond to the various activities and inhalation rates at a given time. In this way, exposure estimates are pretty refined, since it has been found that ambient air peaks do not contribute as expected to the overall daily exposure, since people tend to relax or to sleep at that hour, hence the actual uptake rate is low. In addition, an instrument that provides time-dynamic data, allows the identification of the contribution of specific exposure sources, such as the operation of an open fireplace.

The biomonitoring data on the other hand, provided valuable information on the validation of the PAHs internal dose estimates, calculated starting from ambient air exposure data and accounting for PM size distribution and PAHs TEQ actual uptake. The biomonitoring results confirmed that both (a) children are exposed to higher levels of PAHs compared to adults and (b) people leaving in the west side of Thessaloniki are exposed to higher amount of PAHs, highlighting the socioeconomic component of exposure to biomass burning for space heating emitted toxic components.

3rd case study – PCDDs/PCDFs emitted from accidental fire on plastic recycling plant

Introduction

Waste recycling is one of the main cornerstones of the EU waste management strategy (Farmer et al., 2015). It offers many advantages contributing to the circular economy and the sustainable and efficient use of natural and man-made resources. However, waste recycling facilities may be associated to adverse health outcomes in the aftermath of industrial accidents involving the inadvertent generation of toxic chemicals and their release into the environment. One of the major concerns associated to accident in plastic recycling plants are the emissions of dioxins and furans (PCDDs/PCDFs). These compounds are characterized by a high carcinogenic potency (Cole et al., 2003; Tollefson, 1991). Because PCDDs/PCDFs appears to be acting like a potent and persistent hormone agonist, it appears reasonable to incorporate mechanistic information on receptor-mediated events in risk assessments for TCDD This information may be obtained from steroid receptor action and from molecular data on the Ah receptor (Lucier et al., 1993). This receptor based toxicity, results in sex-dependent sensitivities, as a result of a set of sex-specific PCDDs/PCDFs -responsive genes. In addition, complex interactions between the aryl hydrocarbon and sex hormone receptors may affect the observable differences in sensitivity phenotypes between the sexes (Lee et al., 2015). Moreover, the hepatic transcriptional response of adult male and female C57BL/6 mice diverged significantly after exposure to 500μg/kg TCDD at multiple time-points. Female mice demonstrated a large number of altered transcripts as early as 6h following treatment, suggesting a large primary response, while male animals showed the greatest TCDD-mediated response 144h following exposure, potentially implicating significant secondary responses (Prokopec et al., 2015). Animal model systems provide important insights on PCDDs/PCDFs toxicity, however, it has to be noted that in parallel experiments with human, rat and mice lymphocytes, the human ones showed the most potent CYP1A1 mRNA induction, suggesting that lymphocytes are more sensitive to PCDDs/PCDFs than the lymphocytes of mice and rats (Nohara et al., 2006). However the estimation of the additional probability of cancer due to the additional exposure burden is quite difficult (Dong et al., 2016). A major obstacle is that an elevated short term external exposure associated to the accidental event, has to be translated into long term risk estimates. In addition, recent studies demonstrating a concentration dependence of elimination of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) suggest that previous estimates of exposure for occupationally exposed cohorts may have underestimated actual exposure, resulting in a potential overestimate of the carcinogenic potency of TCDD in humans based on the mortality data for these cohorts (Aylward et al., 2005). Considering the significant persistency and bioaccumulation of PCDDs/PCDFs in human body, the use of biokinetic models for assessing the actual internal dosimetry of this

complex mixture is of particular importance. The pharmacokinetics of TCDD are relatively well understood in adult humans (Kerger et al., 2006; Michalek and Tripathi, 1999; Milbrath et al., 2009). TCDD induces its own elimination at high exposures and its pharmacokinetics are highly influenced by the percentage of body fat at low exposures (Emond et al., 2005). However, the impact of pregnancy and lactation on the elimination of TCDD and other dioxins is not clear (Emond et al., 2016).

Accidental fires in plastic industry comprise one of the major events resulting in contamination of various environmental media to PCDDs/PCDFs of the surrounding area; air samples collected in the 5th day of the event were found to contain over 1000 pg/m^3 TEQ (toxic equivalent quantity) of dioxin, exceeding background levels by 2,500−25,000 fold (Fernando et al., 2014). Based on the above, the current study aimed at calculating the health burden (in terms of cancer risk) of the population living in the Aspropyrgos area (close to Athens, Greece) due to increased exposure to dioxins and furans (PCDDs/PCDFs), emitted by an accidental fire in a plastics recycling plant in June 6, 2015. The fire resulted in significant particle and gaseous emissions of several compounds related to plastic industry. In addition, release of dioxins and furans was a major concern, due to their persistence in environmental and biological matrices, as well as to their carcinogenic potency. In order to face the methodological problem mentioned above, a comprehensive methodology involving both measured data and complex internal exposure modelling was employed.

Summary of existing environmental and biomonitoring data – gaps identification

In order to estimate the risk related to the PCDDs/PCDFs emitted during the fire, it was critical to estimate the long term internal burden of exposure associated to this event. The need for addressing long term exposure is associated to the fact that PCDDs/PCDFs are bioaccumulative and persistent with a half-life time of almost 7.5 years in humans. Hence, it is critical to translate the actual uptake during the accidental event (that lasted for a few days) into a long term (lasting for many years) internal exposure burden. The only scientifically sound way to translate these external doses into internal exposure to the target tissues was carried out with physiology based biokinetic (PBBK) models. To be able to perform this type of calculation, it was critical to be able to identify (a) the background exposure levels to PCDDs/PCDFs and (b) the additional burden of exposure due to this accidental event.

To be able to estimate the additional risk posed by exposure to the accidental event utilizing the INTEGRA platform the following data were needed:

- Data on ambient air levels of PCDDs/PCDFs during the accidental event were obtained by various measurements of PM and analysis of PCDDs/PCDFs in the particle and gaseous phase.

- HBM data for estimating the background exposure to the exposed population. In practice, the HBM data of the local population collected from a previous study (before the accident) were used to estimate the equivalent background exposure that results in the corresponding HBM data; the additional exposure of the measured PCDDs/PCDFs was added to these background levels for a duration of 6 days.

Analysis of ambient air samples (both particle and gaseous phase) showed that the levels of PCDDs/PCDFs in the surrounding area were 1.8 pg/m^3 TEQ WHO (toxicity equivalent concentration in accordance with the methodology of the World Health Organization). These levels are significantly higher than the ones reported in previous studies, where atmospheric background concentration of a typical industrial site in the wider area of Athens was found to be equal to 0.1 pg/m^3 TEQ WHO, but in the same order of magnitude to the levels of landfill fires (Figure 15).

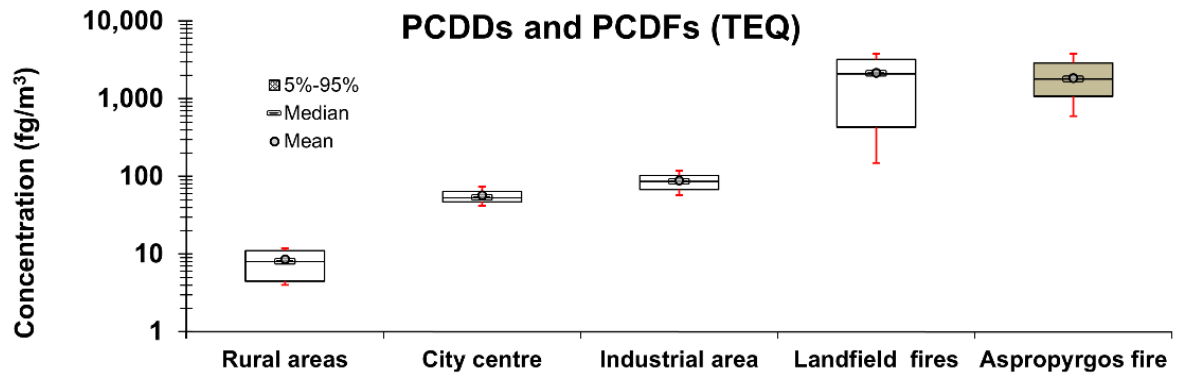


Figure 15. Levels of PCDDs/PCDFs (TEQ) at various Athens sub-areas, as well as during accidental fire events

Additional data collected

Biomonitoring data

Sampling and analysis protocol

Sampling and storage

Blood samples were collected from 60 individuals aged 6 to 58 years old, living in the close proximity of the recycling plant. Approximately 50–80 ml of blood were collected from each individual. Blood samples were collected in polyethylene recipients. Immediately after sampling, blood samples were processed for serum separation, frozen right after separation and transported to the laboratory for measurement of PCDDs/PCDFs. The blood serum samples, remained frozen until they were analyzed at a temperature of -80 °C.

All solvents used were residue analysis picograde and were purchased from Promochem (Germany). The isomers for the preparation of the $^{13}C_{12}$ internal standard solutions were purchased from Wellington Laboratories (Canada). Carbosphere 80–100 mesh was purchased from Alltech (it was cleaned by elution with methanol and consequently with toluene for several weeks before usage). Alumina was Basic activity Super 1 for dioxin analysis, MP Biochemicals GmbH. The sulphuric acid impregnated silica gel was prepared as follows: Silica gel (100 g, 60–200 mesh, Merck) was activated in an oven at 200 °C for at least 2 days and then mixed with concentrated sulphuric acid (44 ml).

Extraction of lipids

Blood serum samples were subjected to a liquid–liquid extraction procedure consisting of mixing with sodium oxalate and methanol, followed by extraction steps with a combination of diethyl ether–petroleum ether. The organic layer of this extraction was evaporated and the lipid residue was dried and weighed in order to calculate the levels of lipids in serum and breast milk.

The labelled quantification standards were added to each sample before the extraction. They were toluene solutions containing a mixture of the $^{13}C_{12}$ isomers of all the 17 PCDDs/PCDFs congeners except OCDF.

Clean up

For the determination of PCDDs/Fs, the samples' clean-up was performed as follow:

Carbon chromatography

A glass column (length 10 cm, 10 mm ID) equipped with mounting ends on both sides was initially filled with glass wool, 2 g of Carbosphere and another plug of glass wool. The column was connected to a glass funnel. The sample residue was dissolved in 50 ml dichloromethane (~5 ml/g fat) and brought onto the top of the Carbosphere column. This volume of dichloromethane was sufficient to remove almost the complete amount of fat (>99%) from the column. The Carbosphere column was inverted in the reflux unit and the PCDD/F fraction was eluted from the column by refluxing with 40 ml of toluene for 16 h. The PCDD/F fraction was concentrated to a volume of about 2 ml and then evaporated to dryness under a gentle stream of nitrogen.

Alumina chromatography

The residue containing PCDDs/PCDFs, was dissolved in 5 ml of hexane and the mixture was brought onto an alumina column prepared as above. The column was washed with 20 ml of hexane/dichloromethane mixture (93:7 v/v) and PCDDs and PCDFs were eluted with 50 ml of a hexane/dichloromethane mixture (60:40 v/v). Finally, the eluate was evaporated to

dryness and redissolved in 50 µl of *n*-nonane containing 2 ng/ml of performance standard ($^{13}C_{12}$ 1,2,3,4 TCDD).

Blood serum samples

The residue obtained by the extraction step was dissolved in 5 ml of hexane and brought onto a column (length 30 cm, 8 mm ID) plugged with glass wool and filled with 2 g 44% H2SO4–silica gel, 5 g alumina and 2 g sodium sulphate. The column was eluted with 50 ml of a hexane/dichloromethane mixture (1:1 v/v). Finally, the eluate was evaporated to dryness and redissolved in 50 µl of n-nonane containing 20 ng/ml of performance standard ($^{13}C_{12}$ PCB 80).

Instrumental analysis

The quantification of PCDDs/PCDFs, was performed by HRGC-HRMS (EI) in MID mode on a Trace GC gas chromatograph (ThermoFinnigan) coupled to a MAT-95 XP mass spectrometer (ThermoFinnigan) equipped with a CTC A 200S autosampler at 10 000 resolving power (10% valley definition). Instrumental conditions and purity control criteria were according to the EPA 1613B and EPA 1668A methods. The quantification of concentration and recovery was carried out by the isotopic dilution method. The limit of detection (LOD) for each congener was determined as the concentration in the extract which produced an instrumental response at two different ions to be monitored with a signal to noise ratio of 3:1 for the less sensitive signal. For TEQ calculations the WHO-98 toxicity equivalent factors (TEF) were used (Van den Berg et al., 1994).

Quality control

A method blank and a quantitative control sample (reference) were included for every eight samples. As reference sample, olive oil spiked with PCDDs/Fs (TEQ value 3.0 pg/g) was used. The reference sample was measured 40 times during the study period. PCDDs/Fs TEQ value showed an average of 3.26 pg/g with a confidence interval of 2.74–3.78 pg/g (confidence level 95%). The LOD was determined for each congener to be between 0.1 and 0.15 pg/g fat. Recovery ranged between 70% and 120%.

Results

The PCDDs/PCDFs levels were calculated as the TEQ values by multiplying with the corresponding WHO-TEF for each congener (Van den Berg et al., 1994). Upperbound total TEQ values were calculated for each category of congeners assuming that non-detected individual congener concentrations are equal to their corresponding limit of detection. The results of the analysis of the 60 samples, indicated that exposure to PCDDs/PCDFs fumes resulted in exposure levels that are higher than the ones identified in previous studies, hence

indicating that the intake of the population was significant. These results could be the basis for further analysis of the toxicokinetic behavior, as well as for cancer risk assessment.

The results of the PCDDs/PCDFs levels in blood of the exposed individuals are presented in Figure 16. The red line (at 6.8 pg/g lipid_TEQ) indicates the mean background concentration in adult blood in the area, as identified in a previous study (Costopoulou et al., 2006).

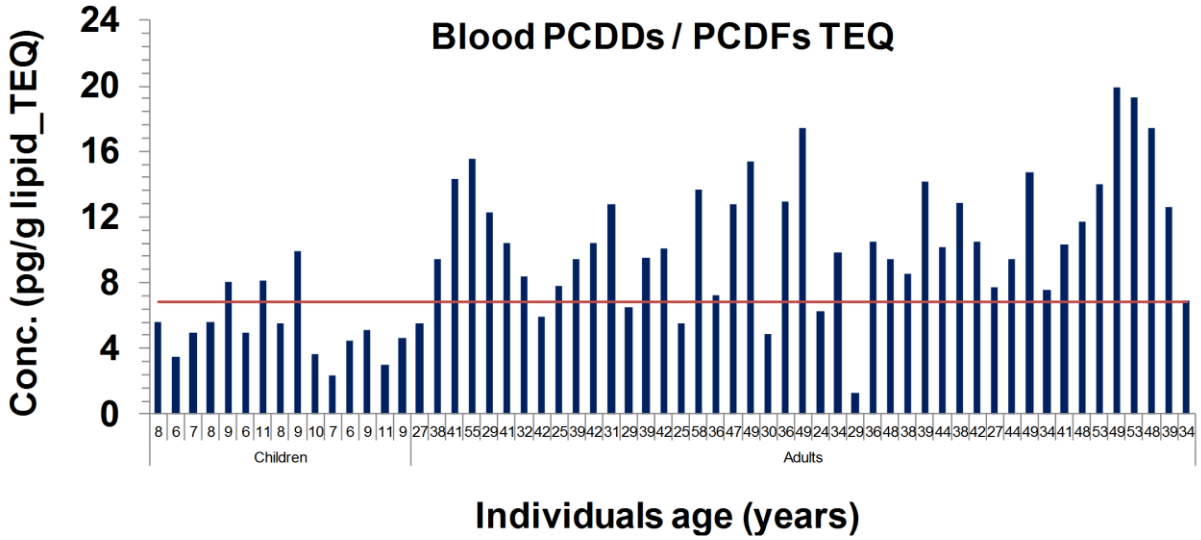


Figure 16. PCDDs/PCDFs blood levels of the Asropyrgos population after exposure to the accidental fire fumes

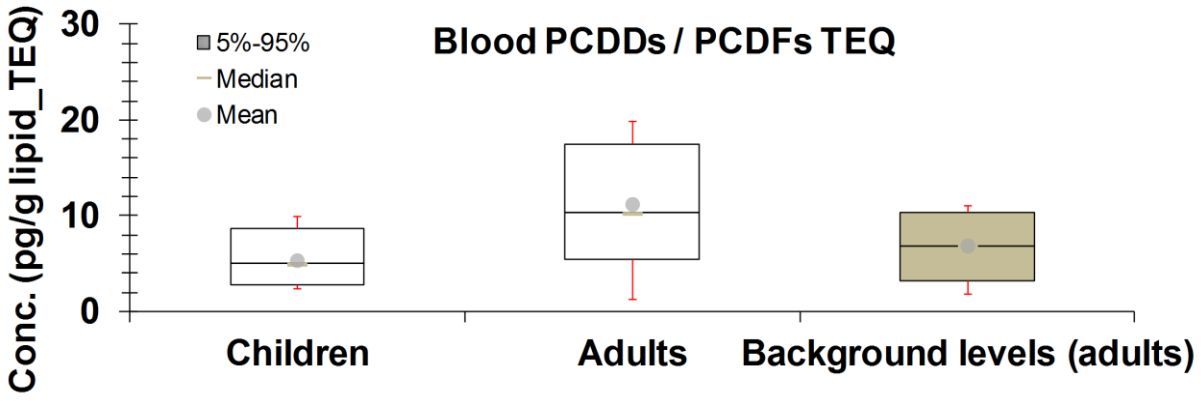


Figure 17. PCDDs/PCDFs blood levels distribution of the Asropyrgos population after exposure to the accidental fire fumes, compared to previous measurements in the area

The distribution of the PCDDs/PCDFs blood levels between children and adults, as well as between exposed and non exposed population are graphically illustrated in Figure 17. The analysis indicated that the accidental event resulted in significant exposure to the population, raising the blood concentrations of adults to almost twice levels. Although the respective change could not be identified in children due to lack of previous measurements, it is

expected that the accidental event would have a higher contribution to children, since newer generations always have lower internal background levels of PCDDs/PCDFs. However, this hypothesis will be further explored through toxicokinetic analysis of this data and development of an internal dosimetry associated cancer risk slope factor.

Conclusions

Although waste recycling is considered one of the most environment friendly and sustainable waste management option, accidental events might result in significant contamination of the surrounding area, affecting adversely the population leaving nearby. The study herein, set the basis for a comprehensive methodological framework for addressing one of the major problems related to accidental events and the release of bioaccumulative, persistent and toxic compounds, which is the translation of a short term exposure event into a long term exposure estimate. Towards this direction, a critical step of the assessment is the evaluation of the internal dose burden of the population exposed to the accidental fire through biomonitoring. From the respective analysis it was found that the additional amount of PCDDs/PCDFs uptaken due to the accidental event resulted in a significant increase of the respective internal dose burden, from 6.8 to 10.2 pg/g lipid_TEQ, which is expected to result in a significant lifetime burden, considering the long half-life time (~7.5 years) of PCDDs/PCDFs.

Case study Slovenia

1st case study – Human Biomonitoring in Slovenia – Hg and other metals

Introduction

Based on the legislation for the implementation of the human biomonitoring (HBM) programme in Slovenia that is defined in the Article 49 Act of Chemicals of the Chapter IX. Protection of Human Health and the Environment (O.J. RS No. 16/2008), the national HBM in Slovenia was conducted in a period between 2007 and 2015 for the first time. The study was funded by the Ministry of health RS, Chemicals Office of the Republic of Slovenia; the implementing organisation was Jožef Stefan Institute Ljubljana and the subcontracting institutions University Medical centre Ljubljana, Regional institutes of public health and regional hospitals and health centres. *Short-term objectives* of the HBM programme are to provide data on exposure of the inhabitants to chemicals and related health impact throughout Slovenia, reference (background) values, and spatial differences in exposure including rural, urban environments and contaminated sites. Provision of institutional framework for the implementation of the programme on a long-term basis is also one of the key objectives to be settled. *Long-term objectives* include the exposure and risk assessment for health, implementation and monitoring of implemented measures, science based risk evaluation (awareness, case-by-case consulting, risk communication …), time trends of exposure, and providing input for policy making, based on surveillance activities.

The objectives of HBM in Slovenia comply well with the main problem targeted by the CROME-LIFE which is the assessment of the impact on human health due to exposure to chemical agents originating either from environmental contamination (air, soil, water), or from consumer products (food contact materials, construction materials, cosmetics, clothes, etc.) through multiple routes.

Summary of existing environmental and biomonitoring data – gaps identification

Parameters measured in the study population are given in **Errore. L'origine riferimento non è stata trovata.**. Residential locations of participants are given in Figure 18.

Table 3:List of biological samples/matrixes and measurements included in the HBM in Slovenia.

Sample	*Individual samples*	*Pooled samples*
Breast milk	*Pb, Cd, total Hg, As, Cu, Zn, Se* *organochlorinated pesticides* *marker PCBs (28, 52, 101, 138, 153, 180)* *triglycerides, cholesterol*	*PCDD,* *PCDF,* *dioxin like PCB,* *PBDE*
Blood - women	*Haemogram* *Pb, Cd, total Hg, As, Cu, Zn, Se* *Creatinine,TSH*	
Blood - men	*Haemogram* *Pb, Cd, total Hg, As, Cu, Zn, Se* *organochlorinated pesticides*	*PCDD,* *PCDF,* *dioxin like PCB,*

	marker PCBs (28, 52, 101, 138, 153, 180) *triglycerides, cholesterol, creatinine, TSH*	*PBDE*
Urine	*Pb, Cd, total Hg, As, Cu, Zn, Se* *Markers of kidney function (albumin, alpha-1-mikroglobulin, IgG, NAG)* *Creatinine*	
Hair	*Total Hg*	

Data obtained from the questionnaire completed by the participants comprises the following variables:

(a) general part: age, body weight and height, education, occupation;
(b) basic characteristic of home: type of building, years the house was built, type of type of heating, type of water supply, traffic density;
(c) health conditions: disease if any, medications, number of amalgam fillings;
(d) life-style and nutritional habits: smoking, passive smoking, use of computer, hobbies, consumption of vegetables, fruit, nuts, milk, cheese, eggs, poultry, game, other meat, fish and seafood, tea, coffee, alcohol, type of oil used, daily water consumption, consumption of supplements;
(e) questions regarding pregnancy and lactation period: any problems during the pregnancy, smoking during pregnancy, alcohol intake, breastfeeding, baby's gender, birthweight, current weight.

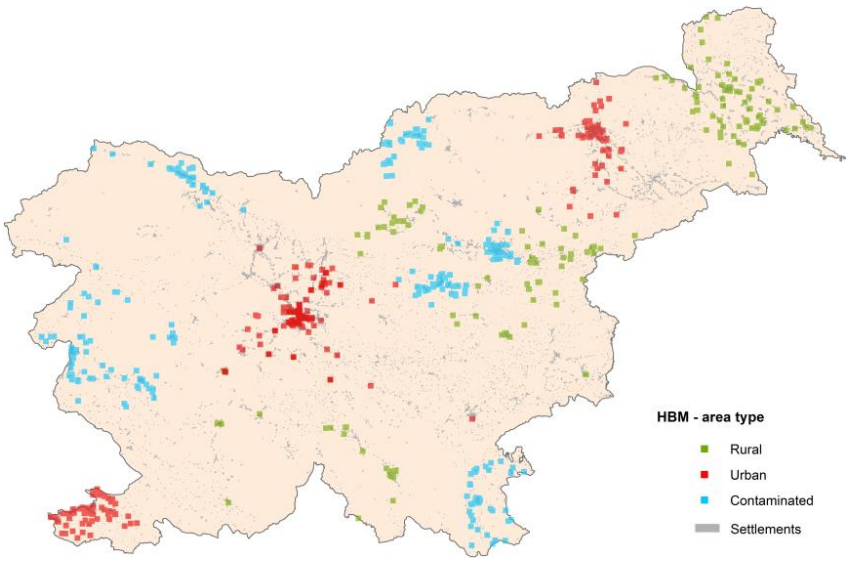


Figure 18: Residential locations of the participants in the Slovenian human biomonitoring, 2007-2015, by the type of areas (rural, urban, contaminated).

Mean participants' age was 30 years, 29 for women and 31 for men, and was similar across the study areas. The majority of the participants had finished secondary or high school, among women there was the highest proportion of those with university education. Among the participants, 11 % were smokers (men-current, women-before pregnancy); the highest

proportion of smokers was in the PCB-contaminated area of Bela krajina and the lowest in the Pb-smelter area of Mežiška dolina.

The levels were mainly below the given HBM values for toxic elements and within the reference intervals for essential elements. Among the potentially toxic elements, Cd levels were higher in women compared to men, while Pb, Hg and Mn levels were in general higher in men than in women. Potential sources of exposure were examined using multiple linear regression. The models for **Cd** showed that the levels in blood were higher in women and in smokers and they increased with age. The urinary Cd levels in women were not influenced by smoking status, while in milk they were influenced by passive smoking. Among the study areas, the levels in urine and milk were higher in rural areas in compare to urban or contaminated.

Lead levels in blood were higher in men, smokers, and in participants consuming water from private water supply; the levels increased with age and were higher in those with lower educational levels. In milk, the levels were higher in women consuming bottled water than in those using water from public or private water supply and also in those smoking before pregnancy.

The levels of **Hg** in blood were influenced by consumption of fresh and canned sea fish and were higher in participants having more amalgam fillings. The levels in blood increased with age and were higher in more educated participants. The levels in hair were influenced only by consumption of fresh and canned fish and were marginally significantly higher in men than in women. Levels in hair are area dependant, and are the highest in the coastal part of Slovenia, where the people consume sea food more frequently than in other parts of the country. The levels in urine were influenced by the presence of amalgam fillings, consumption of fresh and canned sea fish, while the levels in milk by amalgam fillings and consumption of fresh sea fish.

Manganese levels in blood were significantly lower in participants consuming water from private supply and in those consuming poultry more frequently, but were higher in participants consuming game more frequently. Lower values were observed also with higher BMI. In men, poultry didn't have any significant influence on the levels in blood. The levels were lower in participants living in contaminated areas than in those living in rural or urban areas.

Arsenic levels in blood, urine and milk were influenced significantly by consumption of seafood, fresh and canned, but not frozen. Higher levels with higher educational status were observed only in men's blood.

Based on the preliminary findings, environmental data described below was obtained within the CROME project in order to link the internal exposure with potential sources. For example,

area-related Cd levels could be associated with artificial fertilizers, and for this purpose maps of nitrogen and phosphorous surpluses for Slovenia were used. To interpret the presented results on Pb exposure with regard to the water supply, water infrastructure data from Cadastre of public infrastructure together with data on drinking water quality will be used; similarly for Mn and other elements. All environmental data collected within CROME-LIFE project are described below.

Additional data collected

To identify potential sources of exposure that would help interpret the results of the HBM, three major groups of ancillary data were identified and collected: information describing environmental status, information on releases of contaminants from various sources, and data supporting characterization of the built environment in living surrounding of HBM participants. Information gathered is based on variety of national and EU scale sources with different levels of spatial-temporal resolution as described in the following paragraphs and with examples given in the consequent figures.

Environmental data

1) *Drinking water quality*

Sampling and analysis protocol

Drinking water from public water supply was collected from 108 locations across Slovenia, that have been selected among 1283 locations, where water quality is monitored regularly by responsible institutions (Figure 19). The sampling has been conducted as part of a radon (Rn) survey of tap water in Slovenia and was carried out in 2014. The locations have been selected based on the following criteria: (a) number of water supply users, (b) expected Rn concentration, (c) type of water and (d) lithology of the bedrock. The sampling locations have been evenly distributed across the country and more frequent around bigger cities and in areas with relatively higher levels of Rn in air and soil.

Water samples have been collected directly from the pipes after the water was let to run for minimum 10 minutes, in order to collect fresh water. In parallel, tap water was analysed for ^{222}Rn activity concentration using scintillation cell on site. The water samples collected were analysed at JSI for Sb, As, Cu, Ba, Cd, Cr, Ni, V, Pb, Co, Al, Mn, Mo, Fe, Zn, Sn, Sr and Rb. The analysis of total Hg is underway.

Determination of elements in drinking water by ICP-MS

The samples of drinking water were acidified with 1 mL of suprapure HNO_3 per 1 L of sample. The concentrations of selected elements were determined in the samples directly, without any sample pre-treatment, by inductively coupled mass spectrometry (ICP-MS) at optimal measurement conditions. For checking the accuracy SPS-SW1 Quality Control

Material for Surface Water Analysis from SPS Spectrapure Standards AS (Oslo, Norway) was used.

Table 4: Determination of elements in Quality Control Material for Surface Water analysis SPS-SW1 (μg L^{-1}) by ICP-MS. The result represents the mean ± standard deviation of three independent analyses.

QCM	Element	Certified value	Determined value
SPS-SW1	As	10.0 ± 0.1	9.4 ± 0.9
	Cd	0.5 ± 0.01	0.49 ± 0.01
	Co	2.0 ± 0.02	1.95 ± 0.04
	Cr	2.0 ± 0.02	1.97 ± 0.02
	Cu	20 ± 1	19.5 ± 0.8
	Fe	20 ± 1	20.3 ± 0.6
	Mn	10.0 ± 0.1	9.8 ± 0.2
	Mo	10.0 ± 0.1	10.0 ± 0.1
	Ni	10.0 ± 0.1	9.7 ± 0.2
	Pb	5.0 ± 0.1	5.1 ± 0.1
	Se	2.0 ± 0.02	2.04 ± 0.06
	Tl	0.5 ± 0.01	0.51 ± 0.01
	Zn	20 ± 1	19.5 ± 0.7

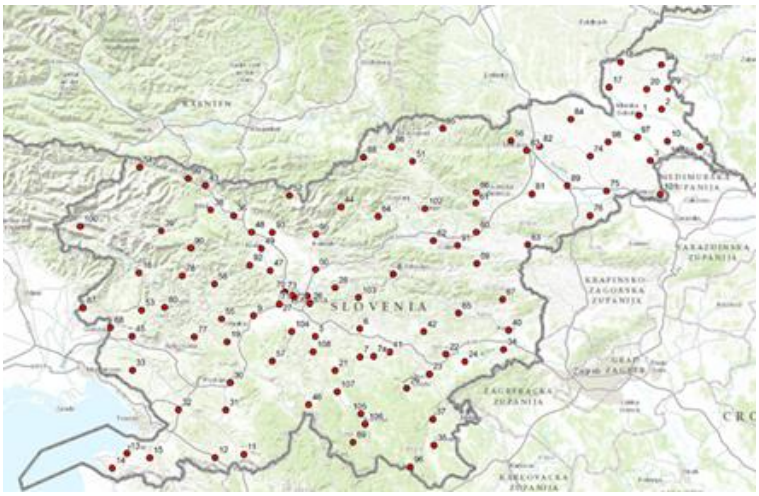


Figure 19: Locations of drinking water measurements.

Results

Database comprises results of concentrations of the selected elements at 108 locations in Slovenia (Sb, As, Cu, Ba, Cd, Cr, Ni, V, Pb, Co, Al, Mn, Mo, Fe, Zn, Sn, Sr, Rb) and Rn level. For each of the sampling sites, number of water supply users, type of raw water for water supply, and information on lithology is available.

1) *Bio-indicators of air quality and deposition*

Sampling and analysis protocol

Moss species *Hypnum cupressiforme* has been collected from 102 locations in Slovenia according to the EMEP[1] grid (50 km x 50 km) (Figure 20) in years 2010 and 2015. The methodology is described in Heavy metals in European Mosses, Survey 2010.

Moss samples collected from the selected locations have been carefully cleaned from all dead material and attached litter, homogenized and freeze-dried.

The samples from the 2010 campaign have been microwave digested and analysed for As, Cd, Cu, Pb, Zn, Mn and Se content by ICP-MS. The analysis of total Hg have been performed by thermal decomposition of the samples, amalgamation and CVAAS detection using Direct Mercury Analyser, DMA-80.

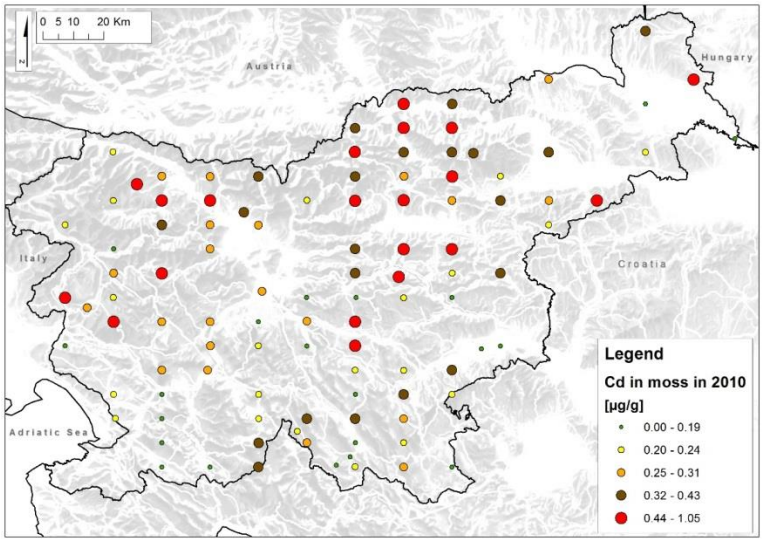


Figure 20: Distribution of elements in moss – an example of Cd concentrations.

[1] Co-operative programme for monitoring and evaluation of the long-range transmission of air pollutants in Europe. http://www.emep.int/

Results

Database comprises the results of measurements of As, Cd, Cu, Hg, Pb, Zn, Mn and Se in moss *Hypnum cupressiforme* at 102 locations in Slovenia. For each of the sampling site, land use/cover and topography of the site is available.

2) *Geochemical map of Slovenia*

Database comprises geochemical soil maps in raster format with 100 m resolution for the following chemical elements: Al, Ca, Fe, K, Mg, Na, P, Ti, Ba, Cd, Cr, Cu, La, Mn, Mo, Nb, Sc, Sr, Th, V, Y, Zn, Zr, and Hg. Maps covering the whole country were created by Geological Survey of Slovenia, based on geodatabase comprising on average 2500 soil analysis, with the exception of Hg (961 analyzes).

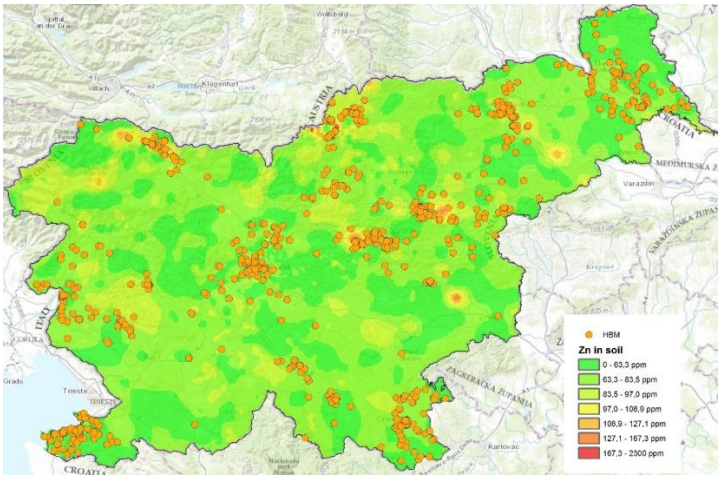


Figure 21: Geochemical map of Slovenia – an example of Zn concentrations in topsoil.

3) *Air Quality data – Country scale*

AQ monitoring network in Slovenia is relatively scarce with approx. 20 stations operating in the country. Therefore, due to a lack of more detailed spatial information or suitable AQ models, interpolated maps showing air quality in Europe will be used. European Environmental Agency (EEA) provides AQ maps for O3, PM10 and PM2.5 at a resolution of 10 km in the 2006-2012 period. Maps are based on combination of Airbase background station monitoring data EMEP station monitoring data (not considering industrial and traffic station types), meteorological ECMWF data and EMEP concentration modelling data.

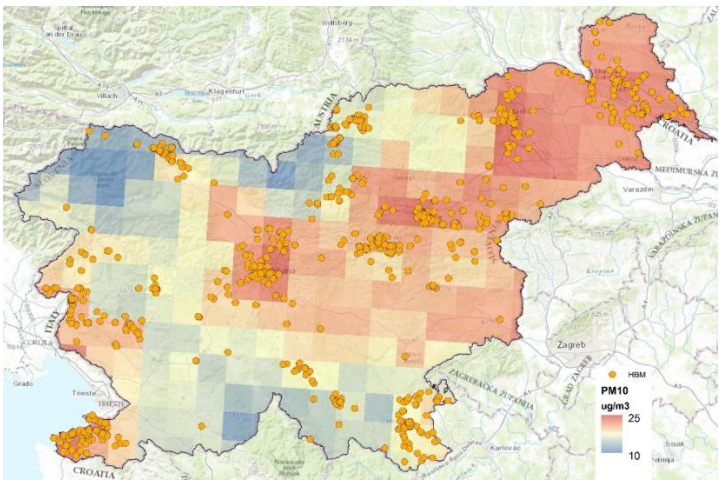


Figure 22: EEA interpolated air quality data – an example of average annual PM10 concentrations for 2012.

exposure assessment AQ data collected (PM, NOx, CO, O3, T, RH, AP) within CITI-SENSE monitoring network will be tested.

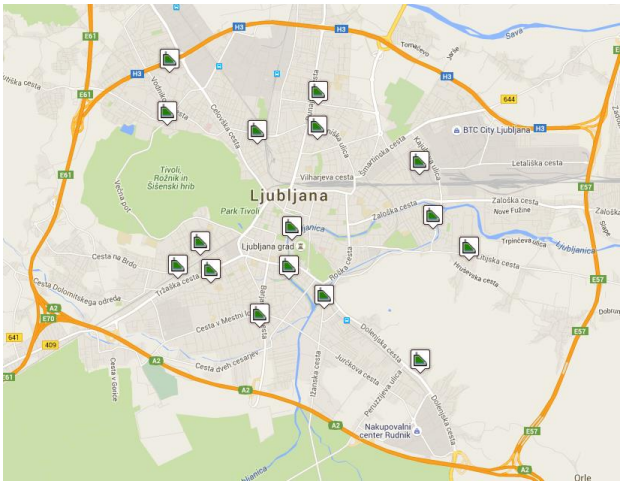


Figure 23: AQ monitoring network in Ljubljana within the CITI-SENSE EU project.

4) Information on releases of contaminants from various sources

Emissions and releases from point sources: In Slovenia there are over 170 facilities reporting releases of various pollutants into the European Pollutant Release and Transfer Register (E-PRTR).

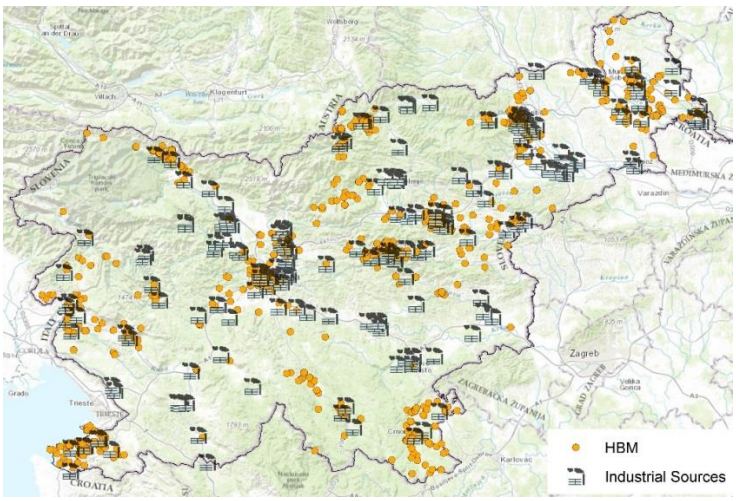


Figure 24: Industrial facilities in Slovenia reporting various pollutant releases to air and water as part of European PRTR.

5) Releases from Diffuse Sources to Air

Within the European Pollutant Release and Transfer Register (E-PRTR) spatial distribution and emission of air pollutants from diffuse sources are also available. The data is based on a scale of 5 by 5 km grid and includes details of NOx, SOx, CO, ammonia NH3, carbon dioxide CO2 and PM10. The following sectors will be considered: industrial releases, non-industrial combustion, road transport and agriculture.

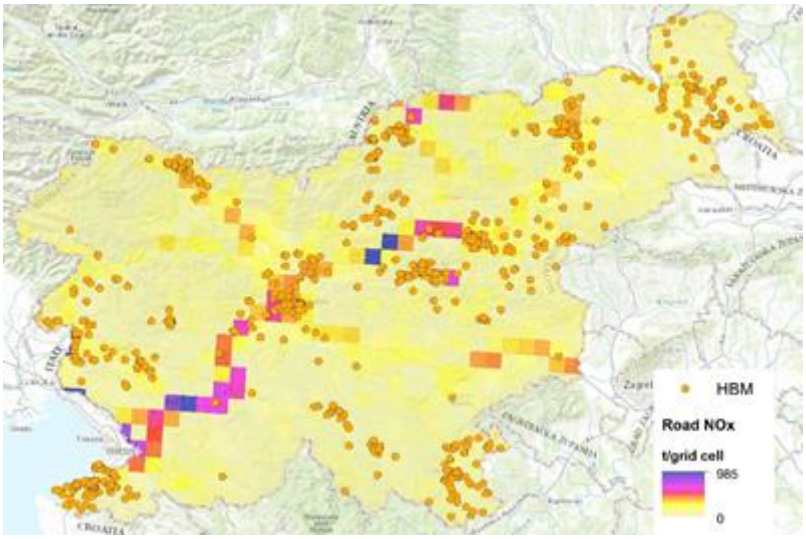


Figure 25: Diffuse sources to air based on E-PRTR – an example of NOx emissions associated with traffic.

6) N and P releases

Modelled nitrogen and phosphorous surpluses were obtained from the Geological Survey of Slovenia. Data covering the whole country of Slovenia is available in raster format with 100 m resolution.

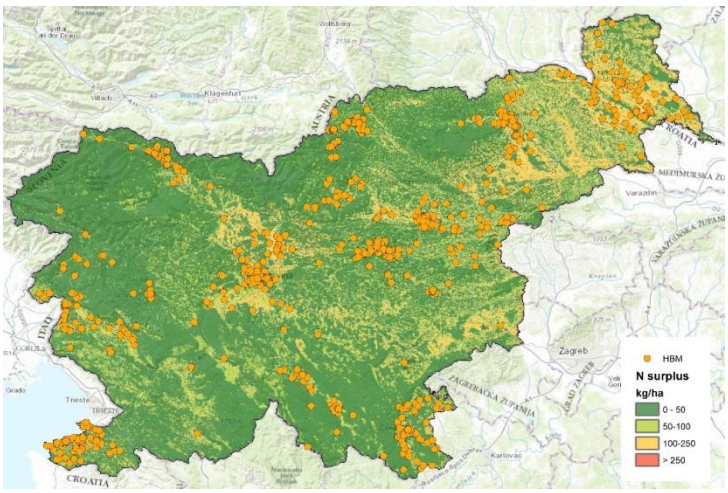


Figure 26: Nitrogen surplus map of Slovenia

7) Build environment

Cadastre of public infrastructure comprises various data on public infrastructure: transport infrastructure, energy infrastructure, utility and water infrastructure. Within CROME, information on road infrastructure will be mainly used as a proxy for exposure to air pollutants from traffic, especially for regions were detailed information is missing.

Building Cadaster is the main record in Slovenia of the data on buildings and parts of buildings. Among others, the database comprise the following graphical and attribute data on buildings that will be used for HBM exploratory data analysis (type, age, heating system, isolation type, type and age of the utilities).

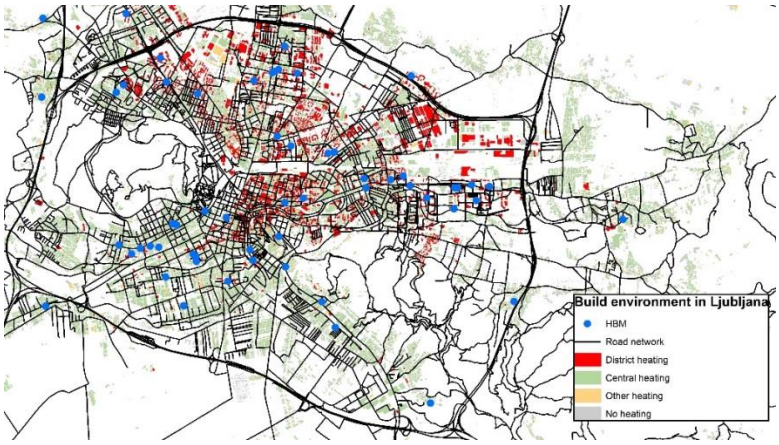


Figure 27: An example of some of the building environment characteristics in Municipality of Ljubljana

Biomonitoring data

Sampling and analysis protocol

The study included lactating *primiparous* women and their partners, 20-45 years of age, from 12 different areas of Slovenia. Among the study areas were 6 contaminated (selected due to recent-smelters, cement factory, power plant, glass factory, or past industrial activities - former Hg mine, former Pb mine, former factory of transformers and capacitors – PCBs pollution), 3 urban and 3 rural areas, in each 50 women and 50 men were planned for recruitment. The mothers who agreed to participate and were eligible for the study donated 16 mL of venous blood, 50 mL of urine, 1 g of scalp hair and max. 80 mL of breast milk; and the eligible partners 23 mL of blood, 50 mL of urine and 1 g of scalp hair. Sampling was carried out in week 2-8 after the delivery. Along with the sampling, mothers and their partners completed a questionnaire to obtain data on residential environment, nutritional habits and life-style in general, their socio-economic status and potential exposure.

Blood, urine and milk samples were analysed for total mercury by CVAAS or CVAFS technique and also for other elements (cadmium, lead, arsenic, selenium, copper, zinc) by ICP-MS. Detailed sampling and analysis protocol is described in the official HBM report by Horvat et al (2015, HBM final report).

Results

The results of the first Slovenian HBM are reported by Horvat et al (2015, Monitoring Kemikalij v Organizmih 2011-2014).

Table 5:Levels of toxic metal(loid)s in blood [ng/mL] of women and their partners from 12 study areas in Slovenia.

Population group	N	N<LOD	GM	95% CI	min-max	P5	P10	P90	P95
Cadmium									
Total	1085	302	0.28	0.27-0.30	<LOD-4.80	<LOD	<LOD	0.72	1.01
Women	536	71	0.35	0.33-0.37	<LOD-3.08	<LOD	0.14	0.71	0.87
Men	549	231	0.23	0.22-0.25	<LOD-4.80	<LOD	<LOD	0.80	1.22
Lead									
Total	1086	0	18.0	17.5-18.5	3.86-116	9.13	10.3	32.7	41.5
Women	537	0	16.7	16.2-17.3	4.25-71.9	8.82	10.3	28.0	33.2
Men	549	0	19.3	18.5-20.1	3.86-116	9.16	10.4	37.4	46.1
Mercury									
Total	1085	2	1.18	1.12-1.24	<LOD-31.0	0.30	0.41	3.41	4.78

Women	536	1	1.11	1.04-1.19	<LOD-10.2	0.31	0.43	3.09	4.06
Men	549	1	1.25	1.15-1.34	<LOD-31.0	0.26	0.38	3.72	5.13
Arsenic									
Total	1086	1	0.89	0.85-0.93	<LOD-28.9	0.35	0.40	2.62	3.74
Women	537	0	0.96	0.89-1.03	0.20-28.9	0.32	0.39	2.90	5.16
Men	549	1	0.83	0.79-0.88	<LOD-22.4	0.36	0.40	2.12	3.40
Manganese									
Total	812	0	13.8	13.5-14.2	5.69-40.5	8.18	9.02	22.1	26.2
Women	410	0	17.1	16.7-17.7	5.83-40.5	10.4	11.5	25.9	28.0
Men	402	0	11.1	10.5-11.0	5.69-29.8	7.64	8.26	15.0	16.4

Table 6:Levels of toxic metal(loid)s in blood [ng/mL] of women and their partners from 12 study areas in Slovenia.

Population group	N	N < LOD	GM	95% CI	min-max	P5	P10	P90	P95
Copper									
Total	1086	0	951	941-961	532-2004	737	766	1200	1272
Women	537	0	1071	1058-1084	657-2004	857	908	1262	1371
Men	549	0	847	839-856	532-1404	708	738	976	1041
Zinc									
Total	1086	0	6607	6550-6665	3010-11733	5150	5518	7809	8298
Women	537	0	6724	6639-6810	3010-11733	5274	5647	8027	8494
Men	549	0	6495	6420-6572	3400-10301	5082	5460	7646	8050
Selenium									
Total	1086	0	105	103-106	53.9-226	74.2	80.5	138	152
Women	537	0	94.7	93.2-96.2	53.9-176	70.7	75.1	119	127
Men	549	0	115	114-117	60.3-226	87.3	91.7	152	166

Table 7: Levels of toxic metal(loid)s in urine [µg/g creatinine] of women and their partners from 12 study areas in Slovenia.

Population group	N	N < LOD	GM	95% CI	min-max	P5	P10	P90	P95
Cadmium									

Total	989	14	0.20	0.19-0.21	<LOD-2.79	0.07	0.09	0.47	0.61
Women	489	12	0.26	0.25-0.28	<LOD-2.79	0.10	0.12	0.58	0.79
Men	500	2	0.16	0.15-0.16	<LOD-1.14	0.06	0.07	0.32	0.42
Lead									
Total	812	249	0.49	0.46-0.51	<LOD-5.31	0.15	0.20	1.19	1.49
Women	410	184	0.49	0.45-0.52	<LOD-3.44	0.15	0.19	1.27	1.57
Men	402	65	0.49	0.46-0.52	<LOD-5.31	0.15	0.22	1.12	1.42
Mercury									
Total	1020	12	0.47	0.43-0.50	<LOD-8.67	0.06	0.11	1.78	2.48
Women	504	10	0.52	0.47-0.58	<LOD-8.67	0.07	0.11	1.92	2.64
Men	516	2	0.42	0.38-0.46	<LOD-7.92	0.06	0.09	1.50	2.19
Arsenic									
Total	812	0	6.70	6.24-7.19	0.37-499	1.71	2.17	26.6	50.5
Women	410	0	7.71	7.02-8.48	0.37-219	2.15	2.53	28.6	48.8
Men	402	0	5.80	5.22-6.43	0.76-499	1.52	1.85	24.5	52.9

Table 8: Levels of metal(loid)s in maternal milk [ng/mL] from 12 study areas in Slovenia.

	N	N < LOD	GM	95% CI	min-max	P5	P10	P90	P95
Toxic elements									
Cd	471	262	<LOD	-	<LOD-0.34	<LOD	<LOD	0.14	0.18
Pb	471	161	0.35	0.32-0.39	<LOD-10.6	<LOD	<LOD	1.05	1.81
Hg	471	18	0.14	0.13-0.16	<LOD-3.39	<LOD	0.04	0.51	0.64
As	471	9	0.18	0.17-0.19	<LOD-3.70	0.07	0.08	0.48	0.79
Essential elements									
Cu	471	0	356	345-366	99-954	221	250	525	578
Zn	471	0	1937	1844-2034	207-7904	740	1011	3701	4404
Se	471	0	12.6	12.3-13.0	5.36-38.1	8.13	9.16	17.6	19.4

Table 9: Total mercury in hair [ng/g] of lactating women and their partners from 12 study areas in Slovenia.

Population group	N	N < LOD	GM	95% CI	min-max	P5	P10	P90	P95

Total	947	0	275	258-292	10.0-7068	48.0	79.5	867	1203
Women	503	0	268	248-290	10.0-1947	52.3	82.0	731	993
Men	444	0	282	256-311	11.0-7068	46.0	77.0	984	1396

Among the elements investigated, Pb blood levels showed the highest levels in Pb-smelter area of Mežiška valley (Figure 28). In this area, blood Pb levels were not associated with smoking nor with type of water supply, but were strongly correlated with soil Pb levels (r=0.44, p<0.001). In other areas of the country, blood Pb levels were not associated with the levels in soil, however the overall correlation was significant (Figure 29).

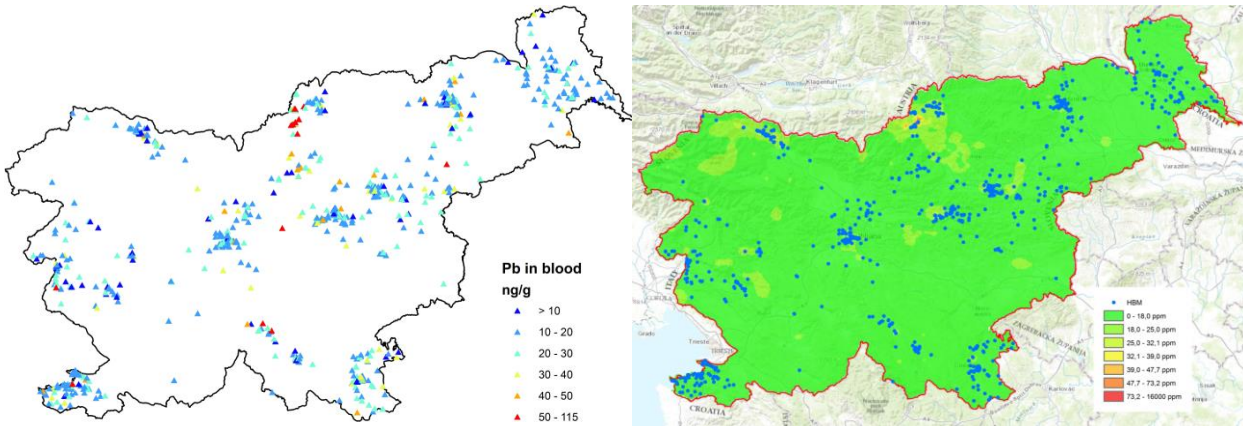


Figure 28: Geo-located Pb levels in blood of HBM study participants (left) and Pb levels in soil (right).

Similarly as blood Pb, milk Pb levels were correlated with soil Pb level significantly, but the milk levels were the highest in the capital, Ljubljana, and not in the Mežiška valley. Increased milk Pb in urban vs. other areas might be due to increased emission of particulate matter in urban environment. Indeed, emission of PM10 from residential sources and from road was the highest in Ljubljana and it associated significantly with milk Pb levels in the model adjusted for other co-variates.

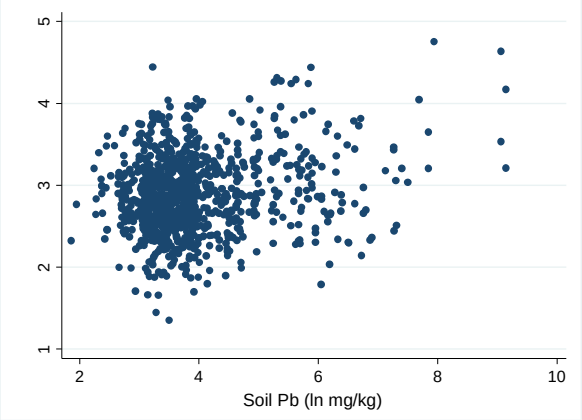


Figure 29: Correlation between Pb in soil and Pb in blood of study population living in Slovenia (r=0.19, p<0.001, n=1043).

Mercury levels in hair and blood were found to be area dependant, and were the highest in the coastal part of Slovenia (Figure 30), where the people consume sea food more frequently than in other parts of the country. In contrary, the levels in urine were not dependant on the geolocation (Figure 30) and were associated with the presence of amalgam fillings, consumption of fresh and canned sea fish. The levels in milk were associated significantly with amalgam fillings and consumption of fresh sea fish.

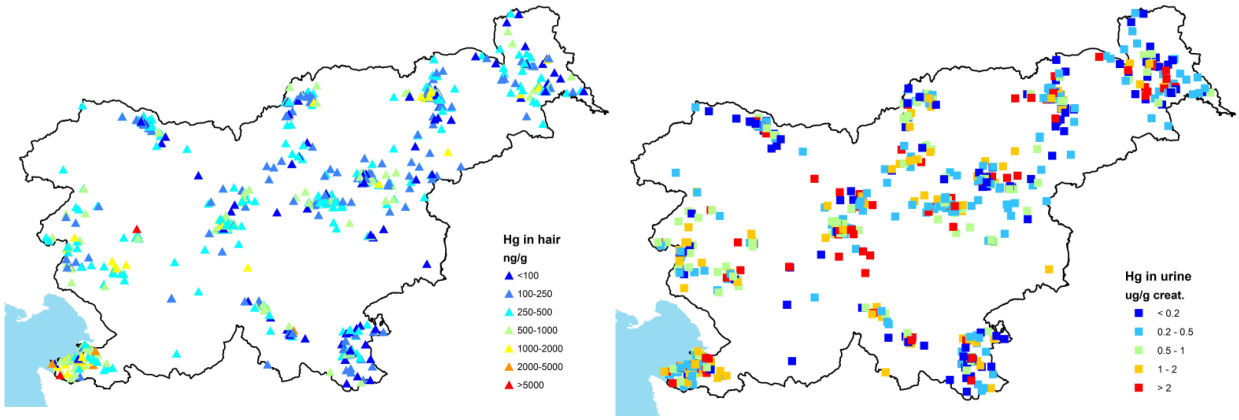


Figure 30: Mercury (Hg) levels in hair (left) and urine (right) of the Slovenian study population.

The Hg levels in blood, urine and hair were in positive correlation with the levels of Hg in soil (Figure 31), the correlation stayed significant also after adjustment for other significant co-variates. The associations observed were predominated by the area of a former mercury mine (the town of Idrija), which is clearly demonstrated from the Figure 31.

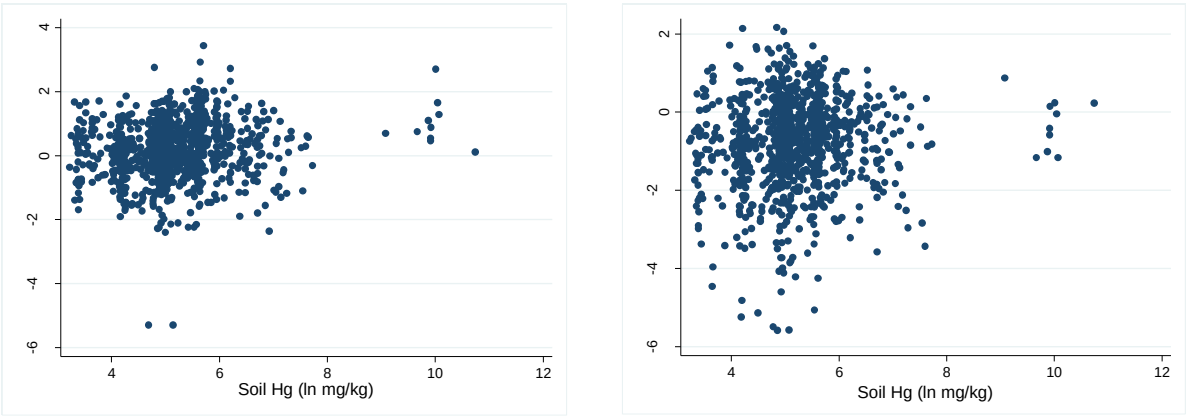


Figure 31: Correlation between Hg in soil and Hg in blood (left) or urine (right) of study population living in Slovenia (r=0.15, p<0.001, n=1042; and r=0.08, p=0.01, n=987, respectively).

The area-related Cd levels were suggested to be associated with artificial fertilizer use that potentially contain Cd. For this purpose, HBM based Cd levels were linked to the maps of nitrogen and phosphorous surpluses used as proxy for fertilizer use. No association was discovered, therefore, based on the collected data higher Cd exposure in rural areas of

Slovenia could not be associated with the use of Cd-containing fertilizers. Certain significance of regional road proximity to participants homes in Cd exposure was observed by univariate correlations, but the association was insignificant after adjustment for other co-variates.

Manganese levels were lower in participants living in contaminated areas than in those living in rural or urban areas. The levels were not associated with concentration of Mn in tap water.

Conclusions

Linking HBM and environmental databases confirmed geo-dependant environmental nature of Pb and Hg exposure in the area of Pb smelter (Mežica Valley) and the former mercury mining town of Idrija (Hg), while the rural area related Cd was not associated with activities related to farming, namely artificial fertilizing. Apart of the Pb-smelter related exposure, public water supplies seem to be an important source of Pb (and also Mn). As a possible source of Pb exposure in the urban environment, emission of particulate matter from residential sources and roads was revealed.

2nd case study – Human Biomonitoring in Slovenia - POPs in serum and milk samples from National human biomonitoring

Introduction

Persistent organic pollutants (POPs) were determined in biological samples (serum and maternal milk) collected within the national Human Biomonitoring programme described above (1st case study) as this data was missing. The data on potential exposure as a consequence of a life-style was obtained through a questionnaire that the participants answered within the HBM study, while the summary of environmental data collected is given below.

Summary of existing environmental and biomonitoring data – gaps identification

The Environmental Indicators in Slovenia report and web presentation have been prepared in accordance with Article 106 of the Environment Protection Act (OJ RS No 41/2004). It comprises 51 environmental indicators, selected from a wider set of indicators proposed by the European Environment Agency. One part is chapter ZR13 Persistent organic pollutants emissions. Main conclusion is that emissions of persistent organic pollutants in Slovenia in the period 1990-2013 decreased primarily due to a combination of targeted legislation, improved control and use of best available technologies.

Slovenian Environment Agency has also published INFORMATIVE INVENTORY REPORT 2015 FOR SLOVENIA, Submission under the UNECE Convention on Long-Range Transboundary Air Pollution. As part of this report there is a chapter 2.6.4 Emission Trends for Persistent Organic Pollutants In Slovenia emission national emission factors are not available; therefore they were taken from EMEP/EEA Emission guidebook 2009 and older issues. Important source of emission factors was research project conducted by Jožef Stefan Institute (Kanduč, 2009), where various scientific literature was examined and adopted for emission calculation. Uncertainty of the applied emission factors is one of the key reasons for the substantial uncertainties in the emission estimates, as most activity data are firmly believed to be far more accurate. Persistent Organic Pollutants have been reported: 1) - Polycyclic aromatic hydrocarbons (PAHs): benzo(a)pyrene, benzo(k)fluoranthene, benzo(b)fluoranthene, indeno(1,2,3-cd)pyrene, 2) Dioxins and furans, 3) - Hexachlorobenzene (HCB), 4) - Polychlorinated Biphenyls (PCB). Emissions of PCB, dioxins and furans, PAH and HCB declined since 1990 as a result of decreased residential use of coal, improvements in abatement technologies for metal refining and smelting, and stricter regulations on emissions from the road transport sector. Implementation of legislation, stricter inspection and use of best available techniques have been responsible for decrease of POPs in last two decades. Emissions of PCB declined substantially from year 1990 to 2013: for PCB (87,3%), dioxins/furans, (32,2 %), PAH (17,5 %) and HCB (98,8 %). Slovenia in 2013 did not exceed

emission levels set in protocol on persistent organic pollutants. Emissions are much below values from the reference year 1990. This report and NFR tables are available to the public on the EIONET central data repository: http://cdr.eionet.europa.eu/si.

Geographic differences in exposure of population due to the specifics of local environment must be taken into account (e.g. locations of past and current industrial activities, spatial distribution of pollutants in different environmental compartments...). Important source about local exposure data are reports on a local level which are mainly in Slovene and in some cases also articles in international journals:

- Poročilo o preiskavah tal, krme, kmetijskih pridelkov in živil živalskega izvora na območju Bele krajine ter rib iz reke Krupe in Lahinje, 131-11/5721-11 (MKGP-IRSKGH), 133-11/4993-11 (MKGP-VURS), Maribor (16.11.2011, april 2012)
- Preiskave vzorcev živil na vsebnost dioksinov in dioksinom podobnih PCB ter dioksinom nepodobnih PCB, Št. 133-11/5485-11, Maribor (julij 2011)
- "Posledice vpliva PCB na okolje v Beli krajini z oceno tveganja za zdravje ljudi zaradi uživanja doma pridelanih živil (jajca, mleko, perutnina) in rib iz reke Krupe, glede na vsebnost PCB". Št. 411-95/11, ZZV Novo mesto, Novo mesto, maj 2011
- V. Polič, Recenzija zaključnega poročila »Posledice vpliva PCB na okolje v Beli krajini v letu 2005« (Zavod za zdravstveno varstvo Novo mesto, št. 421-43/05), Institut "Jožef Stefan", Odsek za znanosti o okoljuEC SEPO, Jamova39, 1000 Ljubljana, Št. IJS -DP –9231, Ljubljana (16. november 2005)
- Polič, A.S., Onesnaženost in ekološke obremenitve okolja kraškega območja reke Krupe s polikloriranimi bifenili –PCB, Doktorska disertacija (izbrana poglavja iz disertacije), Univerza v Ljubljani, Biotehniška fakulteta, Področje Varstvo okolja, Ljubljana (2005).
- Ocena zdravstveno –ekoloških razmer na območju občin Metlika in Črnomelj v Beli krajini v zvezi z ekološko obremenitvijo s polikloriranimi bifenili, UZZSV Ljubljana, drugi zavodi in inštitucije, Ljubljana (28.02.1992)
- 2007–2009 Monitoring kemikalij v organizmih 2007 –2009, MZ/URSK, IJS, ZZV Maribor, ZZV Novo mesto, UKC Ljubljana, št. IJS -DP –10523, Ljubljana (14.07.2010)
- Pregledna ocena stanja obremenitev okolja s pcb v beli krajini, z njimi povezanih tveganj za zdravje ljudi, predlog priporočil in ukrepov za prebivalce bele krajine in za druge deležnike, povezane s prehrano prebivalcev, ZZV Maribor 2012
- Miklavčič Višnjevec, Ana, Stibilj, Vekoslava, Hearth, Ester, Polak, Tomaž, Snoj Tratnik, Janja, Klavž, Janez, Mazej, Darja, Horvat, Milena. Mercury, selenium, PCBs and fatty acids in fresh and canned fish available on the Slovenian market. *Food*

chemistry, ISSN 0308-8146. [Print ed.], 2011, vol. 124, issue 3, str. 711-720, doi: 10.1016/j.foodchem.2010.06.040. [COBISS.SI-ID 23731239],

Additional data collected

Biomonitoring data

Sampling and analysis protocol

Maternal breast milk samples were collected within the national HBM study, 6-8 weeks after birth.

POPs were analysed at National Laboratory for Health, Environment and Food which is part of the European network laboratories for POP and is constantly being controlled by the European Reference Laboratory EURL (http://www.eurl-pesticides.eu/docs/public/home). The lab is using validated (SIST EN ISO/IEC 17025) methods: brominated diphenyl ethers (PBDE), PCB-congeners and chlorinated dibenzodioxins and dibenzofurans (PCDD/F) were determined by HRGC/HRMS based on the EPA methods (EPA 1614, EPA 1668A, and EPA 1613 B modified).

Results

DDT-p,p-DDE was detected in 98 % of all milk samples, but in none of the samples the levels exceeded the reference values for non-contaminated environments (sum DDT).

Hexachlorobenzene (HCB) was detected in 16 % of all milk samples, and 12 % of the samples exceeded the reference value for non-contaminated environments. Trace levels were detected in samples from all participants.

Indicator PCB congeners (PCB 118, PCB 138, PCB 153 in PCB 180) were detected in 55 % of all milk samples, trace levels were present in all samples. The measured levels did not exceed the reference level for non-contaminated environments. In the area of Bela Krajina, where there is historical PCB contamination due to industrial activities, the highest levels of PCBs in milk were observed (max 0.513 mg/kg milk fat), but based on the levels we can say that the inhabitants in this area are not exposed to PCBs in such extent as in the times PCBs were extensively produced and used in the consumers materials.

The presence of organochloride dieldrin was detected in two samples (area of Ljubljana and Maribor), and it exceeded the reference level for non-contaminated environments. Presence of beta-HCH was detected in nine milk samples, while other compounds from the HCH group were not detected.

DDT-p,p-DDE was detected in 43 % of all serum samples; in 3 % of the samples it exceeded the reference level for non-contaminated environments. Median levels showed more or less

equal exposure among the participants from all areas, with some individual cases of elevated levels.

Indicator PCB congeners (PCB 118, PCB 138, PCB 153 in PCB 180) were detected in 11 serum samples. The determined levels did not exceed the reference level for non-contaminated environments. The maximum observed level was from the area of Bela Krajina (1.70 µg/kg). Median levels showed more or less equal exposure among the participants from all areas, with some individual cases of elevated levels.

Gama-HCH was detected in 3 samples (all from Bela Krajina), HCB in 4 samples and dieldrin in 1 sample. The detected level of gama-HCH was significantly higher than the limit of detection (0.20 µg/kg) in all 3 samples, the maximal level determined was 1.0 µg/kg. The HCB levels observed in serum did not exceed the reference level for non-contaminated environments.

Based on the results of individual samples of milk and serum, exposure of the Slovenian general population to DDT-p,p-DDE and indicator PCB congeners was confirmed.

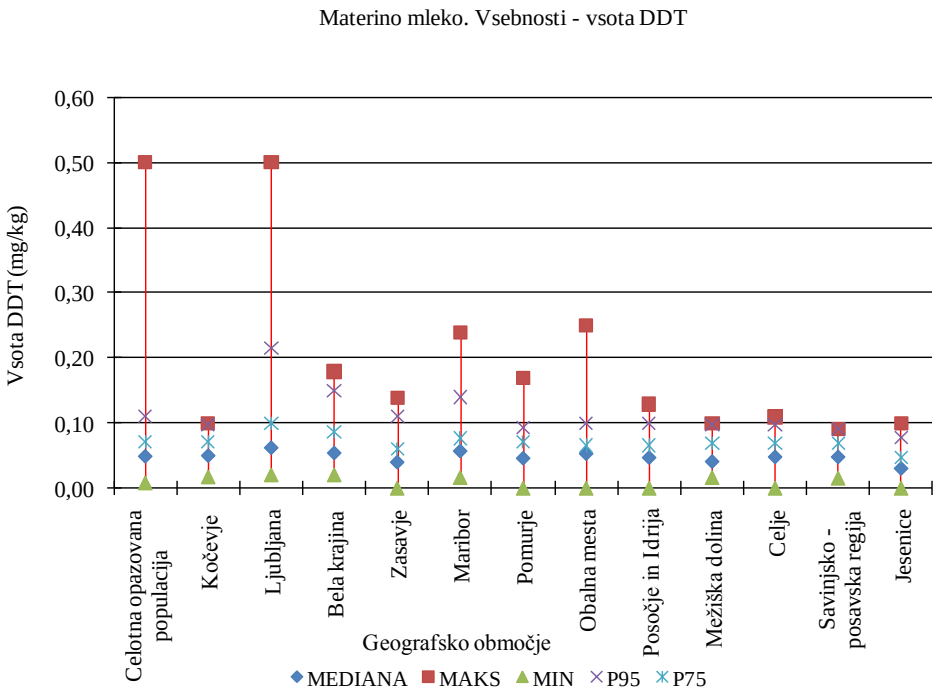


Figure 32:Maternal milk: sum DDT

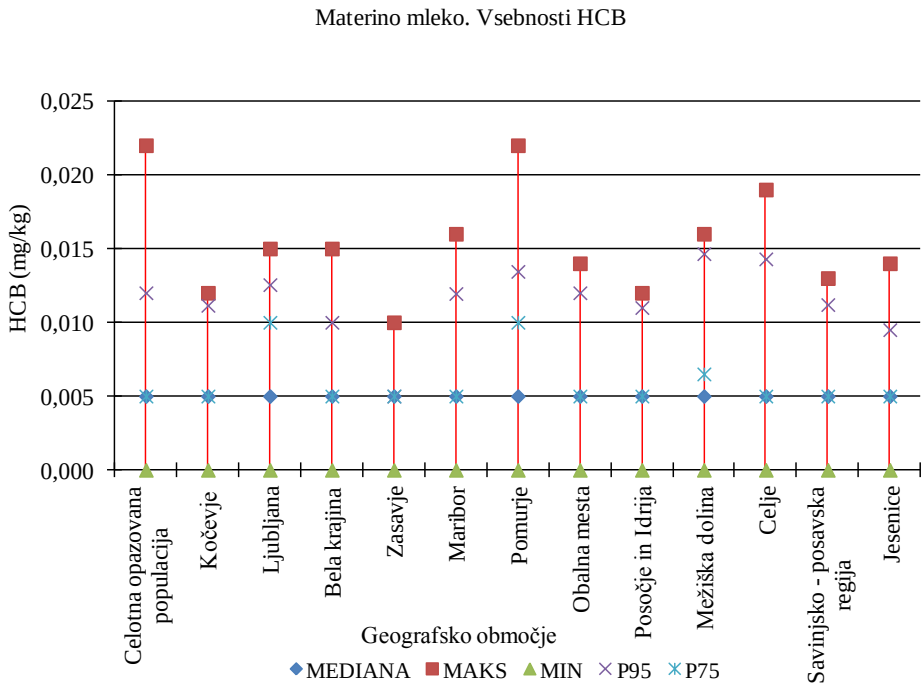


Figure 33: Maternal milk: sum HCB

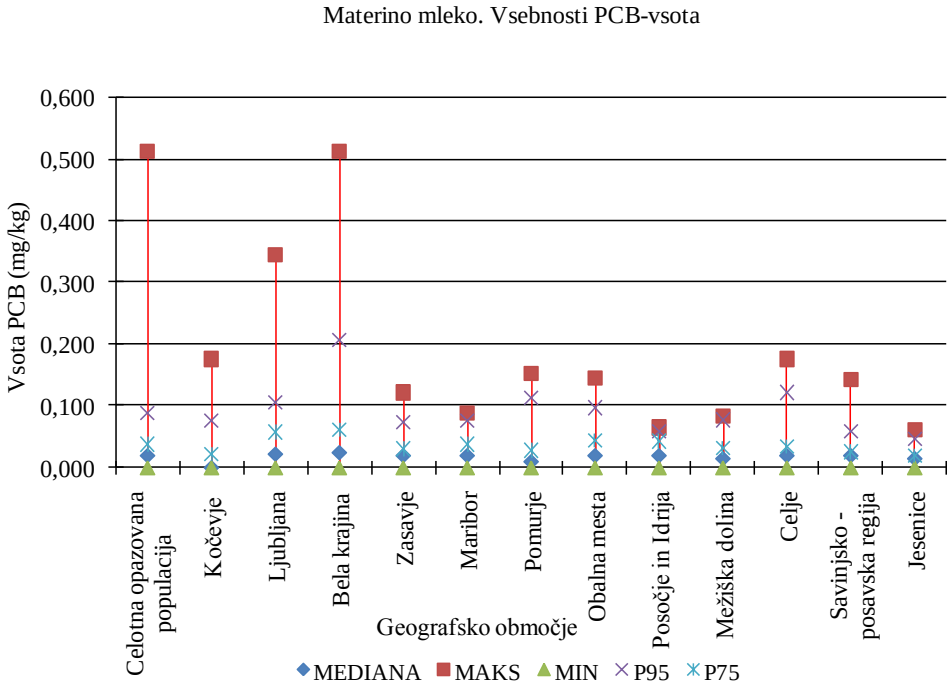


Figure 34: Maternal milk: sum PCB

Conclusions

Based on the results of individual samples of milk and serum, exposure of the Slovenian general population to DDT-p,p-DDE and indicator PCB congeners was confirmed. Based on

the overall results we can conclude that there is a certain exposure of Slovenian population to POPs, but the levels are lower compared with the results from HBM data in the WHO European Region (Human biomonitoring: facts and figures. Copenhagen: WHO Regional Office for Europe, 2015).

Case study Italy - Human Biomonitoring in Italy

1st case study – Human Biomonitoring in Italy – Heavy metals

In the context of the PROBE (PROgramme for Biomonitoring of the Exposure), a biomonitoring campaign of Italian population started in 2008 for several metals in blood, a special survey was addressed to the adolescents and conducted in cooperation with the National Association against Microcytemia (ANMI) during the annual screening for thalassemia among the scholastic population. Children are more vulnerable to environmental stressors than adults, and have less control over their environment. Although evidences indicating impact on child's development are growing, understanding of environmental risks to children remains partial. Environmental stressors may affect children with different vulnerability, at different developmental stages (foetal, infancy, early childhood, adolescence) or even in preconception period. Furthermore, children consume more food and water compared to adults when expressed per kg of body weight, resulting in relatively higher exposures to adverse compounds. Also specific dietary patterns of children may contribute to a higher exposure to contaminants present in food. Apart from having a higher exposure, children also have a different physiology from adults in terms of detoxification capacity.

Introduction

Primary objective of the PROBE adolescent survey was to supply representative data on the metals' internal dose in adolescents in order to highlight the environmental impact on the this group of the population. The activities carried out were devoted to:

- provide a database to derive reference values (RVs) for the assessment of children's and adolescents' environmental exposure.
- examine the possible influences of certain variables (demographics and habits) on the metal level of individuals.

The objective of PROBE adolescent study in the context of the CROME project has been extended to take into account different sources of exposure, lifestyle and diet, their geolocalization, and environmental data to give a holistic vision of the population consistent with the exposome concept as a tool for a more complete exposure assessment in HBM studies.

Methodological procedures

Population

An adolescent cohort of 453 subjects, aged 13-15 years, living in urban and rural areas of Latium region was enrolled for their exposure to metals. The adolescent distribution in the four sampled areas was: 160 from Viterbo (small town), 131 from Fontenuova and 72 from

Monterotondo (rural semi-rural areas), and 89 from Rome (big town). Non-fasting blood specimens were obtained by ANMI during the annual screening for microcytemia in schools (2009). In blood sample 19 metals were investigated: As, Cd, Co, Cr, Hg, Ir, Mn, Mo, Ni, Pb, Pd, Pt, Rh, Sb, Sn, Tl, U, V, and W.

For each donor a questionnaire was also filled in order to collect information on: general data such as gender, piercings, junk jewelry, diet habits, drinking water, lifestyle factors (e.g. exposure to environmental tobacco smoke, alcohol consumption, use of cosmetics, etc.), dental fillings and braces, family occupational status. Socio economic status (SES) of the family was derived merging the educational level and the occupational status of the parents; the International Standard Classification of Occupations (International Standard Classification of Occupations, 2008) was adopted to describe the parental occupations. From the questionnaires administered several information were obtained: 138 adolescents had dental braces and/or fillings while 49 got piercing and 93 adolescents had parents smoking at home. Relating to the diet habits, 265 adolescents consumed fish 1 time a week (1/w) and 81 2 times a week or more (≥2/w) while 241 had milk every day (7/w), 63 from 4 to 6 times a week (4-6/w) and 99 from1 to 3 times a week (1-3/w).

The exposure assessment was completed by geo-referencing all subjects with their residence address location by GIS tools and considering also the lifestyle of the adolescents and their families.

Environmental data

To have a view of the exposure in the exposome sense, metal concentrations were related to environmental data of air and water quality supplied by Regional Environment Authority (Latium Region). Kriging techniques were applied to derive spatially resolved concentration of chemicals in the outdoor air starting from data collected by air monitoring stations. Chemicals considered were NOx, PM10, PM 2,5, benzene, CO, NO2, O3.

Analysis

Blood samples were stored at -20 °C and in the laboratory 1 mL of blood sample out of ca. 1.5 mL collected was microwave digested (Milestone ETHOS MEGA II, FKV, Bergamo, Italy) with ultra-pure nitric acid (Romil Ltd., Cambridge, UK) and subsequently diluted with high purity deionized water (EASY Pure system, Barnstead, Dubuque, USA). The sector field inductively coupled plasma mass spectrometry (SF-ICP-MS, Element 2, Thermo Scientific, Bremen, Germany) analyses were carried out to determine metals content.

The method used for blood analysis was validated by ACCREDIA (the Italian National Accreditation Body) and the following validation performances were assessed: linearity, Limit of Detection (LoD) and Limit of Quantification (LoQ), specificity, accuracy (precision

and trueness), robustness and extended uncertainty (AOAC, 1998; Commission Decision 2002/657/EC; LGC, 2003; NATA, 2009; Thompson et al., 2002).

Statistical analysis

The basic statistics of data relating to 453 adolescents included percentiles, geometric mean (GM) and the 95% confidence interval for the geometric mean (CI, GM). Geometric mean represents better the central tendency of data if the distribution is asymmetric instead of the median index. In the statistical evaluation values below the LoD were taken into account as LoD/2 and extreme values were excluded. This procedure was used to derive Reference Values (RV) where the 95th percentile describes the upper value useful in health care field and environmental policy. The adolescent cohort was also stratified by some characteristics including sex, residence area in turn associated to traffic intensity, presence of dental fillings and/or braces, piercings and tattoos, second hand smoke, fish and milk consumption, SES. Each variable was coded according to the levels applied in the questionnaires: number of dental fillings and/or braces, piercings and tattoos (0: no; 1: yes), second hand smoke (0: no; 1: at home; 2: outdoor), frequency intake of fish (0: never; 1: 1 time/week; 2: >=2 times/week) and milk consumption (0: never; 1: 1-3 times/week; 2: >3 times/week; 3: 7 times/week), SES of the family (0: low; 1: medium; 2: high). For all the comparison and other statistical analysis the data base, included the extreme values, was considered. Differences for each metal concentration among subgroups based on the different variables were tested by Mann-Whitney U test, or Kruskall-Wallis or Wilcoxon test (depending on the number of levels for each grouping variable). Mann-Whitney U test with Bonferroni's correction was used for multiple comparisons, when appropriate. Significance level was set at a $p<0.05$. Statistical calculations were performed by STATA statistical software Release 8.1 (STATA Corporation, TX). To perform geo-statistical analysis all the 453 subjects were geo-referenced on the basis of their residence address in a GIS system and stored in a Geodatabase along with human biomonitoring data, diet habits, environmental data and land cover. Generalized Linear Model (GLM) was used to investigate the associations between human biomonitoring data and diet patters (fish and milk) and land cover. EEA 2006 (http://www.eea.europa.eu/data-and-maps/data/corine-land-cover-2006-raster-3) land cover at high spatial resolution (100 m) was used to analyze possible spatial relationships between the type of land use where adolescents were living and the metals concentration in blood.

Results

The basic statistics derived for 19 metals in the adolescent examined are summarized in Table 10 for all adolescents and in Table 11 for adolescent divided for males and females. Observing the GM values, Pb was the only metal with a different concentration between the two groups, higher in boys than girls (10.7 *vs* 8.73 ug/L). The Wilcoxon or Kruskall-Wallis

tests confirmed these results: blood Hg, Ni and Pb showed differences between sex, Ni at a lower rate and Pb significantly higher in males than girls (Ni: $p<0.05$; Pb: $p<0.0001$) while Hg was higher in girls than males ($p<0.05$). Relating to diet habits, significant differences were observed for fish consumption in As and Hg ($p<0.01$), and for milk consumption in Co ($p<0.05$) (the higher the consumption, the higher the metal concentration). Second-hand smoke was associated to higher levels of As ($p<0.01$) and Pd ($p<0.05$). A group of metals showed significant differences among the residence areas: As, Hg, Mn, Mo, Pb, Sn and V were significantly higher ($p<0.0001$) in the area of Viterbo than in other areas, and Cd, Hg, Ir, Mn, Ni, Pb, Pt, Rh, Sb and U were significantly higher ($p<0.01$) in the area of Rome. Metals related to traffic in the residence zone were As and Pb ($p<0.05$). Lead ($p<0.01$), together with Cr ($p<0.05$), showed significant differences in teens having braces and/or fillings (34%). Finally, socio-economic status of the family was positively associated to As concentration ($p<0.01$).

Table 10: RVs in blood for adolescents of Latium, Italy (µg/L).

Element	Subjects n.	5th	50th	95th	GM	IC 95% GM
As	443	0.13	0.73	2.95	0.71	0.66-0.78
Cd	431	0.13	0.30	0.60	0.29	0.28-0.31
Co	445	0.03	0.09	0.28	0.09	0.09-0.10
Cr	414	0.09	0.30	1.25	0.31	0.29-0.34
Hg	436	0.30	0.83	2.05	0.78	0.73-0.83
Ir *	437	2.50	6.84	15.0	6.71	6.39-7.05
Mn	449	3.36	7.46	16.0	7.22	6.89-7.57
Mo	449	0.54	1.10	2.39	1.11	1.06-1.16
Ni	411	0.18	1.02	2.60	0.94	0.88-1.01
Pb	440	4.16	9.55	21.6	9.60	9.16-10.06
Pd*	440	7.50	22.1	38.6	21.3	20.4-22.2
Pt*	423	5.17	10.9	23.3	10.9	10.4-11.4

Rh*	437	7.50	22.1	35.6	21.2	20.5-22.0
Sb	425	0.17	0.39	0.78	0.37	0.35-0.38
Sn	439	0.18	0.56	1.52	0.57	0.53-0.60
Tl	442	0.02	0.04	0.09	0.04	0.038-0.042
U*	445	2.28	4.85	14.3	5.09	4.83-5.37
V	445	0.03	0.08	0.17	0.07	0.066-0.073
W	444	0.01	0.03	0.08	0.03	0.027-0.030

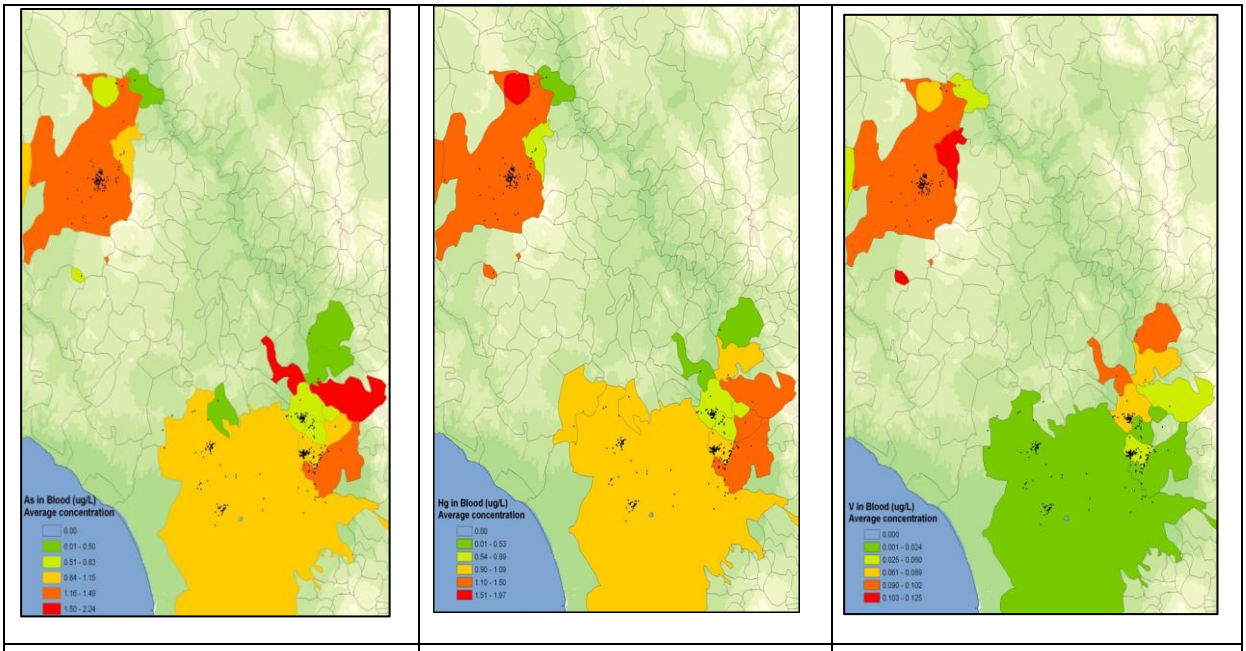
*** ng/L**

Table 11: RVs in blood for adolescents of Latium, Italy (μg/L) by gender.

	Males					**Females**				
		Percentiles					**Percentiles**			
Element	**Subjects n.**	**5 th**	**50 th**	**95 th**	**GM**	**Subjects n.**	**5th**	**50th**	**95th**	**IC GM**
As	205	0.13	0.75	2.82	0.71	238	0.13	0.75	3.08	0.72
Cd	200	0.14	0.31	0.63	0.3	231	0.13	0.3	0.57	0.29
Co	207	0.03	0.1	0.31	0.01	238	0.03	0.09	0.27	0.09
Cr	195	0.08	0.3	1.3	0.31	219	0.09	0.3	1.2	0.31
Hg	207	0.31	0.76	1.96	0.76	229	0.14	0.87	2.05	0.8
Ir *	201	2.5	6.84	15.96	6.94	236	2.5	6.84	14.5	6.52
Mn	210	3.36	7.52	15.33	7.17	239	3.36	7.31	16.25	7.26
Mo	209	0.51	1.08	2.42	1.11	240	0.54	1.14	2.32	1.11
Ni	188	0.18	1.07	2.63	1	223	0.18	0.98	2.5	0.9
Pb	205	4.93	11.1	22.11	10.7	235	3.7	8.73	20.59	8.73
Pd*	203	7.5	22.2	38.49	21.22	237	7.5	21.85	38.65	21.32
Pt*	191	5.07	10.7	22.31	10.6	232	5.7	10.88	25.8	11.17
Rh*	200	7.5	21.9	36.19	21.21	237	7.5	22.21	35.22	21.24
Sb	193	0.17	0.39	0.81	0.38	232	0.08	0.38	0.76	0.36
Sn	205	0.21	0.53	1.56	0.57	234	0.17	0.56	1.47	0.56
Tl	204	0.02	0.04	0.09	0.04	238	0.02	0.04	0.1	0.04

	Males					Females				
		Percentiles					Percentiles			
Element	Subjects n.	5 th	50 th	95 th	GM	Subjects n.	5th	50th	95th	IC GM
U*	208	2.07	5.05	15.51	5.29	237	2.35	4.62	13.74	4.91
V	207	0.03	0.07	0.18	0.07	238	0.03	0.07	0.16	0.07
W	205	0.01	0.03	0.08	0.03	239	0.01	0.03	0.07	0.03

*** ng/L**



As | **Hg** | **V**

Figure 35: Distribution in the Viterbo and Rome areas.

Conclusions

All metals analyzed in this study are environmental pollutants with high concern in field of human health. Their distribution in the environment is a result of natural processes (volcanoes, erosion, spring water, bacterial activity) and anthropogenic activities (fossil fuel combustion, industrial and agricultural processes). However in addition to the environmental sources, also other factors such as individual lifestyles, diet, socio-economic factors contribute to the human levels of metals in the body. Such information derived from the questionnaire administered to all the adolescents enrolled in this study were used to compare HBM data with individual variables which could be important to get an exposome approach. In this study data showed significantly higher concentrations of Pb in the male group. Several authors suggested that lead-related neurotoxic effects seem to be more pronounced in boys than in girls. Considering the diet, arsenic and mercury were found associated to fish

consumption. Arsenic is a common environmental contaminant and can pose a great threat to human health; human exposure comes from contaminated water and soil as well as from food rich in arsenic species, however blood arsenic levels are not as well correlated with drinking water concentrations as are urine arsenic levels. Metals showing significant differences between the Viterbo and Rome areas could be related to natural sources such as As, Cd, Mn, Rh. In particular, it has to be noted that children living in Viterbo area (a known volcanic area) showed higher levels of As, in fact arsenic and some other metals could derive mainly from potential natural sources related to the volcanic activity (Figure 35) The association between HBM data and land cover and with milk and fish consumption was also investigated considering them individually and in combination to explore potential synergies between covariates. To this aim a GLM multivariate analysis has been applied to all the dependent variables (HBM data) and categorical variables (fish and milk consumption and land cover). The form of the model was: intercept + fish + milk + landcover + fish * landcover + fish * milk + milk * landcover + fish * milk * landcover. Results of the GLM analysis in terms of significance (p value) of tests of between-subjects effects are reported in the Manhattan plot shown in Figure 36.

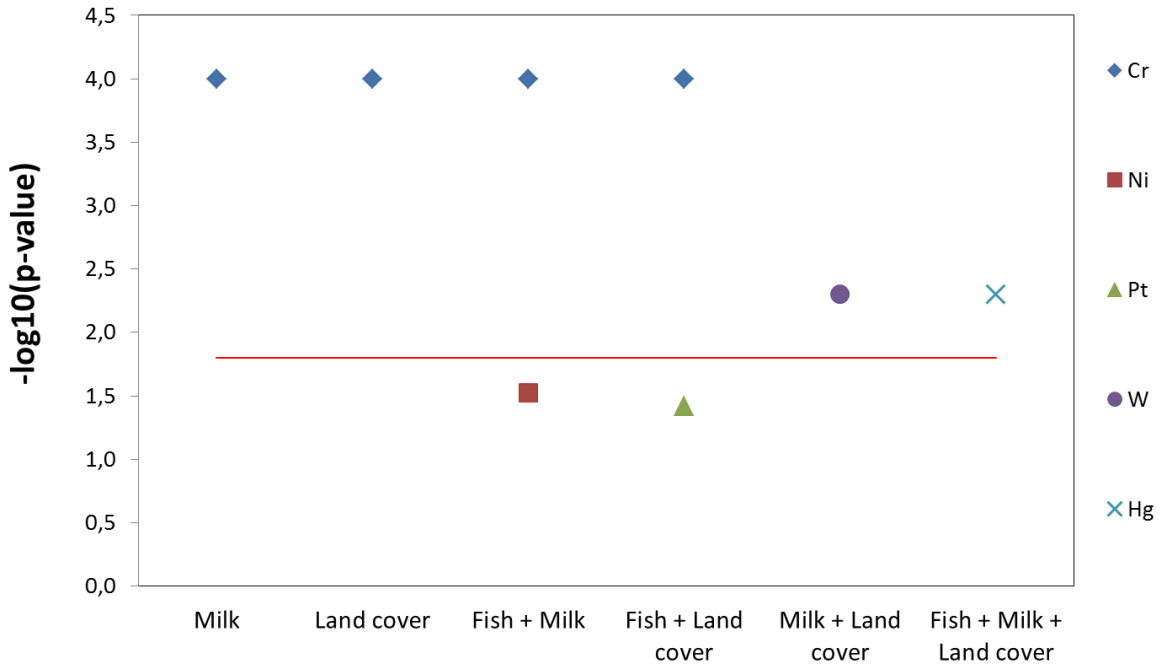


Figure 36: Manhattan Plot.

The figure shows the associations of the environmental factors that have been found to be linked to biomonitoring levels of the metals examined based on all populations studied (i.e. $p < 0.05$). The red line represents a $p < 0.02$, which means a more robust association. A robust statistical associations of Cr with the dietary pathways analysed and land cover, showing that both out-of-region and local sources can be associated with the observed levels in the

population was showed. Other robust associations found are between W and co-exposure to milk and proximity to industrial activities and Hg with a more complex interaction between co-exposure to different dietary pathways (milk and fish) coupled to proximity to industrial activities. No pathway alone is dominant but the combined effect results in statistically significant associations with blood concentration levels. The associations of Ni with dietary pathways (co-exposure to milk and fish) and Pt with fish and industrial activities and enhanced traffic even though statistically significant ($p < 0.05$), they fail to meet the statistical robustness test (they are below the red line in the above diagram). All the other metals do not show statistically significant associations with dietary patterns and/or land cover information. These results support the utility of the exposome approach to evaluate and to have a more accurate and deeper description of association between population exposure and its determinants.

Case studies in Spain

1st case study –Prenatal exposure to trace metals – 489 mothers from Sabadell (Catalonia, Spain)

Introduction

Humans are regularly exposed to metals present in air, water, food, soil and domestic materials. Although the banning of lead from petrol has proven beneficial, many other sources of potentially hazardous metals present in the human environment still remain to be controlled. Whereas certain metals are essential for life, e.g. zinc (Zn), copper (Cu) and iron (Fe), others are toxic, even at low concentrations, e.g. mercury (Hg), lead (Pb), arsenic (As), thallium (Th), chromium (Cr) or cadmium (Cd). Given the increasing use of these toxic metals in new technologies and the increasing inputs of them from road traffic and other sources, there is growing concern over the public health implications of continued exposure to them (Järup 2003; Lauwerys and Lison 1994; Rodriguez and Diaz 1995; Schulz et al. 2007; Wells et al. 2011; Zubero et al. 2010).

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

Mothers constitute a source of heavy metals for their infants during pregnancy and lactation. However, only a few studies on prenatal exposure to trace metals have been published, most of which focused on a small number of these elements (Messiha et al., 1988; Vahter et al., 2008; Wright and Baccarelli 2007; Al-Saleh et al. 2011; Kippler et al. 2009; Shirai et al. 2010). In some cases, animal models have been used to assess the prenatal effects of these pollutants (Liu et al. 2009; Tokar et al. 2010). However, specific measurements at the individual level can enable a better understanding of the possible influence of exposure to metals on health. Such measurements also facilitate the identification of sources and routes of metal contamination at both individual and the general population levels. These aspects are even more important when dealing with prenatal exposure.

Summary of existing environmental and biomonitoring data – gaps identification

There are no generally accepted methods for physiologically assessing exposure to metals. Urine is the preferred source of information for heavy metals biomonitoring, can be collected without invasive methods and has been widely used in large environmental studies such as the

German Environmental Survey for Children (GerES) and the National Health and Nutrition Examination (NHANES) (Esteban and Castaño 2009). However, one basic requirement for biomonitoring of metals with urine analysis concerns the reproducibility of different urine measurements from each individual in samples collected at different time periods. This aspect is particularly significant for women during pregnancy in which children are in-utero exposed to metals and other compounds by maternal transmission. Thus, analysis of metals in urine collected at different pregnancy periods may provide useful information for assessment of the efficacy of the use of urine samples as exposure markers.

Therefore, we aimed: i) to develop an analytical method using acid digestion prior to analysis by inductively coupled plasma quadruple mass spectrometry (Q-ICP-MS) for the simultaneous analysis of 12 metals in maternal urine and ii) to determine the concentrations of 12 metals, namely cobalt (Co), nickel (Ni), Cu, Zn, selenium (Se), As, molybdenum (Mo), Cd, antimonium (Sb), cesium (Cs), Th and Pb, in urine samples of pregnant women living in a highgly industrialized urban town (Sabadell, Catalonia, Spain), which were collected during their first and third trimester of pregnancy. This approach affords an assessment of the steadiness of the concentrations of these metals in urine during the pregnancy period and their usefulness for epidemiological studies, maternal and prenatal exposure estimates.

Biomonitoring data

Sampling and analysis protocol

657 pregnant women were recruited in their 12th week medical visit in the Sant Fèlix Primary Care Center II (Sabadell), between 2004 and 2006. Recruitment involved only those women that lived in Sabadell, were older than 16 years, had a singleton pregnancy, volunteered for the program and wanted to give birth at the Hospitals of Sabadell or Terrassa (a nearby city). Women suffering from chronic diseases, having impaired communication or that become pregnant by assisted reproduction were excluded. After obtaining the consent from the admitted women, questionnaires were administered by trained interviewers in the 12th and 32th weeks of pregnancy.

The urine samples were collected in 100 mL polypropylene containers in the first and third trimester of pregnancy from 489 pregnant women of this cohort. The samples were stored in polyethylene tubes at -20ºC until further processing.

Analysis

Prior to Q-ICP-MS analysis, the samples were digested and diluted to oxidize and remove organic matter and to minimize the concentrations of inorganic solids (Castillo et al. 2008; Krachler 1996). Three mL of each urine sample, 3 mL of Instra-Analysed 65% HNO3 (J.T. Baker, Germany) and 1.5 mL of Instra-Analysed 30% H2O2 (Baker) were introduced in

Teflon vessels. The mixtures were left in an oven at 90ºC overnight. After cooling, the vessels were opened and then placed on a heating plate at 250°C to evaporate off the nitric acid. Once dried, the resulting solid samples were dissolved in 3 mL of 4% HNO3, placed in 7 mL glass bottles and subsequently stored in a refrigerator until instrumental analysis. Before analysis, an internal standard of 10 ppb of In was introduced and depending on sample density they were diluted to 30 mL or 60 mL with MilliQ water to avoid non-spectral interference.

Q-ICP-MS analysis was performed by an X-SERIES II (Thermo Fisher Scientific) instrument. Specific isotope ions for Co and Ni were selected in order to avoid potential calcium interferences from the sample matrix. Cl atoms may also potentially interfere in the determination of As and Se. In these cases the collision/reaction cell technique should be added to the instrumental methods but no interferences were observed in the present samples and these cells were not used. Instrumental limit of detection (LOD) for all metals was 0.2 ng/mL attending to the most reliable lowest calibration point. The two samples corresponding to the first and third trimesters of each subject were digested and analyzed at the same time. One MilliQ water blank was processed in each batch of samples to control for possible contamination. If there was any contamination, thorough cleaning of all material was performed and digestion was repeated. Field samples were also obtained by analysis of Milli Q water which was previously stored in the containers used for maternal urine bottles and transported together with the samples.

A Bio-Rad Level 1 (Lyphochek Urine Metals Control 1-69131; Marnes-la-Coquette, France) urine reference was extensively used to evaluate the developed methodology, as it contains metal concentrations close to those in the urine samples from the study cohort. This reference material provided certified values for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Tl, Zn and Se. Prior to digestion, the lyophilized reference urine samples were reconstituted with 25 mL of MilliQ water as recommended by the manufacturer.

All glassware and polypropylene material was thoroughly cleaned by soaking in 10% nitric acid for 24 h, followed by rinsing three times with MilliQ water. The Teflon vessels were cleaned after each use by rinsing with 10% nitric acid three times, and, following the last rinse, leaving them in the oven at 90ºC overnight. Finally, the vessels were rinsed with a large volume of MilliQ water.

Creatinine was determined at the Echevarne laboratory of Barcelona by the Jaffé method (kinetic with target measurement, compensated method) with Beckman Coulter© reactive in AU5400 (IZASA®).

Statistical analyses

Descriptive statistical parameters were initially computed. Values for mean, standard deviation (SD), median and P91 were calculated for the metal concentrations. Normality was

checked by the Kolmogorov-Smirnov test. The metal concentrations between the first and the third trimesters were compared using Spearman correlations and paired Mann-Whitney hypothesis tests. The individual ratios between the metal concentrations during the third and first trimesters were also calculated. Mean, standard deviation and median values for these ratios were computed. All statistical analyses were performed using Stata 12.0 software (Stata Corporation, College Station, Texas).

Results

Population characteristics

The median age of the mothers at the time of their last menstrual period was 31 years, ranging between 20 and 40 years. Their mean body mass index before pregnancy was 23.77 kg/m2 (standard deviation 4.53 kg/m2, median 22.44 kg/m2, range 16.69-40.77 kg/m2). Overweight and obese women encompassed 18.1% and 8.4%, respectively. The proportion of primiparous mothers was 49%, 41% had another infant and 10.2% had more than two infants.

Method validation

The developed analytical method was tested by analysis of the aforementioned reference material. The analyses were performed in different time periods, on three replicates per period (Table 12). The observed mean results are within the acceptable range of assigned values. The average concentrations of some metals, such as Cd, were close to the lower limit. The relative standard deviations varied between 5% (As and Cd) and 18% (Zn). Repeatibility ranged between 1% (As) and 17% (Zn). Reproducibility ranged between 1% (Tl) and 17% (Cu; Table 12).

Metal concentrations

The metal concentrations and statistics of the samples collected in the first and third trimester normalized to creatinine content (μg/g creatinine) are shown in Table 13. The metal concentrations were not normally distributed but skewed to the right in both trimesters.

Ni, Cu, Zn, As, Se, Mo, Cd, Cs and Pb were detected in more than 90% of samples, whereas Co and Sb were detected in more than 65% in the first and the third trimester (Table 13). Tl was the only element detected in less than 20% of the samples. The differences in metal concentrations between the urine samples collected in both periods were statistically significant for all metals except Ni, As, Tl and Pb (median values: 32 (1st)/35 (3rd) μg/g creatinine for As, 3.9/3.9 for Ni, 0.14/0.13 for Tl, 3.8/3.9 for Pb; Table 13). The concentrations of Co, Cu and Zn were higher during the third trimester (median values: 0.45 (1st)/1.3 (3rd) μg/g creatinine for Co, 12/15 for Cu, 256/290 for Zn; Table 13). The opposite was found for the concentrations of Mo, Se, Cd, Sb and Cs (median values: 55 (1st)/44 (3rd)

μg/g creatinine for Mo, 10/8.7 for Se, 0.61/0.54 for Cd, 0.36/0.28 for Sb and 8.0/6.8 for Cs; Table 13).

Table 12: Concentrations obtained with the developed method for analysis of trace metals in urine samples in the analysis of Bio-Rad Level 1 urine reference standard (ng/mL).

			All values		Repeatibility		Reproducibility	
Element	Reference value	Acceptable range (20%)	Mean ± SD[a]	RSD[b]	Mean ± SD[b]	RSD[b]	Mean ± SD[c]	RSD[b]
As	65	52 - 78	59 ± 3.1	5%	58 ± 0.35	1%	56 ± 2.0	4%
Cd	9.0	7.2 - 11	7.2 ± 0.37	5%	7.1 ± 0.11	2%	7.2 ± 0.55	8%
Co	6.8	5.4 - 8.2	6.8 ± 0.67	10%	6.8 ± 0.67	10%	6.5 ± 0.75	12%
Cu	10	8.1 – 12	10 ± 1.5	16%	9.4 ± 0.81	9%	10 ± 1.7	17%
Ni	4.5	2.3 - 6.7	4.0 ± 0.6	16%	4.3 ± 0.8	18%	3.6 ± 0.36	10%
Pb	14	11 – 16	14 ± 1.6	11%	14 ± 2.2	15%	13 ± 1.6	13%
Sb	9.5	7.5 -11	9.7 ± 1.3	13%	9.1 ± 0.81	9%	10 ± 0.4	4%
Se	72	57 – 86	69 ± 7.5	11%	69 ± 7.5	11%	69 ± 7.5	11%
Tl	8.5	6.8 – 10	8.4 ± 0.52	6%	8.0 ± 0.22	3%	8.3 ± 0.09	1%
Zn	381	305 - 457	363 ± 67	18%	370 ± 65	17%	305 ± 19	6%

[a]mean ± standard deviation (n = 8) .[b]RSD: relative standard deviation (in %)

Consistently with these differences, comparison of the individual concentrations revealed that more mothers had higher concentrations of Se, Mo, Cd, Sb, Cs and Tl in the first than in the third trimester (53-64%; Table 14). On the other hand, more mothers exhibited higher concentrations of Co, Ni, Cu and Zn in the third than in the first trimester (55-82%; Table 14). The concentration ratios between the third and first trimesters were consistent with these observed differences (Table 14). Co, Cu and Zn showed the higher third-first trimester median concentration ratios and Mo, Se, Cd and Sb had the lower.

Table 13: Descriptive statistics of the concentrations of trace metals in the maternal urine samples collected in the first and third trimesters of pregnancy (μg/g creatinine). p-value for difference according to Mann-Whitney paired test.

	% Detected		Mean (SD)		Median		P90		p-value
	1st	3rd	1st	3rd	1st	3rd	1st	3rd	
Co	73.6	84.4	0.73 (1.4)	1.6 (2.5)	0.45	1.3	1.4	2.9	$p < 0.001$

Ni	98.4	98.1	4.9 (5.3)	4.8 (4.5)	3.9	3.9	9.0	8.6	
Cu	100	100	14 (11)	17 (13)	12	15	24	30	$p < 0.001$
Zn	100	100	315 (313)	342 (224)	256	290	552	631	$p < 0.001$
Se	99.8	99.3	12 (11)	9.9 (6.3)	10	8.7	20	16	$p < 0.001$
As	99.8	99.8	69 (120)	62 (79)	32	35	147	136	
Mo	100	100	64 (49)	50 (32)	55	44	106	81	$p < 0.001$
Cd	90.1	87.5	0.77 (0.79)	0.67 (0.48)	0.61	0.54	1.3	1.3	$p < 0.01$
Sb	73.7	64.8	0.81 (1.7)	0.56 (2.0)	0.36	0.28	1.4	0.84	$p < 0.001$
Cs	100	100	9.3 (11)	7.4 (4.1)	8.0	6.8	13	11	$p < 0.001$
Tl	19.7	17.2	0.18 (0.16)	0.18 (0.43)	<LOD	<LOD	0.30	0.30	
Pb	98.9	100	4.8 (4.3)	5.2 (4.9)	3.8	3.9	8.1	8.9	

Table 14:. Comparisons of the metal concentrations in maternal urine collected in the first and third trimesters of pregnancy (µg/g creatinine)

	Spearman coeficients of the correlations	Concentration ratios between the 3rd and 1st trimestres		Concentrations in the 3rd and 1st trimesters	
		Mean (SD)	Median	% 3rd > 1st	% 1st > 3rd
Co	0.39***	4.5 (7.0)	2.2	84	16
Ni	0.39***	1.4 (2.0)	1.1	55	45
Cu	0.21**	1.6 (1.3)	1.2	62	38
Zn	0.60***	1.3 (1.0)	1.1	59	41
Se	0.43***	0.97 (0.71)	0.84	37	63
As	0.24***	2.6 (4.6)	1.1	52	48
Mo	0.16**	1.0 (1.1)	0.80	36	64
Cd	0.57***	1.1 (0.87)	0.92	42	57
Sb	0.40***	1.6 (6.7)	0.75	38	62
Cs	0.26***	0.97 (0.61)	0.89	37	63
Tl	0.22***	1.3 (2.6)	0.91	46	53
Pb	0.46***	1.4 (1.3)	1.0	50	50

** p-value < 0.01; *** p-value < 0.001

The first and third trimester concentrations of all metals were significantly correlated (Table 14). The Spearman coefficients ranged between 0.16 (Mo) and 0.60 (Zn). The degree of significance of these correlations was $p < 0.001$ in most cases (Co, Ni, Zn, Se, As, Cd, Sb, Cs and Pb) and $p < 0.01$ in others (Cu, Mo).

Concentrations of trace metals in the Sabadell cohort

The median concentrations found in the Sabadell cohort (n = 489) were compared with those reported in previous studies worldwide (Table 15). Most literature data are reported in μg/g creatinine but in some cases they are reported in μg/L (Afridi et al. 2009; Link et al. 2007; Minoia et al. 1990; Callan et al., 2013). Despite these unit differences, in healthy populations the average values measured using either of these two methods generally differed by less than 10% (NHANES 2009). Thus, all results in Table 15 should be comparable. Most previous studies have dealt with the general population; only a few specifically encompass pregnant women as in the present study, such as those from Pakistan (Afridi et al. 2009), Korea (Moon et al. 2003) or Germany (Callan et al. 2013). Accordingly, it should be taken into account that, once levels are normalized to creatinine content, the values for women tend to be higher than those for men (NHANES 2009; Schuhmacher et al. 1994). Furthermore, pregnancy may involve increases or decreases in the concentrations of certain metals, as discussed later.

The concentrations of Ni and As were higher than those reported in previous studies (Link et al., 2007; Ohashi et al., 2006; NHANES 2009; Banza et al., 2009; Alimonti et al., 2000; Seifert et al., 2000; Minoia et al., 1990; Callan et al, 2013; Table 15). On the other hand, Zn, Se and Tl showed lower urine concentrations in Sabadell than in studies from other population groups (Banza et al., 2009; Paschal et al., 1998; Schuhmacher et al., 1994; Minoia et al., 1990; Afridi et al., 2009; Callan et al., 2013; Table 15). The other metals showed intermediate concentrations when comparing with previous studies.

The median concentrations of Co in the first trimester, 0.45 μg/g creatinine, were intermediate between those reported in previous studies (Table 15) but those in the third trimester, 1.3 μg/g creatinine, were higher than in most previous studies except one from a mining polluted area of Congo (mean 15.7 μg/g creatinine) (Banza et al. 2009) and were similar to those described in a maternal population from Australia (Callan et al., 2013). The strong difference in the concentrations of this metal in the first and third trimesters of pregnancy levels is consistent with a significant increase in Co excretion during pregnancy. These concentrations during the last stage of pregnancy may not be comparable to those from general population.

The observed concentrations of Pb, 3.8-3.9 μg/g creatinine (Table 15), were significantly lower than those reported in populations from Italy from a time period in which Pb was still used as additive in gasoline, 17 μg/L (Minoia et al. 1990), or Korea (5.1 μg/g creatinine; Moon et al. 2003). However, they were higher than those recently reported in the US, 0.63-

0.72 μg/g creatinine (NHANES 2009), or 1.9 μg/g creatinine (Paschal et al. 1998). These concentrations were consistent with the effects of Pb withdrawal from gasoline, although the Sabadell levels may be consistent with predicted increases of Pb mobilization from the bone tissues during pregnancy (Gulson et al. 2004).

Comparison of levels in the first and third trimesters

Some previous studies have considered changes in metal concentrations during pregnancy but they essentially compared blood or serum samples and only analyzed a few metals such as Cu and Zn (Huang et al. 1999; Liu et al. 2010; Izquierdo-Álvarez et al. 2007; Hernandez et al. 1996), Se, Cu and Zn (Kilinc et al. 2010) or Pb and Cd (Bonithon-Kopp et al. 1986). A very small number of studies on metals during pregnancy have considered urine samples, e.g. As (Gardner et al. 2010) or Cd (Hernandez et al. 1996).

Table 15: Comparison of trace metal concentrations from the Sabadell cohort and previous studies (concentrations in µg/g creatinine unless otherwise indicated)

Reference	Sampling Years	Location	N	Co	Ni	As	Zn	Sb	Cd	Pb	Cu	Cs	Tl	Se
Present work[a]	2004-06	Sabadell – 1st trim	489	0.45	3.9	32	256	0.36	0.61	3.8	12	8.0	0.13	10
Present work[a]	2004-06	Sabadell – 3rd trim	489	1.3	3.9	35	290	0.28	0.54	3.9	15	6.8	0.13	8.7
Link et al., 2007[a]	2002-03	South Germany	500	…	…	4.6[d]	…	…	…	…	…	…	…	…
NHANES report, 2009[a]	1999-00	USA (99-00)	2500	0.35	…	…	…	0.12	0.18	0.72	…	4.1	…	…
NHANES report, 2009[a]	2001-02	USA (01-02)	2500	0.36	…	…	…	0.13	0.20	0.64	…	4.5	…	…
NHANES report, 2009[a]	2003-04	USA (03-04)	2500	0.31	…	8.2	…	…	0.21	0.63	…	4.6	…	…
Ohashi et al., 2006[b]	2000-05	Japan	1000	0.60	1.8	…	…	…	…	…	13	…	…	…
Banza et al., 2009[b]	2006-07	DR Congo	179	15.7	3.3	18	306	0.07	0.75	3.2	17	…	…	17
Paschal et al., 1998[b]	1988-94	USA	496	0.78	…	…	…	0.67	…	1.9	…	1.0	0.24	…
Moon et al., 2003[b]	2000	Korea	38	…	…	…	…	…	1.6	5.1	…	…	…	…
Batáriová et al. 2006	2001-03	Czech Republic	160	…	…	…	…	…	0.33	…	…	…	…	…
Alimonti et al. 2000[b]	nr	Rome	131	…	0.39	…	…	…	…	…	…	…	…	…
Schuhmacher et al., 1994[b]	nr	Tarragona	434	…	…	…	699	…	…	…	27	…	…	…
Seifert et al., 2000[b]	1990-92	Germany (1990/92)	4000	…	…	4.6	…	…	0.21	…	9.5	…	…	…
Zubero et al., 2008[b]	2006-08	Bizkaia	29	…	…	…	…	…	0.36	…	…	…	…	…
Minoia et al., 1990[b]	nr	Italy	11-900	0.57[d]	0.9[d]	17[d]	456[d]	0.79[d]	0.86[d]	17[d]	23	…	0.42[d]	22[d]
Afridi et al., 2009[c]	nr	Pakistan	93 (pregnant)	…	…	…	1150[d]	…	…	…	…	…	…	…
Afridi et al., 2009[c]	Nr	Pakistan	115 (non	…	…	…	850[d]	…	…	…	…	…	…	…

			pregnant)											
Callan et al., 2013[ad]	2008-11	Australia	173	1.2	2.3	13	396	…	…	…	…	10.4	…	26
Hinwood et al., 2013[ae]	2008-11	Australia	173	…	…	…	…	…	<0.3	0.7	…	…	…	…

[a]Median. [b]Geometric mean. [c]Arithmetic mean. dμg/L [e]Pregnant women (3rd trimester)

As mentioned above, the concentrations of all metals except Ni, As, Th and Pb were statistically different between the first and third trimesters. Most of the metals with differences between the two stages of pregnancy showed higher concentrations in the urine collected in the first trimester than in the third trimester, except in the case of Co, Cu and Zn. Some previous studies considering the Zn and Cu in plasma or blood samples also found significant differences in concentration at different periods of pregnancy (Izquierdo-Álvarez et al. 2007; Liu et al. 2010; Hernandez et al. 1996), although in one study on Zn, no difference was found (Huang et al. 1999). On the contrary, no significant differences for Se in serum samples during pregnancy were observed in a southeastern Mediterranean region of Turkey (Kilinc et al. 2010) nor for Cd in Aragon (Spain) (Izquierdo-Álvarez et al. 2007) or Southern Catalonia (Spain) (Hernandez et al., 1996), while significant differences in the concentrations of these metals in the first and the third trimesters were found in our study. Some of these studies did not take into account paired samples of the same mothers at different stages and they involved a lower number of samples.

Different changes and adaptations occur in the women during pregnancy, such as increase of plasma volume. This may explain why most metals show a decrease in concentration during pregnancy. However, despite of this higher volume the concentrations of some minerals and vitamins also increase (King 2000). These metabolic changes may also influence the amount of metal released into urine. For instance, the increase in glomerular filtration rate during pregnancy has been related with observed concentration increments of Zn in urine during this maternal period (Swanson and King 1987). This observation is also consistent with the increase of Zn between the first and third trimesters found in the Sabadell cohort (Table 13) and could also explain the significant increases of other metals such as Co and Cu (Table 13). In any case, there must be specific factors affecting each metal separately that must be considered in specific studies. This is the first study in which differences in exposure during pregnancy have been studied for a large number of metals over a large number of paired samples (n = 489).

The concentrations of trace metals in urine collected in the first and third trimesters of pregnancy were significantly correlated in all metals studied, with high statistical significance in most cases. These significant correlations likely reflect an absence of major changes in metal exposure during pregnancy and the differences between both trimesters observed for most metals may reflect metabolic changes during this period. Accordingly, the findings from this studied cohort indicate that the measurements of these trace metals at any stage of pregnancy provide a representative estimate of the exposure to these compounds during the whole period. Sampling in specific stages may be chosen according to logistics or specific study purposes and the observed concentrations must always be interpreted in reference to this selected phase.

Conclusions

Exposure to metals in pregnant women has been assessed from their urine composition collected in the first and third trimesters of pregnancy using a newly developed digestion protocol for Q-ICP-MS analysis. All metals except Ni, As, Th and Pb showed statistically significant concentration differences between these two periods. The concentrations of all metals in the first and third trimesters were significantly correlated which reflect the absence of major changes of metal inputs in the studied women during pregnancy. The significant concentration differences between these two sampling periods may respond to metabolic pregnancy changes. Accordingly, the measurements of the studied trace metals in urine provide representative estimates of exposure during the whole pregnancy period.

2nd case study – Food sources of arsenic in pregnant Mediterranean women with high urine concentrations of this metalloid (Catalonia, Spain)

Introduction

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

Humans are exposed to arsenic by consumption of food and water (Abernathy et al. 2001). In some areas drinking water is an important source of exposure to inorganic arsenic (Vahter et al., 2006a). Nevertheless, food is generally the main contributor to the total arsenic daily intake. Rice may be one of the most important dietary sources. This cereal has higher efficiency for the assimilation of this metalloid from soils than other crops (Williams et al., 2007). Accordingly, areas with high arsenic in the water incorporate this metalloid by consumption of this cereal, e.g. Bangladesh (Vahter et al. 2006b). On the other hand, seafood is one of the most important sources of organic arsenic. In some populations the dietary load of this metalloid depends on the amount and kind of seafood consumption (Meltzer et al. 1994).

Exposure to arsenic has been found to be associated with diverse health effects such as skin lesions (Argos et al., 2011) and immunotoxicological (Ahmed et al., 2011; Andrew et al., 2011), cardiovascular (Chen et al., 2011; Yuan et al., 2011) and endocrine disorders (Chen et al., 2007). Arsenic is carcinogenic (non-threshold, class I human carcinogen; ATSDR 2007). It has been associated to skin, lung, bladder and liver cancer (International Agency for Cancer Research, 2004) and can cross the placental barrier (Concha et al., 1998). Prenatal exposure to arsenic from drinking water has been associated with reduction of birth weight in highly exposed populations (Rahman et al. 2009), as well as neurodevelopmental defects (Hamadani et al., 2011; Parajuli et al., 2013). Increased blood pressure and anemia during pregnancy has also been related to arsenic exposure (Hopenhayn et al. 2006, Kwok et al. 2007).

Biomethylation is the most important way for detoxification of inorganic arsenic, MMA and DMA are less harmful and rapidly eliminated by urine. During pregnancy and lactation there is increased methylation, which may be a way to protect the fetus from high exposure to toxic

arsenic. Nevertheless, during the first stages of pregnancy, when these processes are not fully enhanced and fetal susceptibility to possible harm is higher, prenatal exposure may be more important and may induce changes that could become apparent much later in life (Vahter 2009).

Summary of existing environmental and biomonitoring data – gaps identification

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

Total urinary arsenic was determined in two different stages of pregnancy, representing the first and third trimesters, from a cohort of Mediterranean mothers. The influence of diet, with special emphasis in seafood consumption, on the observed concentrations of arsenic in urine was evaluated. Differences in exposure or excretion that eventually could have effects on prenatal exposure or future children development were assessed by urinary arsenic during both trimesters.

Biomonitoring data

Sampling and analysis protocol

Sampling and analysis was performed as described in the previous case. Arsenic concentrations during first and third trimester of pregnancy were compared using chi-squared tests and Spearman correlations. When the distributions were not Gaussian, Spearman correlation rates were calculated in order to define possible associations between different variables. Mann-Whitney and Kruskall-Wallis testing was therefore used to compare between groups of categorical variables. Univariate linear regression between log-transformed arsenic levels and different maternal variables was performed. Those variables showing $p<0.20$ in univariate modeling were included in multivariate linear regression models.

Results

Concentrations of total urinary arsenic during the first and third trimesters

Descriptive statistics are shown in Table 16. Arsenic was above the limit of detection in 99.8% of the urine samples. Geometric mean concentrations were 34 μg/g creatinine (55 μg/g creatinine of interquartile range) and 37 μg/g creatinine (55 μg/g creatinine of interquartile range) during the first and third trimesters, respectively. The concentrations were not statistically different between both periods and individual measurements between both

trimesters were significantly correlated (p<0.001). The distributions of concentrations were not normal because they were skewed to the right. P90 were 150 and 136 μg/g creatinine during the first and third trimesters, respectively.

Table 16: Descriptive statistics of arsenic concentrations in urine from the first and third trimesters of pregnancy.

	% Detection	Arithmetic mean (SD)	Geometric mean (IQR)	P90	p-Value[a]	Spearman's correlation rho[b]
ng/mL						
1st trimester	99.8	60 (141)	28 (44)	132	0.179	0.187
3rd trimester	99.8	57 (83)	31 (51)	128		(p<0.001)
μg/g creatinine						
1st trimester	99.8	69 (121)	34 (55)	150	0.731	0.239
3rd trimester	99.8	62 (79)	37 (55)	136		(p<0.001)

[a]significance of the mean differences. [b]significance of the correlations.

Dietary factors influencing arsenic concentrations

No significant differences in seafood intake were found between the first and third trimesters (Table 17). Mean total seafood consumption during the first and third trimesters was 5.1 servings/week (69 g/day). 41% and 43% of this consumption corresponded to lean fish, 41% and 38% to fatty fish and 19% and 18% to shellfish, respectively.

According to the univariate models, the only dietary items having significant positive associations to arsenic levels in both trimesters were lean and fatty fish and total seafood consumption (first trimester linear beta coefficients 0.011, 0.0060, 0.0070; third trimester linear beta coefficients, 0.0084, 0.0055, 0.0046; p<0.001, p<0.05 and p<0.001, respectively; Table 18). Shellfish intake was significantly associated to arsenic concentrations in the urine of the first trimester (linear beta coefficient 0.013, p<0.05). On the contrary, rice consumption showed significant negative associations with arsenic but only during the first trimester (linear beta coefficient -0.0039, p<0.05). Other dietary items with significant negative associations were cakes/sweets during the first trimester (linear beta coefficient -0.0025, p<0.05) and nuts during the third trimester (linear beta coefficient -0.021, p<0.01). Eggs consumption showed a positive association with arsenic concentration in the urine collected in the first trimester (linear beta coefficient -0.014, p<0.05).

Influence of seafood consumption

The trends found in the univariate models for lean fish and total seafood consumption were

also observed upon quartile categorization of these variables (Table 19). Thus, lean fish consumption was significantly associated to arsenic concentrations in the urine collected in the first ($p<0.001$) and third ($p<0.01$) trimesters. Shellfish consumption was also associated to arsenic concentrations of urine collected in the first ($p<0.01$) and third ($p<0.05$) trimesters and fatty fish was associated to arsenic in the urine from the third trimester ($p<0.05$). Total seafood consumption was associated to arsenic in urine from both trimesters ($p<0.001$).

Table 17: Descriptive statistics of fish consumption (g/day) during the first and third trimesters.

	Arithmetic mean (SD)	Median	Max	p-value[a]	Spearman's correlation rho[b]	% total seafood (mean)
Lean Fish						
1st trimester	31 (22)	29	94	0.554	0.463	43
3rd trimester	32 (23)	29	180		(p<0.001)	44
Fatty fish						
1st trimester	26 (18)	21	100	0.453	0.453	38
3rd trimester	26 (21)	21	182		(p<0.001)	38
Shellfish						
1st trimester	12 (9.7)	10	70	0.030	0.427	19
3rd trimester	11 (12)	10	185		(p<0.001)	18
Total seafood						
1st trimester	69 (35)	65	198	0.302	0.526	-
3rd trimester	69 (40)	64	399		(p<0.001)	-

The quartile parametrization shows that the main differences were found when comparing the arsenic concentrations of the first quartile with the rest (Table 19). No significant differences were found between the third and fourth quartiles.

The results of a multivariate model considering diet, environmental exposure and maternal characteristics are shown in Table 20. Non dietary factors did not show significant associations in both trimesters. Lean fish consumption showed positive associations with arsenic in urine from the first ($p<0.001$ and $p<0.01$ in the third and fourth quartiles, respectively) and third trimesters ($p<0.01$, $p<0.05$ and $p<0.001$ in the second, third and fourth

quartiles, respectively). No significant associations were found for fatty fish. Shellfish consumption showed positive associations with arsenic in the urine from the first ($p<0.01$ in the second, third and fourth quartile) and third trimesters ($p<0.05$ in the third quartile).

Elaboration of a multivariate model considering total seafood consumption instead of these seafood groups, resulted in significant associations between this diet component and arsenic concentrations in urine from both trimesters ($p<0.001$). The significant positive association between total seafood consumption and urinary arsenic can be represented by GAM plots (Figure 37). These plots show that the main increase was found between 0 and 100 g/day of total consumption and that further increase did not result into higher urinary arsenic concentration.

Table 18: Univariate modeling of the arsenic concentrations in urine from pregnant women with different items from their diets. Beta coefficients (95% confidence interval) between log-transformed concentrations of arsenic in both trimesters and consumption of each dietary item (g/day) are shown.

	1st trimester	3r trimester
Dairy products	-0.00014 (-0.00059, 0.00031)	-0.00041 (-0.00079, 0.000031)
Eggs	0.014 (0.0013, 0.027)*	0.00011 (-0.0010, 0.010)
White meat	0.0024 (-0.0080, 0.0031)	-0.0041 (-0.0097, 0.0016)
Red meat	-0.0011 (-0.0047, 0.0024)	-0.0025 (-0.0059, 0.00094)
Lean fish	0.011 (0.0061, 0.015)***	0.0084 (0.0043, 0.013)***
Fatty fish	0.0060 (0.00048, 0.011)*	0.0055 (0.00097, 0.010)*
Shellfish	0.013 (0.0027)*	0.0027 (-0.0056, 0.011)
Total seafood	0.0070 (0.0041, 0.010)***	0.0046 (0.0022, 0.0069)***
Vegetables	0.000033 (-0.00092)	0.00064 (-0.00021, 0.0015)
Fruit	-0.00023 (-0.00071, 0.00026)	-0.00037 (-0.00079, 0.000044)
Nuts	-0.0092 (-0.023, 0.00044)	-0.021 (-0.034, -0.0074)**
Legumes	-0.0012 (-0.0057, 0.0033)	-0.0018 (-0.0053, 0.0017)
Pasta/Cereal	-0.0020 (-0.0057, -0.0017)*	-0.0016 (-0.0051, 0.0020)
Rice	-0.0039 (-0.0071, -0.00076)*	0.0021 (-0.0048, 0.00053)
Potatoes	0.00059 (-0.0021, 0.0033)	-0.0024 (-0.0053, 0.00052)
Bread	0.00084 (-0.0017, 0.0034)	-0.0018 (-0.0042, 0.00051)
Cakes/sweet things	-0.0030 (-0.0057, -0.00028)*	-0.0024 (-0.0047, 0.0000017)

Coffee/infusions	-0.00029 (-0.00085, 0.00027)	0.000031 (-0.00048, 0.00054)

* p<0.05, ** p<0.01, *** p<0.001

Table 19: Mean (standard deviation) of the concentrations of urinary arsenic (in µg/g creatinine) during the first and third trimesters of pregnancy grouped by quartiles of fish consumption (g/week) (p of significance according to the Kruskal-Wallis test).

Consumption quartile	White fish		Fatty fish		Shellfish		Total seafood	
	1st trim	3rd trim	1st trim	3rd trim	1st trim	3rd trim	1st trim	3rd trim
1st	50 (72)	44 (45)	56 (69)	56 (78)	50 (94)	48 (55)	45 (62)	41 (46)
2nd	56 (73)	63 (68)	86 (188)	65 (106)	78 (141)	65 (81)	52 (75)	61 (64)
3rd	97 (168)	67 (92)	69 (110)	58 (57)	66 (75)	82 (108)	103 (195)	74 (112)
4th	88 (159)	85 (111)	64 (76)	69 (73)	78 (145)	56 (68)	75 (92)	71 (77)
p	0.0001	0.001	0.45	0.022	0.001	0.040	0.0001	0.0004

Table 20: Multivariate modeling of the urine arsenic concentrations in both trimesters of pregnancy and maternal characteristics and different variables of exposure. Beta coefficients (95% confidence interval) between log-transformed concentrations of arsenic in both trimesters and maternal characteristics are shown. Fish consumption was analyzed by separation of different types of fish (* p<0.05, ** p<0.01, *** p<0.001).

	1st trimester	3r trimester
Exposure to heavy truck traffic		
Practically never	1	1
Rarely	-0.0035 (-0.27, 0.26)	0.067 (-0.31, 0.17)
Regularly	-0.23 (-0.55, 0.10)	-0.29 (-0.58, -0.0057)
Heavily	-0.085 (-0.35, 0.18)	-0.086 (-0.33, 0.15)
Body mass index (kg/m^2)	-0.024 (-0.048,-0.00084)*	-0.033 (-0.054, -0.0.11)**
Maternal origin		
Spain	1	1
Latin America	-0.26 (-0.72, 0.20)	-0.036 (-0.45, 0.39)
Rest of Europe	-0.17 (-0.87, 0.52)	-1.1 (-1.7, -0.45)**
Rest of the world	0.68 (-0.91, 2.3)	-0.27 (-1.7, 1.1)
Social class (3 cat)		
I+II	1	1
III	0.072 (-0.22, 0.37)	-0.010 (-0.27, 0.25)
IV+V	0.19 (-0.087, 0.48)	-0.11 (-0.36, 0.14)
Parity		
No previous children	1	1
1 children	-0.18 (-0.41, 0.037)	-0.055 (-0.25, 0.14)
2 or more children	-0.24 (-0.63, 0.16)	-0.11 (-0.48, 0.25)
Age of the home (yr)	-0.015 (-0.095, 0.066)	-0.092 (-0.16, -0.020)*
Physical exercise		
Sedentary / low activity	1	1
Moderate	-0.16 (-0.42, 0.088)	-0.22 (-0.44, -0.011)*
Quite a lot / very active	-0.046 (-0.33, 0.24)	0.047 (-0.20, 0.29)
Dietary items (g/day)		

Dairy products	-0.000073 (-0.00053, 0.00038)	-0.00036 (-0.00075, -0.00002)
White meat	-0.0033 (-0.0091, 0.0026)	-0.0043 (-0.010, 0.0014)
Red meat	-0.00040 (-0.0040, 0.0031)	0.00067 (-0.0028, 0.0041)
Eggs	0.015 (0.0021, 0.028)*	0.00033 (-0.0096, 0.010)
White fish		
1st quartile	1	1
2nd quartile	0.13 (-0.18, 0.43)	0.34 (0.093, 0.59)**
3rd quartile	0.62 (0.32, 0.93)***	0.31 (0.037, 0.57)*
4th quartile	0.51 (0.22, 0.79)**	0.51 (0.24, 0.79)***
Fatty fish		
1st quartile	1	1
2nd quartile	-0.010 (-0.30, 0.28)	-0.12 (-0.39, 0.16)
3rd quartile	0.074 (-0.24, 0.39)	-0.10 (-0.37, 0.17)
4th quartile	0.0048 (-0.30, 0.31)	0.046 (-0.25, 0.34)
Shellfish		
1st quartile	1	1
2nd quartile	0.49 (0.20, 0.77)**	0.11 (-0.14, 0.35)
3rd quartile	0.48 (0.15, 0.80)**	0.34 (0.056, 0.63)*
4th quartile	0.45 (0.14, 0.76)**	0.12 (-0.16, 0.41)
Vegetables	-0.00024 (-0.0012, 0.00074)	0.0011 (0.00021, 0.0021)*
Fruit	-0.00031 (0.00083, 0.00021)	-0.00043 (-0.00086, 0.000033)
Potatoes	0.0011 (-0.0016, 0.0039)	-0.00035 (-0.0034, 0.0027)
Bread	0.00015 (-0.0040, 0.0027)	-0.00069 (-0.0030, 0.0016)
Pasta / cereal	-0.0016 (-0.0040, 0.00084)	-0.0011 (-0.0032, 0.0013)
Nuts	-0.0091 (-0.023, 0.0048)	-0.024 (-0.037, -0.010)**
Cakes/sweet things	-0.0029 (-0.0057, -0.00016)*	-0.0012 (-0.0037, 0.0012)
r^2	0.16	0.21

The multivariate model also shows the significant negative associations between concentrations of arsenic and consumption of cakes/sweets ($p<0.05$, first trimester) and nuts ($p<0.001$, third trimester). Eggs showed a positive association with arsenic concentrations

(p<0.05, first trimester). All these significant associations were also observed in the univariate analysis. In contrast, the negative association of arsenic concentration and pasta/cereal in the univariate analysis was not retained in the multivariate model (Table 20). On the other hand, a positive association between arsenic concentration and consumption of vegetables was found (p<0.05, third trimester) which was not identified in the univariate analyses (Table 18).

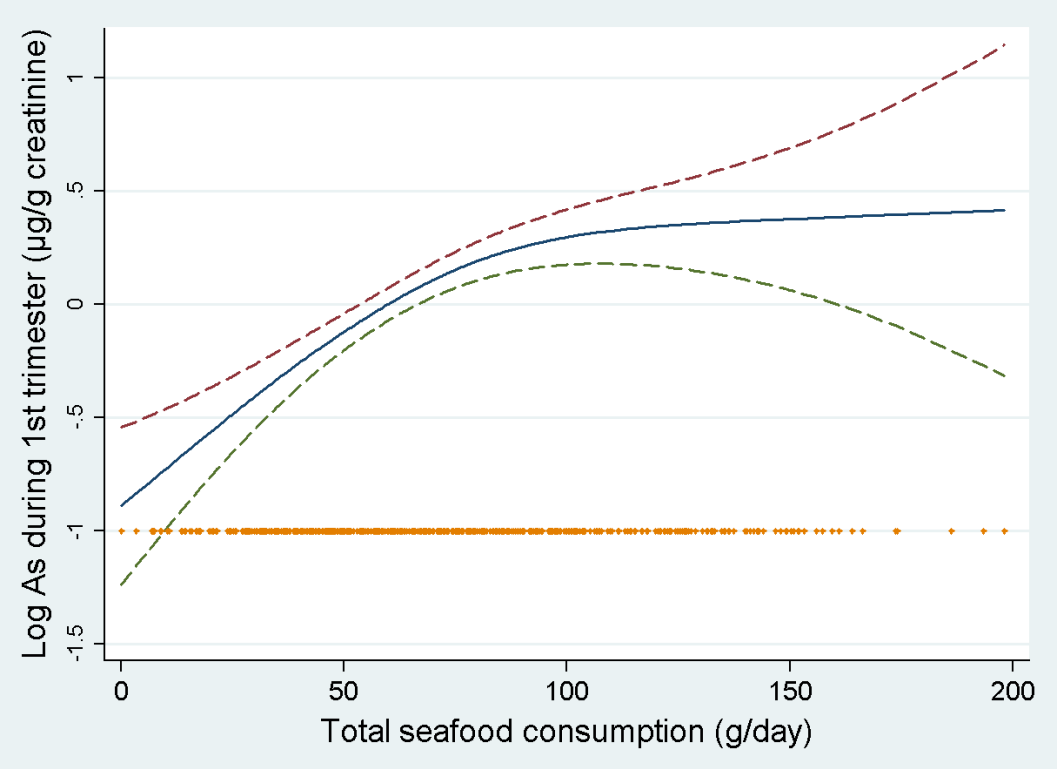


Figure 37: Graphic representation of a general additive model showing the relationship (and 95% confidence levels) of total arsenic levels in urine (Y axis: µg/g creatinine; log-transformed) and total seafood consumption (g/day) taken as continuous variable (X-axis). The symbols (+) on the X-axis show total seafood consumption of each subject.

Arsenic concentrations

The arsenic urine concentrations in the mothers of the Sabadell cohort were lower than those found in populations exposed to waters contaminated with arsenic, such as Bangladesh (Gardner et al. 2010) or Antofagasta (Chile; Hopenhayn et al. 2003) (Table 21). The concentrations of the present study were also lower than in occupationally exposed individuals (Spain; Domingo et al. 2011). On the contrary, the levels for non-exposed population from European countries, Australia and USA were generally lower than those found in the present study (Brender et al. 2006, Caldwell et al. 2009, Callan et al. 2013, Link et al. 2007, Saoudi et al. 2012, Seifert et al. 2000). Nevertheless, studies from Mediterranean countries such as Italy, Croatia or Greece show arsenic concentrations in urine that are close to those in the present study (Miklavčič et al. 2012, Minoia et al. 1990), indicating that the

Mediterranean populations have similar exposure to total arsenic in urine as in the Sabadell cohort.

Table 21: Comparison of the arsenic concentrations in urine of pregnant women from Sabadell with urine concentrations from other populations.

Reference	Sampling years	N	Location	As[k]
Present work[a]	2004-06	489	Sabadell – 1st trim	28
Present work[a]	2004-06	489	Sabadell – 3rd trim	31
Link et al. 2007[ah]	2002-03	500	South Germany	4.6[d]
Caldwell et al., 2009[ag]	2003-04	2500	USA	8.2
Banza et al. 2009[bg]	2006-07	179	DR Congo	18
Seifert et al., 2000[bg]	1990-92	4000	Germany	4.6
Saoudi et al., 2012[bi]	2006-07	1515	France	12
Hopenhayn et al., 2003[ae]	1998-00	29	Chile	58
Garner et al., 2010[be]	2001-03	500	Bangladesh	94
Brender et al., 2006[ae]	1995-2000	74	Texas (Mexican origin)	9.0
Minoia et al., 1990[bg]	Nr	540	Italy	17[d]
Miklavcic et al., 2012[ae]	2007-2009	900	Italy	18
Miklavcic et al., 2012[ae]	2007-2008	234	Croatia	24
Miklavcic et al., 2012[ae]	2006-2009	484	Greece	37
Domingo et al., 2001[bf]	Nr[j]	28	Spain [f]	76
Callan et al., 2013[ae]	2008-2001	173	Australia	13

[a]Median. [b]Geometric mean. [c]Arithmetic mean. [d]µg/L [e]Pregnant women.

[f]Occupational exposure. [g]General population (including children). [h]Children. [i]General population (only adults). [j]not reported. [k]µg/g creatinine.

Dietary arsenic sources

Rice consumption did not show any significant association with arsenic concentrations in urine. Consumption of this cereal has been associated with total arsenic levels in some studies concerning populations from sites with arsenic contaminated waters, e.g. Bangladesh (Vahter et al., 2006), but this is not the case in the Sabadell cohort. The associations between seafood consumption and arsenic concentrations observed in the present study are consistent with previous studies on general population. In a study of pregnant women from different

Mediterranean countries, mean total seafood consumption was 40.4, 43.4 and 39.4 g/day in Italy, Croatia and Greece, respectively (Miklavčič et al. 2012). Considering that shellfish intake was not included in these estimations, the observed values are similar to the consumption reported by the mothers from Sabadell. The median concentrations of urinary arsenic in the women from these countries were 18, 24 and 37 µg/g creatinine for Italy, Croatia and Greece, respectively. The geometric means of the Sabadell cohort are 34-37 µg/g creatinine (Table 16), in the high end of values observed in these Mediterranean countries, as it has been mentioned in the previous section. The significant differences in medians from these three countries were attributed to fish consumption (Miklavčič et al., 2012). In agreement with these results, other studies on populations with lower fish consumption have reported lower arsenic content in the urine samples. Thus, in France more than 50% of participants reported fish consumption between once a week and twice a month (in our study only 11-13% of women would be included in this category) and found median levels of urinary arsenic that were 12 µg/g creatinine (Saoudi et al. 2012). In USA, the NHANEs study indicated that 57.2% of population reported consuming fish between 1 and 4 times per month (Navas-Acien et al. 2011) and the concentrations of total urinary arsenic were 8.2 µg/g creatinine (Caldwell et al. 2009). The results of the Sabadell cohort and these previous studies provide a consistent relationship between fish consumption and arsenic intake that is general for western countries (Abernathy et al., 2001; Fontcuberta et al., 2011; Caldwell et al., 2009; Saoudi et al., 2012; Seifert et al., 2000; Brantsaeter et al., 2010; Meltzer et al., 2002).

In the Sabadell cohort, the highest association of urinary arsenic was found with ingestion of lean fish. This association is consistent with the results of one previous study considering the observed associations between food items, namely lean and fatty fish, and blood arsenic concentrations from Norwegian population (Brantsæter et al. 2012). Analyses of arsenic in different fish species have reported high concentrations in shark, monkfish, common dab, ray or sole and lower concentrations in fatty fish species such as anchovy, sardine, tuna, salmon or mackerel (Fontcuberta et al. 2011, Muñoz et al. 2000, Sirot et al. 2009). Studies of arsenic content in mollusks and some shellfish, such as crabs, shrimps, lobsters or octopus have also found high concentrations of this metalloid (Brooke and Evans 1981, Fontcuberta et al. 2011, Lunde 1973, Sirot et al. 2009). Shellfish has been reported to have higher influence than fish in urinary arsenic levels in one study from US population (Navas-Acien et al. 2011). On the contrary, in other cases the influence of shellfish in the concentrations of total arsenic, either in urine or blood, has been reported to be lower than fish (Brantsæter et al. 2012, Muñoz et al. 2000, Saoudi et al. 2012). Local differences in shellfish, fish and their ecosystems, determining arsenic accumulation rates in the consumed species, may explain the differences between these studies.

Other dietary items, such as rice (Fontcuberta et al. 2011) and some vegetables, have been

associated with arsenic ingestion but these cases are related with irrigation with water of high arsenic content (Vahter 2009), e.g. in Bangladesh the high content of arsenic in groundwater involved that local rice was an important source of this metalloid for the population (Gardner et al. 2010, Vahter et al. 2006a). In the Sabadell cohort seafood is the main source which far exceeds the other diet components as arsenic supplier.

It has been long demonstrated that arsenic coming from seafood is organic and non-toxic. In general, inorganic arsenic corresponds to less than 1% of total arsenic content (Fontcuberta et al. 2011). In some bivalves and crustaceans this proportion is higher but the organic forms are still predominant (Fontcuberta et al. 2011, Muñoz et al. 2000, Sirot et al. 2009). Most arsenic present in marine animal organisms is in the form of arsenobetaine which is inert and rapidly excreted (Borak and Hosgood 2007). Some studies have shown that subjects having ingested seafood in the past 24 hours had 10-fold higher levels of arsenobetaine in urine than those who had not (Navas-Acien et al. 2011; Caldwell et al., 2009). In some cases, an increase of inorganic arsenic is observed besides the organic compounds, (Miklavčič et al., 2012; Saoudi et al., 2012; Buchet et al., 1996) but the increase is small or negligible as found in some controlled experiments (Hsueh et al., 2002). Anyway the increase is considered biologically not significant due to its low level (Buchet et al. 1996). Accordingly, the high levels typically found in Mediterranean populations, such as the Sabadell cohort, should not be of concern for public health if it can be demonstrated that they originate from seafood ingestion.

Changes in urinary arsenic along pregnancy

The concentrations of total urinary arsenic during both trimesters were not significantly different. Along pregnancy, diverse changes and adaptations occur in the woman body, such as a 40-fold increase in plasma volume (King 2000) and increases of the glomerular filtration rates (Swanson and King 1987). In the Sabadell cohort, these two metabolic modifications did not have any influence on arsenic urinary excretion. Increases of arsenic along pregnancy have been reported in studies on populations from sites contaminated with this metalloid which were attributed to increasing methylation as detoxification mechanism (Hopenhayn et al. 2003). In the present study, urinary arsenic was clearly related with seafood ingestion during the two periods of pregnancy.

As a matter of fact, grouping of the mothers by higher arsenic concentrations in the first or third trimesters shows a strong correspondence with seafood consumption (Table 22). Thus, the differences in mean consumption of white fish are statistically different among mothers with higher arsenic in the first than in the third trimester ($p<0.01$) and vice versa ($p<0.05$). These differences always involve highest intake in the group of highest arsenic concentration (Table 22). Significant differences are also observed among mean consumption of shellfish between mothers with higher arsenic in the third than in the first trimester ($p<0.05$; Table 22)

and total seafood in the mothers with highest arsenic in the first than in the third trimesters ($p<0.01$; Table 22). Thus, in the Sabadell cohort the urine levels are closely associated to fish consumption which was the main contributor to the concentration differences between the first and third trimesters and the main predictor of arsenic levels in both periods.

Table 22: Mean (standard deviation) of the fish consumption (in g/day) during the first and third trimesters of pregnancy grouped for differentiation of women with higher arsenic concentrations in these periods.

Consumption period	White fish		Fatty fish		Shellfish		Total seafood	
	$1^{st} > 3^{rd}$	$3^{rd} > 1^{st}$	$1^{st} > 3^{rd}$	$3^{rd} > 1^{st}$	$1^{st} > 3^{rd}$	$3^{rd} > 1^{st}$	$1^{st} > 3^{rd}$	$3^{rd} > 1^{st}$
First	34 (22)	29 (22)	27 (21)	26 (18)	12 (9.2)	12 (10)	73 (36)	66 (34)
Third	31 (24)	32 (23)	25 (21)	28 (21)	12 (8.9)	11 (14)	68 (40)	70 (40)
P[a]	0.0039	0.0497	0.38	0.51	0.31	0.042	0.0072	0.26

[a]p of significance according to Kruskal-Wallis test.

An important limitation of the present study is the lack of arsenic speciation since it provides useful information on the origin and toxicity of this metalloid When fish consumption is reported to be the main determinant of arsenic concentrations in urine it can be assumed that this metalloid is in its organic non-toxic forms but further chemical species assessment is recommended. In any case, the results of the present study proof that the urine concentrations from the Sabadell mothers are closely associated to diet. They do not reflect metabolic changes along pregnancy and this may be a feature of general population from sites not locally polluted with arsenic.

Conclusions

Mediterranean populations, such as the one represented by the Sabadell cohort, have higher total urinary arsenic concentrations than other European or North American populations. Consistently, the pregnant women from the Mediterranean region considered in this study have high arsenic levels. The high seafood consumption typical of these populations is the main cause for these high arsenic concentrations. A strong association between total urinary levels of arsenic and total seafood consumption is observed in the two different stages of pregnancy examined (first and third trimesters). Specifically, lean fish consumption is the main contributor to the intake of this metalloid. No significant differences in arsenic excretion are observed between these two periods and the observed small differences are mainly related to changes in lean fish consumption between the first and third trimesters. Seafood arsenic has been demonstrated to be mainly composed of organic species that are inert, not toxic and rapidly excreted. Therefore, these high levels of urinary arsenic found in Mediterranean populations are not cause of health concern. Assessment of the origin of arsenic in general

population is important for evaluation of possible health effects in the exposed population. The approach described in the present study provides the necessary information for estimation of the possible toxic effects associated to arsenic accumulation.

3rd case study – Interdependence between urinary cobalt concentrations and hemoglobin levels in pregnant women (Catalonia, Spain)

Introduction

Cobalt is a transition metal of widespread environmental occurrence. It is a minor component in a huge amount of minerals (Kim et al., 2006). It has been used for different applications such as pigments, catalysts in oil and gas production, battery electrodes, orthopedic prostheses and others (NHANES, 2009). It is then present in an important amount of manufactures, though human exposure to this metal depends mainly on diet. Its main sources are fish, green vegetables and fresh cereals (Unice et al., 2012). Cobalt is an essential trace metal used in the formation of vitamin B12 (also named cobalamin). 85% of the human body content of cobalt is this form, although only a small fraction of human cobalt intake is used for this purpose and most of the ingested cobalt is in inorganic form (Kim et al., 2006). This inorganic form has not an essential function and is not required in human diets. Cobalt deficiency has never been described in human metabolism (Simonsen et al., 2012). Remarkably, cobalt supplements are available and the manufacturers claim that this metal is useful for fat and carbohydrate metabolism, protein synthesis, red blood cell production and myelin sheath repair in the central nervous system (Finley et al., 2012). Cobalt has also been used as a homeopathic element to correct for eventual excessive excretion of estrogen during female hormone replacement therapy (Pausterbach et al., 2013). It is also suspected to have been used as doping agent due to its erythropoietic and angiogenetic properties (Lippi et al., 2006).

Occupational and accidental exposures to cobalt have been reported to originate asthma and respiratory problems (Nemery et al., 1992; Swennen et al., 1993), alterations of thyroid hormones (Prescott et al., 1992) and other effects. An oral reference dose of 0.03 mg/kg-day has been recently proposed as the maximum cobalt intake for non-cancer health effects in general population over lifetime exposure (Filey et al., 2012). This dose corresponds to 2.1 mg/day for a 70 kg adult, which is 50–400 fold higher than the average daily dietary cobalt intake of the US population (5–40 μg/day) (Finley et al., 2012). However, toxicological effects have been attributed to inorganic cobalt in its free ionic state, not bound to albumin, at lower concentrations than usual in subjects with albumin alterations such as anephric patients, sepsis patients or sickle cell children (Pausterbach et al., 2013).

Summary of existing environmental and biomonitoring data – gaps identification

The maternal concentrations of metals, including cobalt may change along pregnancy which may also be related to variations in fetal exposure. Measurements of trace metal changes along pregnancy have been considered in some cases but these studies did not include cobalt. Iron depletion is one of the most relevant changes during pregnancy (Goonewardene et al., 2012). Barany et al. (2005) demonstrated that iron status has an influence in the concentration

in blood of several metals such as cobalt. Moreover, animal studies have shown that iron depletion is associated with an increase of the intestinal absorption of divalent metals such as cobalt (Flanagan et al., 1980).

Gastrointestinal absorption of dietary cobalt can typically range from 10 to 35% (Unice et al., 2012). Intakes of 20% and 45% in males and females, respectively, have been considered as standard reference values in human biokinetic models (Unice et al., 2014). These gender differences are due to iron status. Menstrual losses in women may lead to lower iron which has been associated to higher levels of cobalt intake (Meltzer et al., 2010).

Toxicokinetic modeling and cobalt intake studies have long demonstrated that urinary cobalt is a good measure for cobalt concentrations in the human body. CoCl2 intake and absorption is reflected in the urine cobalt concentrations (Christensen et al. 1993). Furthermore, urinary cobalt excretion was found to represent two thirds of daily intake in a group of women who self-measured their dietary intake (Harp and Scoular, 1952). Correspondences between decreases of iron and increases of cobalt have been observed when comparing differences in concentrations of this metal in subjects with abnormal and normal iron status (Barany et al., 2005). Hereditary hemochromatosis patients were found to accumulate both iron and cobalt (Nichols and Bacon, 1989).

Accordingly, urine is the preferred source of information for cobalt biomonitoring because it can be collected without invasive methods. It has been widely used in large environmental studies with trace metals such as the German Environmental Survey for Children (GerES) and the National Health and Nutrition Examination (NHANES).

The present campaign was devoted to compare the levels of cobalt in urine of pregnant women in the first and third trimester of pregnancy and for assessment of the possible relationships of iron decrease occurring along pregnancy with the observed changes.

Biomonitoring data

Sampling and analysis protocol

Sampling and analysis was performed as in the previous case. Hemoglobin in blood during the 12th and 32nd weeks of pregnancy was analyzed as a marker of iron status. Analysis was performed using a Sysmex XE-2100 system, where hemoglobin is determined by the sodium lauryl sulfate (SLS)-hemoglobin method.

Cobalt 3rd-1st trimester individual ratios were calculated by division of the 3rd trimester by the 1st trimester concentrations. Furthermore, individual cobalt concentrations differences between both trimesters were also calculated.

Cobalt concentrations between the first and the third trimesters were compared using chi-

squared tests and Spearman correlations. Spearman correlation rates were calculated to identify possible associations between different variables in non-parametric distributions. Mann-Whitney and Kruskall-Wallis testing was used to compare between groups of categorical variables. Univariate and multivariate linear regression models were performed to investigate which maternal factors were associated to cobalt concentrations.

Results

Cobalt levels, either in ng/mL or μg/g creatinine in the first and third trimesters are shown in Table 23. The distributions of individual concentrations were not gaussian but skewed to the left. Descriptive statistics in ng/mL or in μg/g creatinine were not significantly different. Accordingly, μg/g creatinine concentrations were generally used in the study of the associations. Median concentrations were 0.45 and 1.3 μg/g creatinine during the first and third trimesters, respectively. P90 was 1.4 and 2.9 μg/g creatinine during the first and third trimesters, respectively. The cobalt concentrations during the third and first trimesters were significantly correlated (Spearman coefficient 0.39; $p<0.001$).

The concentrations during both trimesters were significantly different ($p<0.001$). Calculation of the individual ratios between third and first trimesters provided an arithmetic mean and median of 4.5 (SD 7) and 2.2 μg/g creatinine, respectively (Table 23). Most pregnant women from the studied population (84%) had higher cobalt levels during the third than the first trimesters.

Vitamin B12 intake showed no statistically significant association with cobalt levels in both trimesters involving Spearman's correlation rates of 0.0471 ($p = 0.36$) and -0.0690 ($p = 0.19$) during the first and third trimesters, respectively. Moreover, women taking supplementation either during the first or third trimesters did not show significant differences in cobalt concentrations when comparing with those who did not. Thus, in the first trimester, the mean urine concentrations of the two groups were 0.60 μg/g creatinine, standard deviation -SD- 0.58 μg/g creatinine, vs. 0.84 μg/g creatinine, SD 1.8 μg/g creatinine, ($p = 0.107$) and in the third trimester they were 1.7 μg/g creatinine, SD 2.8 μg/g creatinine, vs. 1.5 μg/g creatinine, SD 1.2 μg/g creatinine ($p = 0.624$).

Table 23:Metal urine concentrations of cobalt in the first and third trimesters of pregnancy, in ng/mL and μg/g creatinine. Correlation rate between concentrations in both trimesters and p-value of the difference.

	% Detection	Arithmetic mean (SD)	Median (IQR)	P90	p-Value	Spearman's correlation rho
ng/mL						
1st trimester	73.6	0.57 (0.56)	0.42 (0.74)	1.2	p<0.001	0.41

3rd trimester	84.3	1.4 (1.4)	1.2 (1.2)	2.8		(p<0.001)
3rd/1st ratio		4.6(6.3)	2.1(4.3)	12		
3rd – 1st difference		0.88 (1.2)	0.70 (1.3)	2.1		
µg/g creatinine						
1st trimester	73.6	0.73 (1.4)	0.45 (0.70)	1.4	p<0.001	0.39
3rd trimester	84.3	1.6 (2.5)	1.3 (1.3)	2.9		(p<0.001)
3rd/1st ratio		4.5 (7.0)	2.2 (3.8)	10		
3rd – 1st difference		0.90 (2.5)	0.72 (1.2)	2.2		

Table 24: Urine cobalt concentrations in the present cohort and in other populations, in ng/mL (µg/g creatinine in brackets)

Reference	Origin	N	Co
Our study (1st trim)	Sabadell	345	0.42(0.45)[b]
Our study (3rd trim)	Sabadell	345	1.2(1.3)[b]
Callan et al., 2013	Australia	173	(1.17)[b d]
Ohashi et al, 2006	Japan	1000	0.68[a]
Banza et al 2009	DR Congo (mining area)	179	(15.7)[a]
Yokota et al 2007	Japan (battery plant)	16	28[c e]
Paschal et al 1998	USA	496	(0.78)[a]
Minoia et al 1990	Italy	11-900	0.57[a]
NHANES 03-04	USA	2500	(0.314)[b]
Komaromy et al., 2000	USA	1000-16000	(1)[a d]

[a]Geometric mean. [b]Median. [c]Arithmetic mean. [d]Pregnant women [e]Only men, just after leaving the plant

The mean hemoglobin concentrations during the first and third trimesters were 12.63 g/dL and 11.55 g/dL, respectively. These differences were statistically significant (p<0.001). Women with higher hemoglobin decrease along pregnancy had significantly higher cobalt increase between both trimesters (p<0.05). This correlation was statistically significant (Figure 38). No statistically significant association was found for cobalt and hemoglobin

during the first trimester but the association was statistically significant in the third trimester (-0.12; $p<0.05$).

Table 25:. Cobalt concentrations (µg/g creatinine) in anemic and non-anemic pregnant women (under and over 11 g/dL hemoglobin in blood) during the first and third trimesters of pregnancy.

	% women	Mean (SD)	Median (IQR)	P90	p-Value
1st trimester					
>11 g/dL Hb	96	0.85(0.71)	0.44 (0.69)	1.3	0.16
<11 g/dL Hb	4.2	0.72 (1.4)	0.69 (0.71)	2.1	
3rd trimester					
>11 g/dL Hb	72	1.5 (2.9)	0.93 (1.2)	2.6	0.016
<11 g/dL Hb	28	1.8 (1.4)	1.2 (1.5)	3.4	

Table 26:. Multivariate regression model for maternal urine cobalt concentrations during the third trimester of pregnancy and its possible associated factors.

	β (IC)	p-Value
Last measurement of hemoglobine in g/dL blood	-0.11 (-0.22, -0.00093)	0.048
Maternal origin		
Spain	1	
Latin America	-0.081 (-0.54, 0.38)	0.731
Europe	0.13 (-0.58, -0.85)	0.710
Rest of the world	-2.5 (-4.6, -0.49)	0.020
Exposure to heavy truck traffic		
Practically never	1	
A few	0.11 (-0.18, 0.41)	0.450
Quite often	0.36 (-0.010, 0.72)	0.057
Continuous	0.22 (-0.18, 0.51)	0.146
Smoking during 3rd trimester		
No	1	
Yes	0.33 (-0.0029, 0.67)	0.052

White meat consumption [a]	-0.0063 (-0.013, 0.00066)	0.076
Blue fish consumption [a]	0.0056 (-0.00077,0.012)	0.084
Vegetables consumption [a]	0.00098 (-0.00012, 0.0021)	0.081
Legumes consumption [a]	-0.0050 (-0.010, 0.00054)	0.077
Coffee/Tea comsumption [a]	-0.00070 (-0.0013, -0.00093)	0.048
R^2	0.094	

[a]gr/week of consumption

A multivariate linear regression model was built taking into account different dietary and social maternal factors (Table 26). This model confirmed the negative significant association between cobalt and hemoglobin levels ($p<0.05$). Compilation of the same multivariate linear regression model on anemic (< 11 g/dL hemoglobin in blood) and non-anemic (> 11 g/dL)

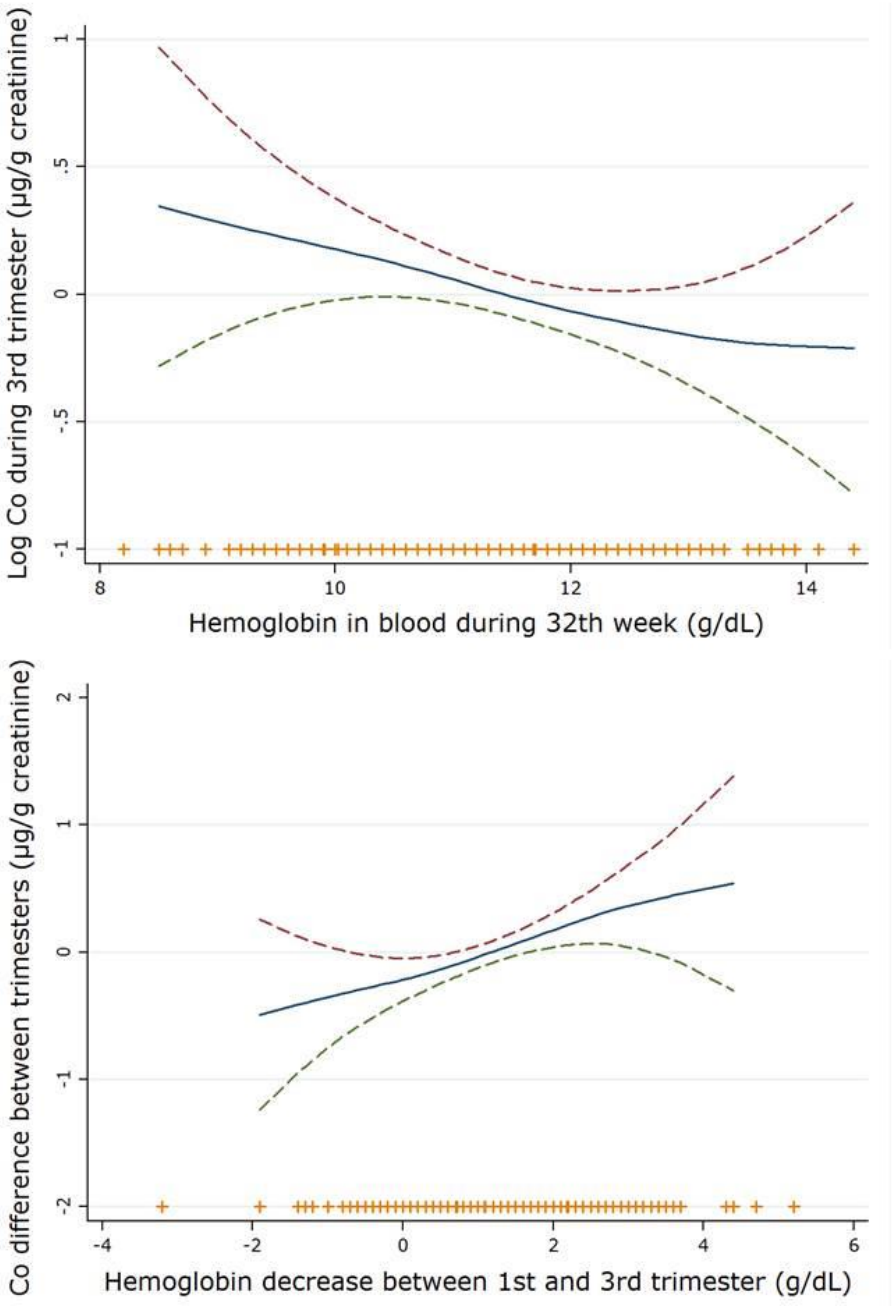


Figure 38: Graphic representation of generalized additive models (GAM) showing association (and 95 % confidence levels) between maternal blood hemoglobin levels and the logarithm of urine cobalt concentrations in the first and third trimesters of pregnancy and the increases of cobalt at decreasing hemoglobin. The symbols (+) on the X-axis show the hemoglobin levels of each subject (g/dL).

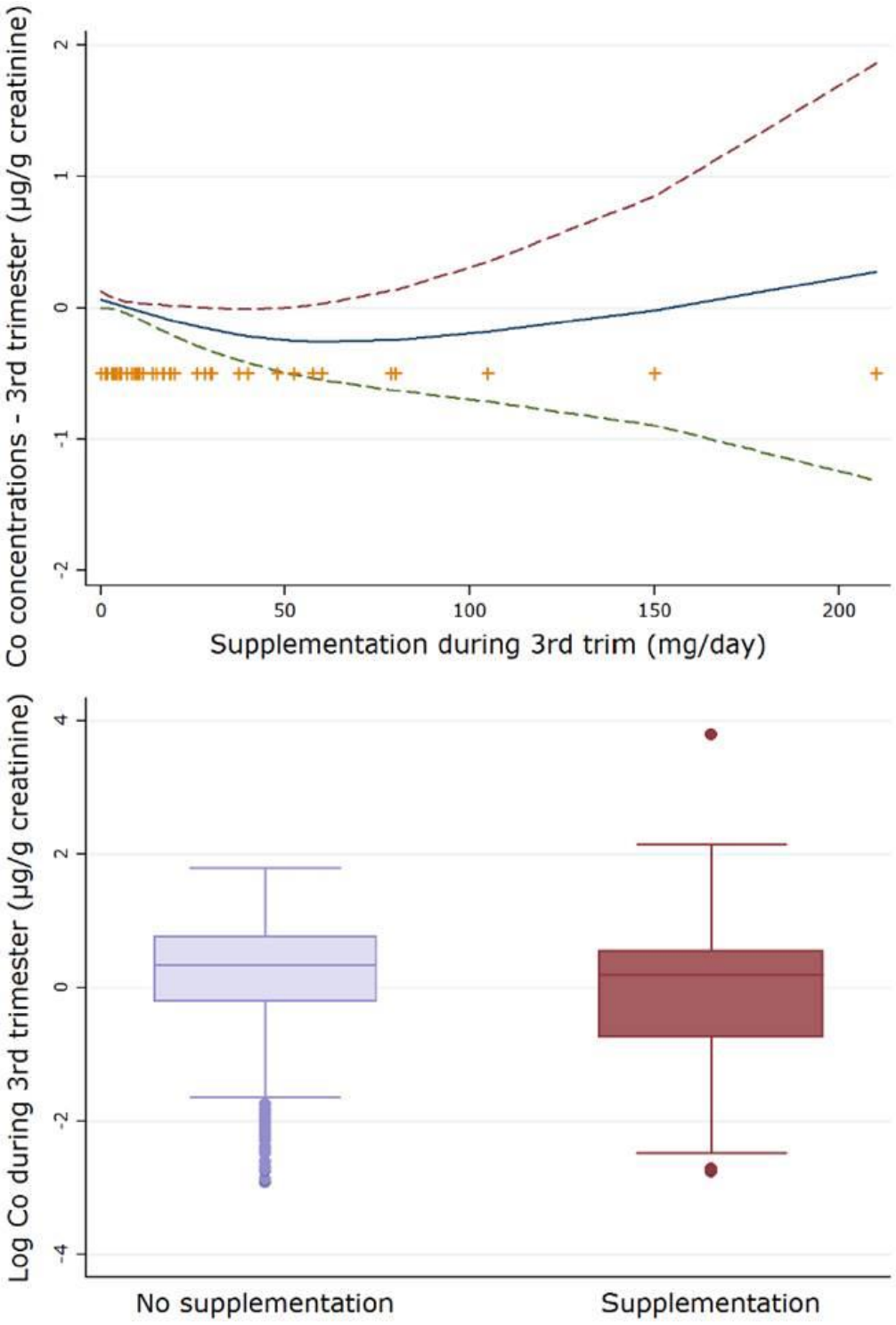


Figure 39: Association between iron supplementation intake and cobalt concentrations during the third trimester of pregnancy.

(Goonewardene et al., 2012) women showed that the former had significantly higher concentrations of cobalt. Consumption of coffee or tea also showed significant negative association with cobalt but with a lower beta coefficient ($p<0.05$). Therefore, during third

trimester cobalt concentrations were negatively associated to hemoglobin levels and the trend was stronger among anemic women.

During the first trimester only 4.2% of pregnant women were anemic and this proportion rose up to 28% in the third trimester (Table 25). Anemic women had higher concentrations of cobalt than women with normal levels in both trimesters, but the differences were only significant during the third trimester ($p<0.05$; Table 25).

Iron supplementation was taken by 8.1% (mean intake 19 mg/day) and 35% (mean intake 30 mg/day) in the first and third trimesters, respectively. A significant negative association between cobalt levels and iron supplementation was observed during the third trimester ($p<0.01$; Figure 39, but not during the first. When supplementation was high, this trend was not so well defined. Nevertheless, women taking supplementation had lower levels of cobalt during third trimester than those who were not taking it ($p<0.05$; Figure 39).

The urine concentrations of cobalt in the studied cohort are similar to those found in general population from Europe (Minoia et al., 1990), USA (Komaromy-Hiller et al., 2000; NHANES, 2009), Japan (Ohashi et al., 2006) and Australia (Callan et al., 2013). In contrast, they are lower than levels reported in a mining area (Banza et al., 2009) or in occupational studies (Yokota et al., 2007). The levels during the third trimester were similar to those reported for pregnant women in Australia (Callan et al., 2013) (Table 24). According to the biokinetic model of Unice et al., (2014), assuming an inorganic cobalt intake of 10-20 µg/day during 30 years and 10% of gastrointestinal absorption, a urine concentration range between 0.6 and 1.2 ng/mL should be observed, which is in the same range as our data (Table 24). Cobalt intakes may change according to dietary differences between countries, e.g. 0.12 mg/day (United Kingdom), 29 µg/day (France) and 5-40 µg/day (USA) (Kim et al., 2006). Analysis of cobalt in 10-day representative diet items of an industrialized area of Spain led to estimate a mean intake of 9.8 µg/day (Domingo et al. 2012), which is consistent with these previous ranges. Thus, the observed cobalt concentrations of the present study are consistent with toxicokinetic data from general population of western countries in which the main exposure and intake is due to the diet (Gál et al., 2008) and not to specific sources. The correlation between the concentrations in the first and third trimester of pregnancy is consistent with a constant exposure scenario along pregnancy for the whole studied population.

Cobalt is part of the vitamin B12 molecule. The absorption mechanisms of this vitamin are different from those of inorganic cobalt (Kim et al., 2006). Various studies have described mean daily losses of 0.1% of the B12 vitamin pool which may be enhanced when intake is high (IOM, 1998). No significant associations between intake of B12 supplements and urine cobalt concentrations either during the first or third trimesters of pregnancy have been

observed in the present study. Accordingly, most of the ingested cobalt is in inorganic form and vitamin B12 intake has not an influence on the observed cobalt urinary levels (IOM, 1998; Kim et al., 2006).

Few previous studies have considered the changes in trace metal concentrations along pregnancy (Kilinc et al., 2010; Liu et al., 2010) and none of them included cobalt. Callan et al. (2013) analyzed cobalt concentrations in urine of pregnant women during the third trimester and the results were similar to those corresponding to the third trimester in the Sabadell cohort, but they did not describe results at other stages of pregnancy. Considering other metals concentration decreases of Cd, Se or Cs have been reported (Kilinc et al., 2010;) and have been attributed to the 40-50% increase in plasma volume along pregnancy (Hytten, 1985). In contrast, urinary Zn excretion was reported to increase along pregnancy due to the higher glomerular filtration rate that occurs along this process (Swanson and King, 1987). In the case of cobalt, the two-fold higher median increase between the first and third trimesters (Table 23) reflects the individual increase of this metal in 84% of women from this population. Although glomerular filtration rate increase could explain this change, we consider that a metabolic process may be responsible for an enhancement of cobalt absorption

Basal metabolic rate rises up to 60% during the third trimester of pregnancy (Hytten, 1985), increasing iron demand to transport sufficient oxygen for aerobic processes. Moreover, there is an increased need for transport to the fetus for hematopoiesis. Actually, 80% of the iron present in the newborn term infant is accreted during the third trimester of pregnancy (Baker et al., 2010). Accordingly, requirements of absorbed dietary iron increase from 0.8 mg/day during the first trimester to 7.5 mg/day during the third (Milman, 2006). This increase in iron consumption leads to iron decrease along pregnancy, e.g. a mean hemoglobin decrease of 1.10 g/dL in the present study, including 28% of anemic women (less than 11 g/dL hemoglobin) in the third trimesters.

The observed urine cobalt increase may respond to a compensatory mechanism of iron decrease (Table 23, Figure 38 and Figure 39). Accordingly, a statistically significant negative correlation is observed between cobalt and hemoglobin concentrations in the samples collected in the third trimester. The difference is defined even better when considering anemic women as they have significantly higher cobalt concentrations than women with normal hemoglobin levels. Hemoglobin decrease during pregnancy reflects iron depletion and leads to cobalt increase.

This relationship is consistent with previous studies. A correspondence between decrease of iron and increase of cobalt has been reported in adolescent girls and boys, particularly in girls, either by comparing serum ferritin or transferrin transporter and serum cobalt or from the differences of cobalt concentrations between subjects with abnormal and normal iron status

(Bárány et al., 2005). Hereditary hemochromatosis patients were found to accumulate not only iron but also cobalt (Nichols and Bacon, 1989). Associations between cobalt blood concentrations and ferritin or hemoglobin status were reported in Norwegian women (Meltzer et al., 2010). Women with normal and high iron concentrations have been observed to retain less cobalt than women with iron depletion (Christensen, 1995; Christensen et al., 1993) Women are also known to retain more cobalt than men which may be related to iron losses by menstruation (Christensen, 1995; Christensen et al., 1993; Unice et al., 2012). Indeed, women have been observed to increase three times more cobalt in urine than men at equal intake of this compound in a short term study of gastrointestinal uptake of different inorganic cobalt compounds (Christensen, 1995; Christensen et al., 1993).

Increased intestinal cobalt absorption as consequence of iron deficiency by bleeding or diet was reported in mice experiments (Flanagan et al., 1980). Cobalt absorption has been demonstrated to be enhanced not only in iron depletion but also during adolescence or during the last stage of pregnancy when there are higher iron body demands (Barany et al., 2005). The increase of cobalt absorption may be mediated by the divalent metal transporter 1 (DMT1), an intestinal active transporter for inorganic Fe in its oxidized from Fe(II). This transporter has been demonstrated to be associated with the intestinal transport of other divalent cations such as Mn(II), Cu(II), Zn(II) or Co(II) (Gunshin et al., 1997). In vitro experiments with DMT1 animal transfected cells showed that this enzyme is able to transport cobalt into the cell (Garrick et al., 2006, Forbes and Gros, 2003), together with other divalent ions. This protein is up-regulated by iron status, either in iron depletion or when iron body demands are enhanced (Garrick et al., 2003; Mackenzie and Garrick, 2005). Hence, iron decrease together with higher demand occurring during the third trimester of pregnancy may enhance the DMT1 expression leading to a higher absorption of cobalt and higher concentrations of this metal in urine. This mechanism has already been proposed to justify observed associations between iron status and metal concentrations in human populations (Meltzer et al., 2010; Barany et al., 2005) and further studies are needed for a more comprehensive understanding of the processes involved. The present study provides evidence of the correspondence between iron status and cobalt in pregnant women.

Cobalt induces erythropoietin transcription, stimulating red blood cell production (Unice et al., 2012). In fact, it has been hypothesized that this metal may eventually be used by some athletes to increase erythropoietin activity (Lippi et al., 2006). The higher levels of cobalt during the third trimester may stimulate the production of red cells during late pregnancy to fulfill the O2 requirements for the high metabolic rate occurring at this stage, as well as to provide sufficient iron for red cell production for the fetal growth.

Women taking iron supplementation had significantly lower levels of cobalt during the third trimester (Figure 39). As their iron requirements were fulfilled and hemoglobin levels were

generally higher, lower cobalt absorption and lower cobalt levels in urine were observed.

The observed results may also be significant in populations occupationally or environmentally exposed to cobalt. Hence, in populations with daily cobalt intake close or higher to 0.03 mg/kg·day in women with albumin alterations (Pausterbach et al., 2013) or during pregnancy, the proposed chronic oral reference dose for maximum cohort intake (Field et al., 2012) could have health effects in the mothers and, most importantly, in their children. Dietary and control measures for avoiding iron depletion should be emphasized in these cases.

One of the main limitations of our study is the use of hemoglobin as the only measure of iron status. Hemoglobin reflects mass of circulating red blood cells. It should be complemented with other measurements such as serum ferritin or serum ferritin transporter in order to diagnose and iron-defficiency anemia status, although during pregnancy iron deficiency is difficult to confirm (Goonewardene et al., 2012). However, hemoglobin is a rapid and cheap parameter which do not require costly laboratory equipment and testing, thus it can be useful to have a first approach (Cameron and Neufeld, 2011).

Conclusions

Cobalt levels in maternal urine have been observed to rise significantly from the first to the third trimesters, probably due to the iron decrease along pregnancy. A significant negative correlation has been found between hemoglobin and urine cobalt concentrations in the third trimester, as well as between the differences between hemoglobin levels and urine cobalt concentrations between these two trimesters. This association has been previously reported in adolescents, women and hemochromatosis patients, but the present study is the first in which this trend is observed during pregnancy. Cobalt enhances transcription of erythropoietin, leading to higher red cell production. Higher absorption of this metal may tend to counterbalance iron depletion during last stages of pregnancy, when basal metabolic rate is high and 90% of fetal growth occurs and iron requirements are increased. This mechanism may be useful to contribute to fulfill the oxygen demand of these processes that are crucial for proper fetus development. However, the present results recommend the implementation of monitoring programs of cobalt concentrations in pregnant women from populations occupationally or environmentally exposed to this metal. This strategy could allow to anticipate possible deleterious effects for the mother or fetus as consequence of enhanced cobalt accumulation.

4th case study – Evaluation of atmospheric inputs as possible sources of antimony in pregnant women from urban areas

Introduction

Atmospheric particles in urban areas have been linked to several health outcomes such as oxidative stress, inflammation, epoc and cardiovascular or cerebrovascular stroke (Perez et al., 2009; Pope and Dockery, 2006). Significant correlations between daily mortality and ambient air particulate matter (PM) have been identified in Barcelona (Ostro et al., 2011). However, urban particles constitute very complex mixtures. Insight on the origin of these deleterious health effects and possible remediation actions depend on the association of specific properties such as size, chemical composition and others to specific toxic outcomes.

Summary of existing environmental and biomonitoring data – Gaps identification

Sb is a toxic metalloid that is present in the diet at low concentrations (Arnich et al., 2012). The intestinal absorption of this element in humans is 5-20% (Lauwers et al., 1990). Its high volatility involves high affinity for atmospheric PM which constitutes a potential pollution source of this compound by particle inhalation (Belzile et al., 2012). Few studies on concentrations of Sb in humans are available (Filella et al., 2013a; Filella et al., 2013b) and very limited information is found on prenatal and children exposure but its presence in amniotic fluid has been observed (Caserta et al., 2011).

Cu is an essential metal that is necessary for the function of some enzymes such as ceruloplasmine or cytocrom c oxydase. It is present in a wide variety of foods (Mason, 1979). Besides diet and the gastrointestinal system, it may also be incorporated through respiration (Wiseman and Zereini, 2014). To date, industrial activity is the main source of this metal to the environment but vehicular traffic has also become a potential source because of its current use in brake linings (Amato et al., 2009). This metal is also known for its toxicity at high concentrations, children are more susceptible to deleterious effects than adults (Mason, 1979).

Sb and Cu are present in urban particulate material (Amato et al., 2011). Inhalation of PM may be a source of these metals for human populations. Urine is an adequate matrix for heavy metals biomonitoring (Fort et al., 2014) and can be collected without invasive methods. The concentrations of Sb and Cu in women at any pregnancy stage provide representative results for the whole pregnancy period (Fort et al., 2014). In the present study urban atmospheric pollution is evaluated as potential source of prenatal exposure to these metals.

Biomoniroring data

Sampling and analysis protocol

Sampling and analysis was performed as in the previous case. Median age of the mothers at the time of their last menstrual period was 31 years, ranging between 18 and 42 years (Table 27). The mean BMI of these mothers before pregnancy was 23.6 kg/m^2 ranging between 14.9 and 53.8 kg/m^2. Among these, 18.4 and 7.6% of them were overweight and obese, respectively. Concerning parity, 53.3% of the mothers were primiparous, 39.1% had another infant and 7.5% had more than two infants. During the third trimester of pregnancy, 21.4% and 43.6% of women considered themselves quite-very active and sedentary-not active, respectively. Concerning vehicular traffic exposure, 40.9%, 25.4%, 24% and 9.5% of women reported living in streets with heavy, frequent, moderate and rare traffic, respectively. 88.7% of the women worked during all pregnancy and 24.2% of them worked in manufacturing or transport. Paternal occupation in manufacturing or transport was 46.3% of total. Height of the housing was grouped as between ground and 4th floor and above, encompassing 87.5% in the first case.

Results

Sb and Cu concentrations

The concentrations of Sb and Cu, in ng/mL and μg/g creatinine, are shown in Table 28. The distributions of concentrations of Sb and Cu were not parametric but skewed to the left. Descriptive statistics in ng/mL or μg/g creatinine were not significantly different (Table 2). Thus, description of results and discussions are referred to μg/g creatinine. The geometric mean Cu and Sb concentrations were 13 μg/g creatinine (interquartile range 9.8 μg/g creatinine; P90 27 μg/g creatinine) and 0.28 μg/g creatinine (interquartile range 0.35 μg/g creatinine; P90 0.85 μg/g creatinine), respectively. The concentrations of both metals were significantly correlated (Spearman's correlation rho = 0.30, $p < 0.001$).

Table 27: Main characteristics of the participating pregnant women

Characteristics	**N**	**%**
Age (years)	[a] 30.9 (17-42)	
<25	36	7.7
25-29	149	32.0
30-35	199	42.7
>35	82	17.6
Pre-pregnancy BMI (kg/m^2)	[a] 23.6 (14.9-53.8)	
<20	74	16.0
20-25	267	57.9

25-30	85	18.4
≥30	35	7.6
Parity		
0	248	53.3
1	182	39.1
≥2	35	7.5
Social class		
Non manual	239	51.2
Manual	228	48.8
Origin		
Spanish	419	90.3
Latin American	33	7.1
Rest of Europe	10	2.2
Others	2	0.43
Physical activity		
Sedentary / little active	202	43.6
Moderately active	162	35.0
Quite / very active	99	21.4
Smoking		
Never	231	50.2
Smoking at the beginning of	132	28.4
Smoking throughout pregnancy	71	15.3
Cotinine		
< 4 ng/mL	199	42.8
≥ 4 ng/mL	265	57.1
Traffic intensity near the		
Heavy	190	40.9
Frequent	118	25.4
Moderate	112	24.1
Rare	44	9.5
Worked all pregnancy		

Yes	409	88.7
No	52	11.3
Maternal occupation		
Manufacturers - transporters	113	24.3
Rest	352	75.7
Paternal occupation		
Manufacturers - transporters	251	46.3
Rest	216	53.7
Height of housing		
0-4th	407	87.5
5th-12th	58	12.5
Season		
Winter	125	27.0
Spring	114	24.6
Summer	125	26.0
Autumn	99	21.4

[a]Arithmetic mean (Range)

Seasonal differences

The geometric mean concentrations of both metals were highest in the urine samples collected in winter (17 μg/g and 0.31 μg/g creatinine of Cu and Sb, respectively; Table 28). The geometric mean concentrations of Sb were lowest in the urine collected in autumn (0.23 μg/g creatinine). Those of Cu were lowest in the urines collected in summer (9.7 μg/g creatinine; Table 28). In the univariate models the concentrations of Sb were significantly different between winter and spring or autumn ($p < 0.05$) while the levels of Cu were significantly lower in summer ($p < 0.001$) and spring and autumn ($p < 0.01$) than in winter Figure 40.

Table 28: Statistics of the concentrations of Sb and Cu in general population and in the groups defined by influence of traffic pollution, season and physical activity.

	Sb	**Cu**
General cohort		
% detection	65	100
Concentration (ng/mL)		

Arithmetic mean (SD)	0.45 (1.2)	14 (9.6)
Geometric mean (IQR)	0.25 (0.31)	11 (11)
P90	0.68	26
Concentration (μg/g creatinine)		
Arithmetic mean (SD)	0.56 (2.0)	16 (11)
Geometric mean (IQR)	0.28 (0.35)	13 (9.8)
P90	0.85	27
Traffic intensity near the homeplace (μg/g creatinine)		
Rare	0.21[a] (0.35)[a]	12[a] (9.5)[a]
Moderate	0.27 (0.33)	12 (10.4)
Frequent	0.30 (0.37)	14 (10.8)
Heavy	0.29 (0.29)	14 (9.3)
Season (μg/g creatinine)		
Winter	0.31 (0.43)[a]	17 (12)[a]
Spring	0.26 (0.28)	14 (7.2)
Summer	0.30 (0.40)	9.7 (7.6)
Autumn	0.23 (0.23)	13 (9.8)
Physical activity (μg/g creatinine)		
Sedentary / little active	0.25 (0.33)[a]	14 (9.3)[a]
Moderately active	0.28 (0.27)	12 (8.4)
Quite / very active	0.35 (0.44)	14 (15)
Worked during all pregnancy (μg/g creatinine)		
Yes	0.28 (0.32)	16 (9.6)
No	0.32 (0.46)	13 (13)
Height of housing (μg/g creatinine)		
Ground to 4th	0.29 (0.37)	13 (10)
$_{5}$th-$_{12}$th	0.23 (0.24)	13 (9.4)
Maternal occupation (μg/g creatinine)		
Manufacturer – transporter	0.24 (0.25)	14 (10)

Rest	0.30 (0.39)	13 (7.8)
Paternal occupation (μg/g creatinine)		
Manufacturer – transporter	0.28 (0.32)	14 (9.0)
Rest	0.29 (0.36)	12 (11)

[a]geometric mean (interquartile range)

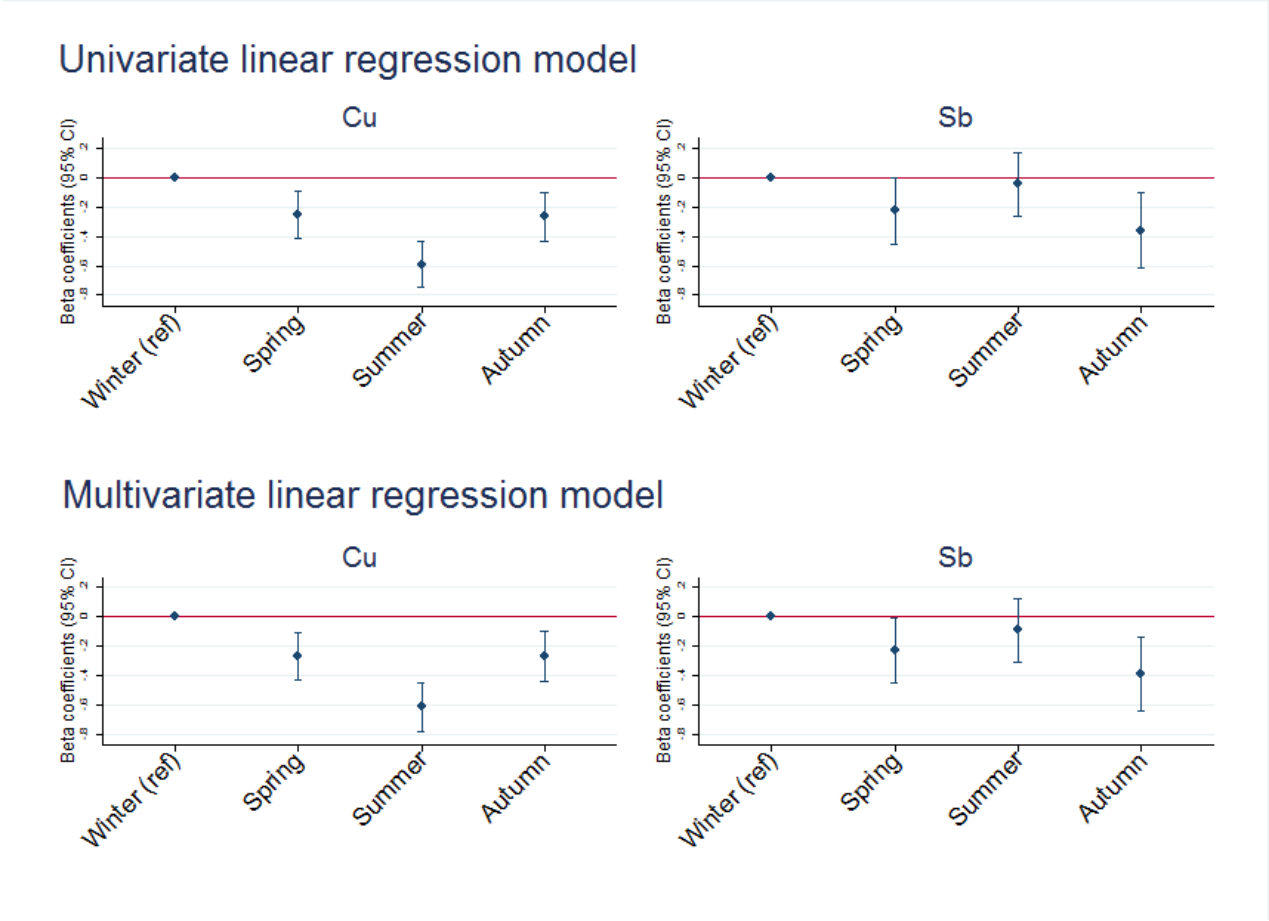


Figure 40: Results of the univariate and multivariate models for the influence of sampling season in the concentrations of Cu and Sb in the urine of pregnant women. The reference category is indicated.

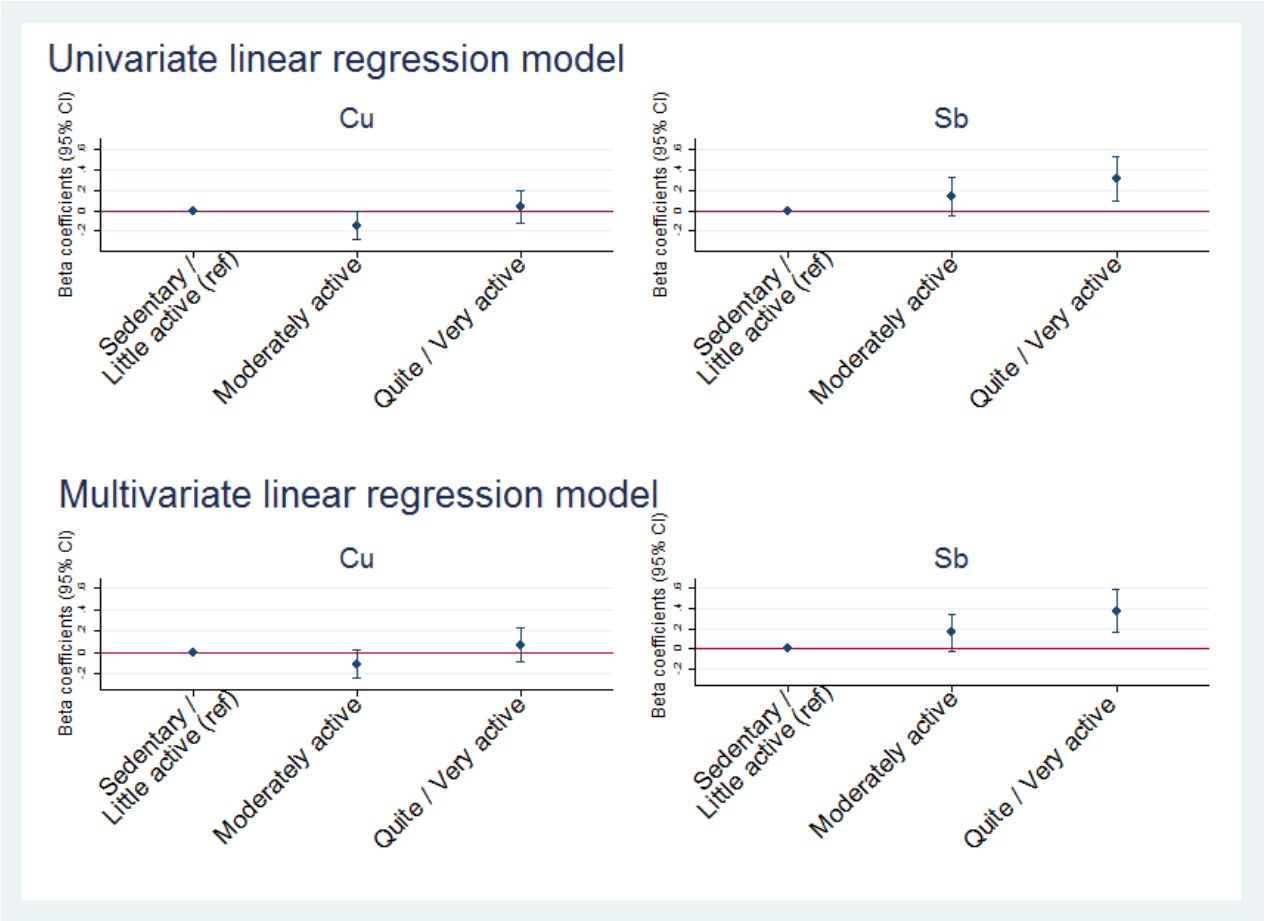


Figure 41: Results of the univariate and multivariate models for the influence of maternal physical activity in the concentrations of Cu and Sb in the urine of pregnant women. The reference category is indicated.

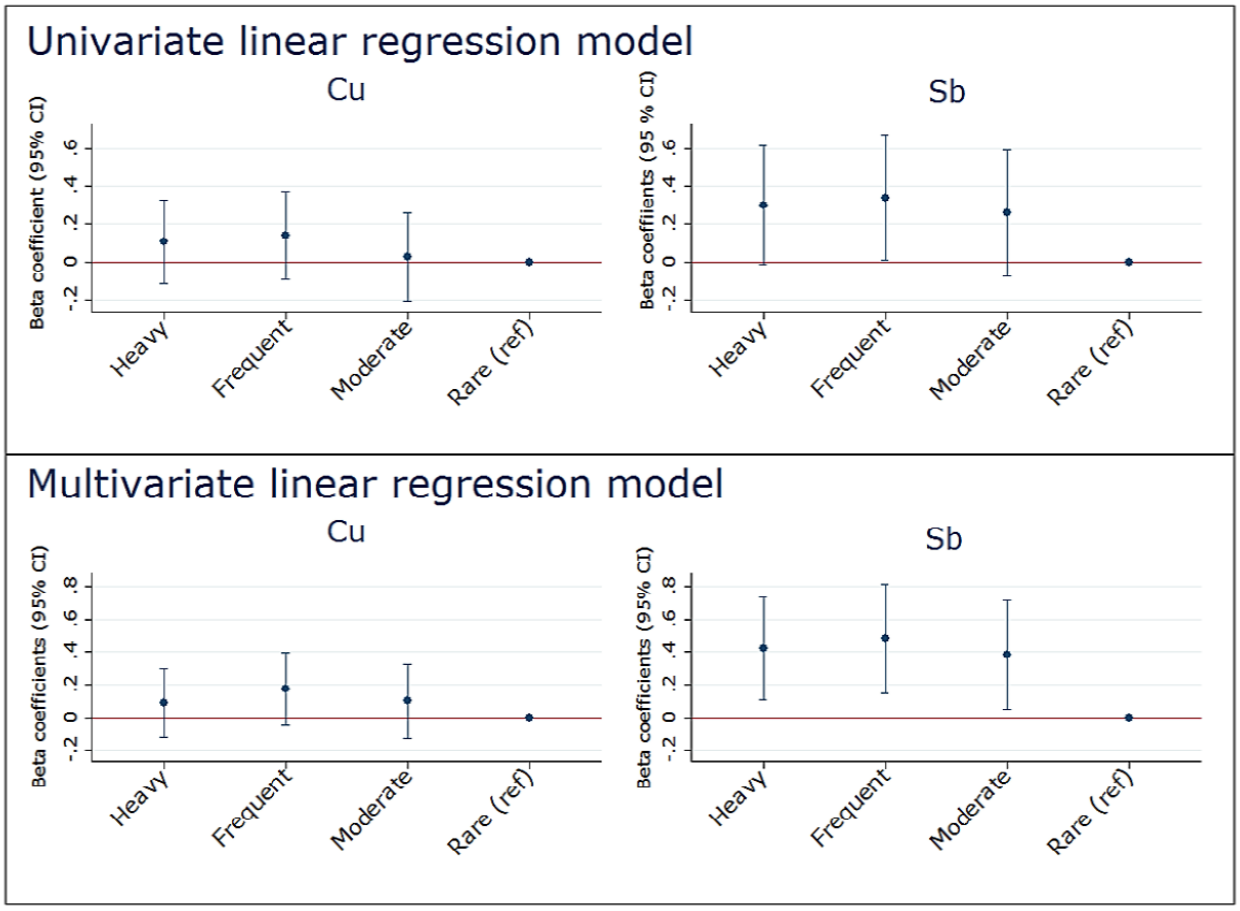


Figure 42: Results of the univariate and multivariate models for the influence of traffic in the concentrations of Cu and Sb in the urine of pregnant women. The reference category is indicated.

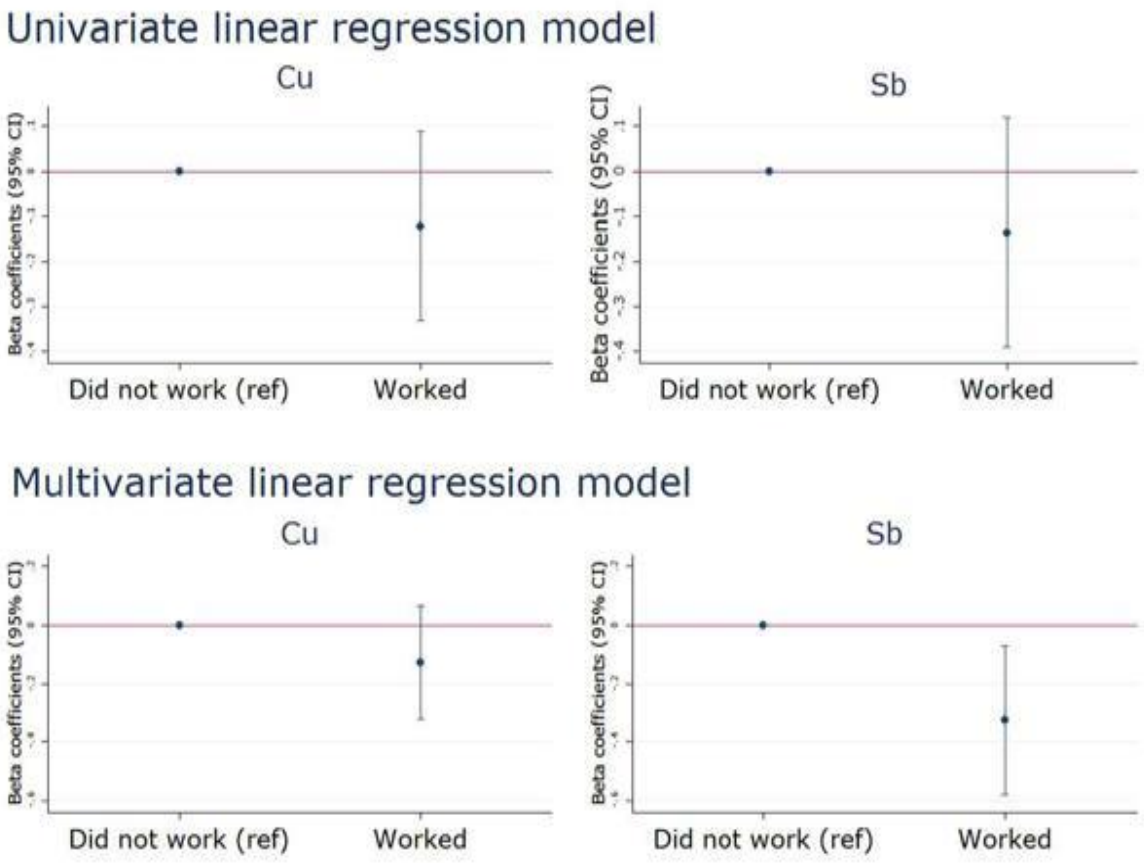


Figure 43: Results of the univariate and multivariate models for the influence of working during pregnancy in the concentrations of Cu and Sb in the urine of pregnant women. The reference category is indicated.

Physical activity

The geometric mean Sb urine concentrations were higher in quite or very active women than in those with more sedentary habits (0.35 and 0.28 μg/g creatinine vs 0.25 μg/g creatinine, respectively; Table 28). These differences were significant in the univariate models ($p < 0.01$; Figure 41. On the other hand, the geometric mean Cu levels in sedentary women were higher than in moderately active women but lower than in quite or very active women. Only the difference between sedentary and moderately active women was significant in the univariate models ($p < 0.05$; Figure 41).

Table 29: Comparison of the urine Sb and Cu concentrations in this cohort with previous studies (μg/g creatinine)

Reference	Sampling years	Location	N	Sb	Cu
Present work[b]	2004-06	Sabadell	461	0.28	13
NHANES report, 2009[a]	2001-02	USA	2500	0.13	…

Ohashi et al., 2006[be]	2000-05	Japan	1000	…	13
Banza et al., 2009[b]	2006-07	DR Congo	179	0.07	17
Paschal et al., 1998[b]	1988-94	USA	496	0.67	…
Schuhmacher et al., 1994[b]	nr	Tarragona	434	…	27
Seifert et al., 2005[b]	1990-92	Germany	4000	…	9.5
Alimonti et al., 2005[a]	nr	Italy	50	0.68	…
Heitland et al., 2006[b]	2005	Germany	87	0.037	5
Minoia et al., 1990[b]	nr	Italy	306/507	0.79[d]	23
Callan et al., 2013 [af]	2008-11	Australia	173	…	10.4
Lüdersdolf et al., 1987 [ag]	nr	Germany	109	1.9	…

[a]Median. [b]Geometric mean. [c]Arithmetic mean. [d]µg/L. [e]Women only. [f]Pregnant women. [g]Men from a glass-producing plant

Traffic pollution

The geometric means of the Sb urine concentrations were higher in women with homes in street categorized in the heavy or frequent traffic density groups than in those of streets with very low or rarely any traffic (0.29 and 0.30 µg/g creatinine vs 0.27 and 0.21 µg/g creatinine, respectively; Table 28). The geometric means of the Cu concentrations were slightly higher for women living in streets with heavy traffic than in streets with practically no traffic (14 µg/g creatinine vs 12 µg/g creatinine). In the univariate models for car traffic the Sb concentrations were significantly higher in the women group exposed to heavy or frequent vehicular traffic than in those rarely exposed ($p < 0.05$; Figure 42).

Other population characteristics

Women who worked during the whole pregnancy had lower concentrations of Cu and Sb than those who did not (13 µg/g and 0.28 µg/g and vs. 15 µg/g and 0.32 µg/g creatinine of Cu and Sb, respectively), but these differences were not statistically significant in the univariate linear

regression models (

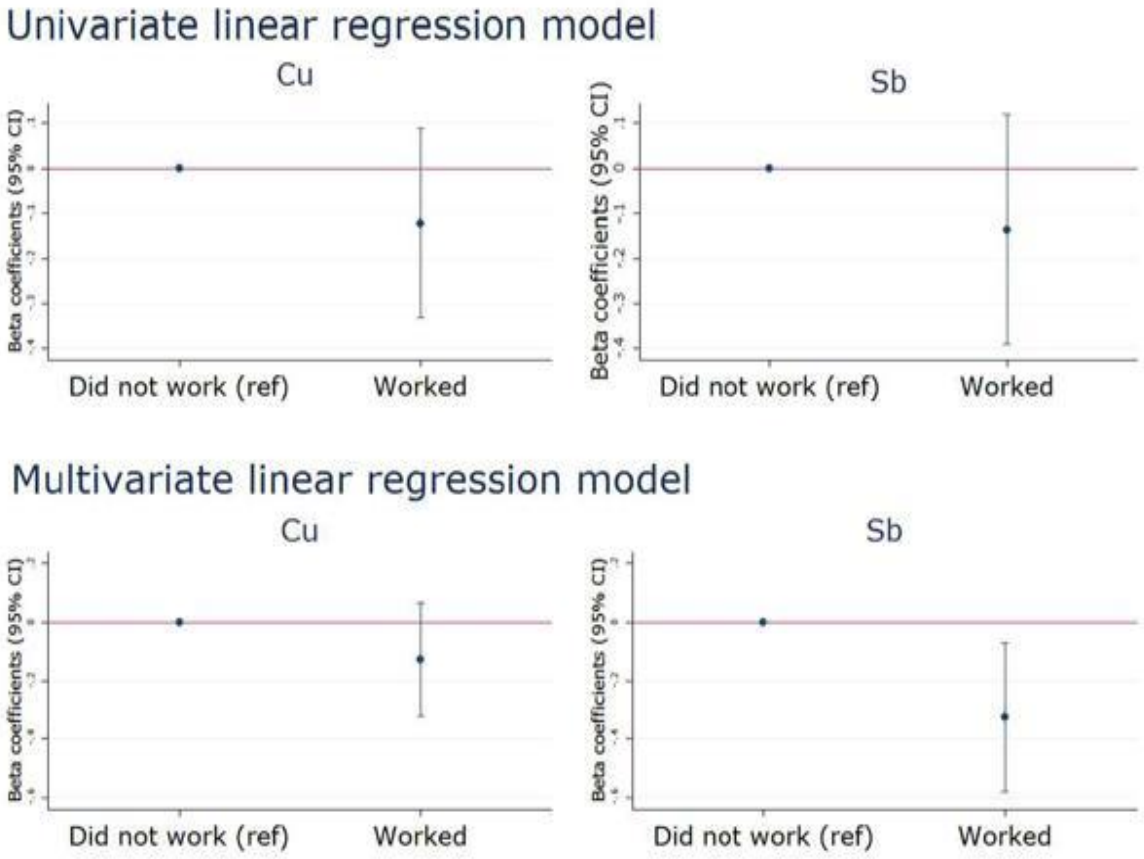


Figure ***43***).

Mothers whose apartments were below the fifth floor had higher geometric mean Sb concentrations than those living above, between the 5th and the 12th floors (0.29 μg/g vs. 0.23 μg/g; Table 28). No difference in Cu geometric means was observed in relation to home altitude. Different Sb and Cu means were observed for maternal and paternal occupation but without statistically significance.

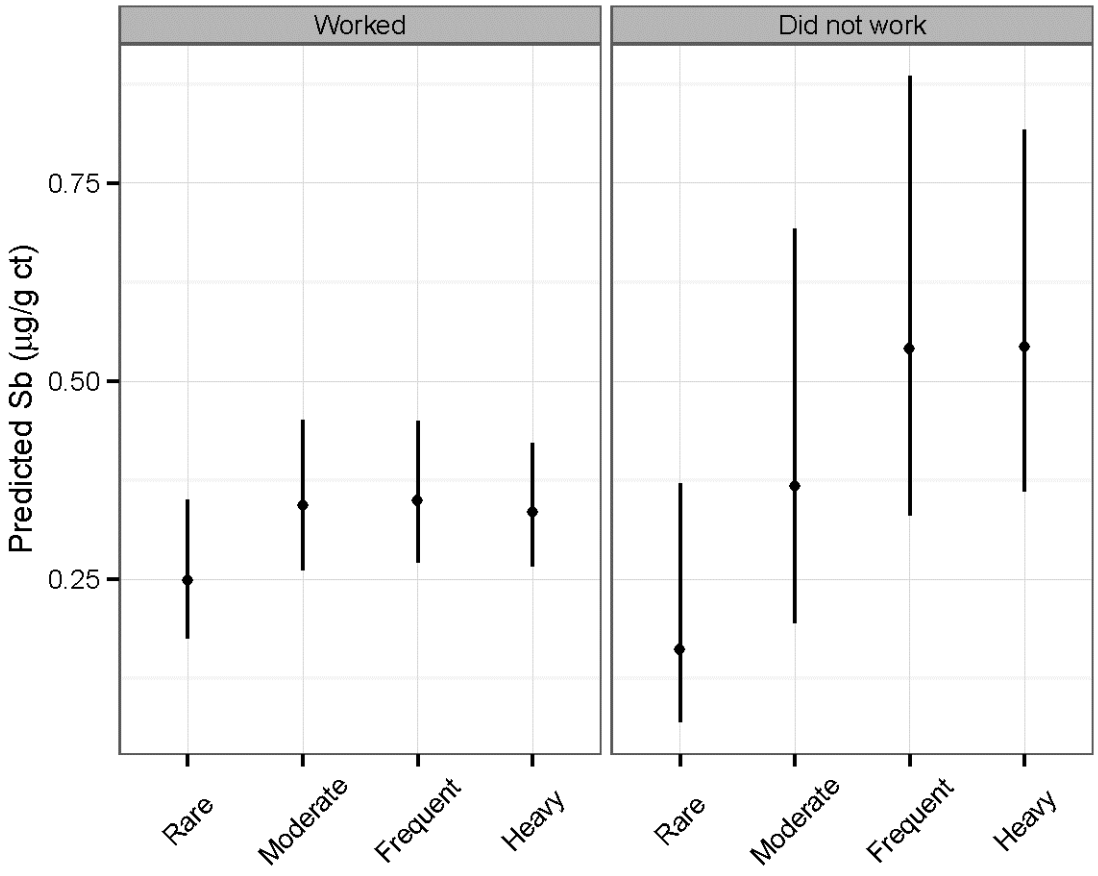


Figure 44: Predicted values of Sb (interval: 95% CI) from the multivariate linear regression models representing the interaction between working during pregnancy and car traffic exposure.

Multivariate analysis

Multivariate linear regression models were built considering all above mentioned variables. According to the backward stepwise selection, pre-conception BMI, social class, cotinine, and consumption of food items such as bread, cereal/pasta and candies were also included in final models for Sb. In the case of Cu, age, parity, height of the housing, paternal occupation, cotinine and consumption of fruits, nuts, potatoes, coffee or infusions and alcohol were the selected variables. The adjusted R^2 for final models was 0.10 and 0.14 for Sb and Cu, respectively.

Sb concentrations were again higher during winter than in spring and autumn ($p<0.05$ and 0.01, respectively). For Cu the multivariate models also showed higher significant concentration differences in winter than in the other seasons (Figure 40).

The multivariate models for physical activity also showed significantly higher Sb concentrations for active and moderately active women than for those with sedentary habits ($p<0.01$; Fig.2). Cu showed the same trend but without statistical significance (Figure 41).

Vehicular traffic density showed a statistically significant association with the urine Sb levels (Figure 42). Women from streets with rare traffic had significantly lower urine concentrations than those living in streets with continuous and frequent traffic ($p < 0.01$) and those living in streets with moderate traffic ($p < 0.05$). The beta coefficients and significance levels were higher in the multivariate than in the univariate models. Concerning Cu, the multivariate models also showed lower urine concentrations for women from streets with low traffic intensity than from moderate, frequent or heavy traffic but the differences were not significant.

According to the multivariate models, women that did not work during pregnancy had higher significant Sb concentrations than those who worked ($p<0.05$;

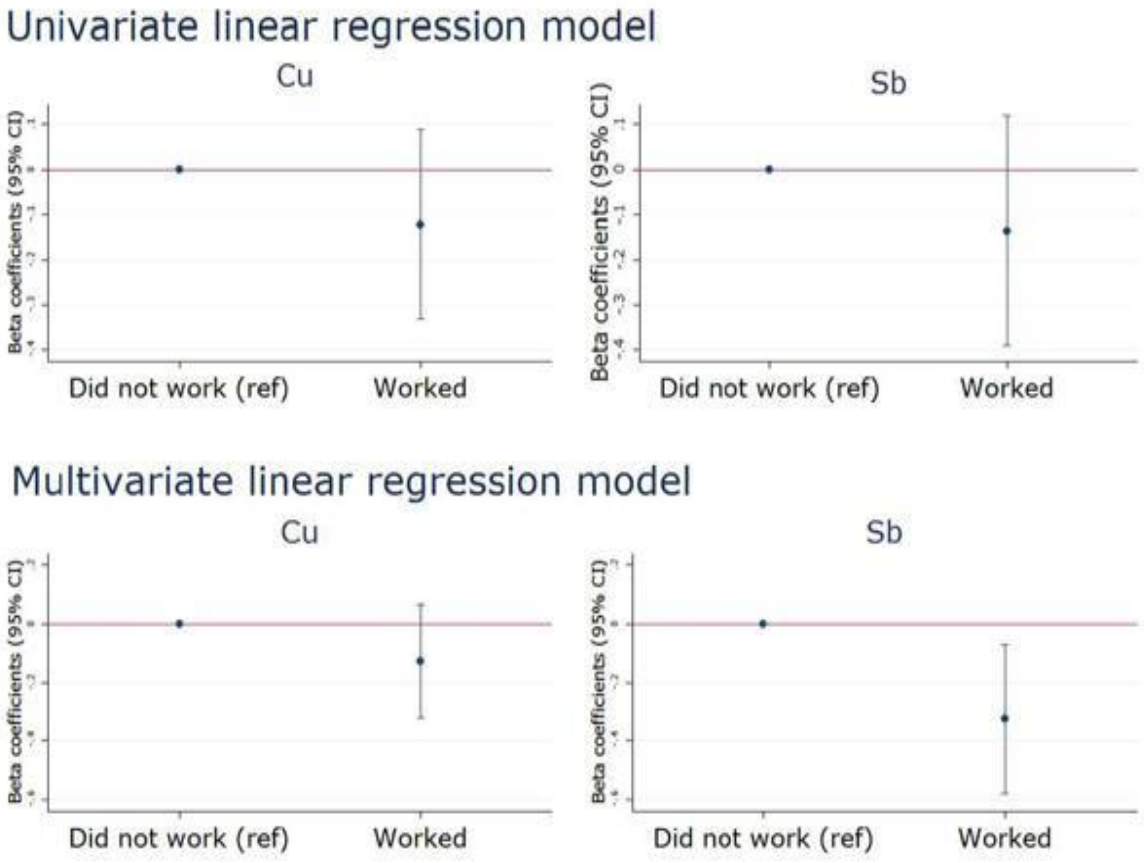


Figure ***43***). The same difference was observed for the Cu concentrations but the differences were not statistically significant. No significant differences were found for height of housing and type of maternal or paternal occupation.

Finally, Cu concentrations did not show any significant association with diet items, while Sb showed a positive association with the tertiles of intake of pasta/cereal. The beta coefficients

of this association using the first tertile as the reference category were 0.32 (SD: 0.10; p<0.01) and 0.25 (SD: 0.10) for the second and third tertiles, respectively.

Variable interactions

Interactions between vehicular traffic exposure and working during pregnancy were also evaluated for both metals. No significant interactions were found for Cu but in the case of Sb they were significant. Accordingly, women who did not work had a more marked association between Sb content and vehicular traffic than working women (Figure 44).

Calculation of the Spearman correlations for Sb and Cu concentrations over all samples showed a rho coefficient 0.3025 (p<0.001). However, calculation of these correlations per season separately only showed a significant correlation between Sb and Cu for the samples collected in winter (rho: 0.569; p<0.001).

Urinary Sb and Cu concentrations in other cohorts and environments

The urine concentrations of Sb in the Sabadell cohort were slightly higher than those reported in populations from Congo (Banza et al., 2009), Germany(Heitland and Köster, 2006) or USA (NHANES, 2009) but lower than those found in workers from a glass-producing plant (Lüdersdorf et al., 1987) as well as those in general population from Italy (Alimonti et al., 2000; Minoia et al., 1990) or USA between 1988 and 1994 (Paschal et al., 1998) (Table 29). The urine Cu levels were similar to those found in Japan (Ohashi et al., 2006), lower than those reported in Tarragona (Catalonia, Spain) (Schuhmacher et al., 1994), and higher than those in general population from Germany (Lüdersdorf et al., 1987; Seifert et al., 2000) or in pregnant women from Australia (Callan et al., 2013) (Table 29).

Sb and Cu have been previously analyzed in atmospheric $PM_{2.5}$ in Sabadell (Minguillón et al., 2012). In summer, concentrations of 2.5 and 5.5 ng/m^3 in a suburban background area were observed, respectively. These concentrations increased to 3 and 20 ng/m^3 in a dense traffic street, respectively. In comparison to other concentrations these results were higher than the annual means of regional forest environments (Montseny; Pey et al., 2010b) or in an urban background from Birmingham during spring (Taiwo et al., 2014) but they were lower than in an urban background from Barcelona (Pey et al., 2010b). Sb was markedly lower than those reported in atmospheric samples from Tijuana area (Minguillón et al., 2014).

Seasonality

The highest concentrations of Sb and Cu in Sabadell have been found in the atmospheric particles collected in winter (Minguillón et al., 2012) which reflects winter anti-cyclonic episodes and thermal inversion in the area (Pey et al., 2010a). The configuration of the geographic depression where Sabadell is located makes dispersion of the air pollutants particularly difficult when wind is not oriented along the depression axis (Minguillón et al.,

2012). The higher Sb and Cu concentrations found in the maternal urine samples collected in winter are consistent with these observations.

The significant correlation of the urine concentrations of Sb and Cu in the samples collected in winter is consistent with the finding of highest concentrations of these metals in the atmospheric particles. The Sb and Cu urine concentrations from the samples collected in the other seasons shows no correlation. This difference is consistent with the above reported inputs and metabolic role of these two metals. Both metals have been demonstrated to be highly soluble in pulmonary fluid (Wiseman and Zereini, 2014). Sb has been reported to be more absorbed through the respiratory than the gastrointestinal track (Iavicoli et al., 2002) and can therefore be incorporated from air pollution. Cu may be incorporated from this source and also from diet and is retained by metabolic needs. The excretion behavior of this last metal may only reflects environmental exposure in conditions of high atmospheric pollution such as winter thermal inversion.

Physical activity

Associations between physical activity and increased metal excretion have been reported (Campbell and Anderson, 1987; Kovacs et al., 2012), but some of these studies showed that the most important way of excretion of trace metals during physical exercise was sweat (Genuis et al., 2011). During pregnancy women have higher nasofaringeal and faringeal capillarity which increases the absorption capacity of air pollutants (Plaat and Arrandale, 2012). Higher intake of metal pollution should be reflected in higher urine excretion of these elements as observed in the present study for Sb (Figure 41). The more intense respiration during physical activity may lead to higher inhalation of particles and its components.

Conversely, urine Cu excretion did not show significant associations with physical exercise. As mentioned above, diet is a more important source of this metal into humans than Sb. The lack of association of Cu excretion with physical activity suggests that the statistically significant cases may reflect higher intake by inhalation of atmospheric pollution and not general mobilization processes of all stored metals at higher metabolic activity.

Influence of traffic pollution

Since the late 90s, antimony (III) sulphide, Sb_2S_3, is used in brake linings after elimination of asbestos which led to an increase of Sb in the atmospheric PM (Garg et al., 2000; Wåhlin et al., 2006). Braking at traffic lights and stop signs enhance brake lining wear (Apeagyei et al., 2011). The high temperatures reached in this action enhance the oxidation of Sb_2S_3 into antimony oxide that is much more soluble in water than the original sulfide. Sb_2O_3 is classified as possible carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (Sundar and Chakravarty, 2010). As mentioned above, previous atmospheric pollution studies have shown significant contributions from road traffic to the Sb

and Cu content of PM (Minguillón et al., 2012) and the atmospheric occurrence of Cu and Sb has been attributed to brake lining metal emissions (Adachi and Tainosho, 2004; Amato et al., 2011; Amato et al., 2009; Hjortenkrans et al., 2007). Brake lining wear has been considered to be responsible for 90 and 99% of airborne Cu and Sb, respectively (Thorpe and Harrison, 2008).

The observed dependence of urine Sb from traffic activity (Figure 42) is consistent with these observations on the metal composition of urban particles. Accordingly, the women living in homes with higher vehicular traffic nearby showed higher concentrations of Sb in urine (Figure 42). Cu showed the same trend but the differences were not statistically significant (Figure 42).

In the case of Cu, traffic pollution may influence much less than diet in the intake of this metal in pregnant woman. Thus, although pulmonary solubility of Cu in PM was reported to be above 80% (Wiseman and Zereini, 2014), an occupational study on electrolytic department workers presumably exposed to this metal as consequence of emissions to the air did not show high concentrations of Cu in urine (Nieboer et al., 2007).

In the present study, the exposure to car traffic was specifically considered in the area where the homes of the pregnant women were located (Figure 42). Accordingly, the association was stronger for the women who did not work during the pregnancy period as they remained more time near their homes than the working women. This difference was observed for both Sb and Cu.

Dietary and atmospheric apportion of Sb and Cu.

The average concentrations of Sb and Cu in the supply waters of the city are 0.86 μg/l and 3.6 μg/l, respectively (Casas et al., 2001). These values are far below the public health goals of 6 μg/l and 1300 μg/l, for Sb and Cu, respectively, of the National Primary Drinking Water Regulations from the US EPA (EPA, 2009).

Calculation of the total dietary ingestion of Sb for mean weight women of 76 kg using reported data from UK (Rose et al., 2010) assuming an intestinal absorption of 5-20% (Lauwers et al. 1990) results into estimated of 0.15-0.61 μg/day. The equivalent Cu intake assuming a mean intestinal absorption of 30-40% (Wapnir, 1998) is 390-520 μg/day.

On the other hand, taking into account the reported concentrations of Sb and Cu in $PM_{2.5}$ in the suburban background area of Sabadell (Minguillón et al., 2012) and a daily inhalation of 22 m^3 of air in pregnant women (Brochu et al., 2006) estimates of Sb inhalations of 0.1 and 0.055 μg/day during winter and summer, respectively, are obtained. The estimates for Cu are 0.12 and 0.44 μg/day during these two seasons, respectively.

According to these values, the dietary contribution of both metals is higher than the theoretical atmospheric input. Nevertheless, this difference is much broader for Cu than for Sb, which is consistent with the previously reported lack of statistical significance of the Cu atmospheric inputs when comparing urine concentrations of this metal and determinants of atmospheric pollution intake. In the multivariate analysis, no dietary item was associated with Cu and consumption. In the case of Sb there was an association of urine concentrations and consumption of cereal/pasta involving 5% of total daily intake, but the beta coefficients of the tertiles of consumption were lower than those of traffic exposure, physical exercise and seasonality.

Strengths and limitations of the study

Although the associations between Sb and atmospheric inputs are found to be statistically significant, the current study has some limitations. Vehicular traffic exposure was only evaluated at home and through questionnaire variables. Deployment of a network of aerosol samplers for monitoring specific exposures to traffic particles in the different home areas would increase the robustness of the associations but this approach was beyond the technical and economic possibilities of this study. In addition, 35% of the samples had non-detectable levels, which may be a cause for bias. Nevertheless, final models included different variables that modulate exposure to atmospheric pollutants and the results for all of them were consistent. Further studies considering more markers of traffic exposure, including not only those registering exposure at home but also a complete picture of daily exposure to vehicular traffic should be performed for a better assessment of these findings.

Conclusions

Atmospheric inputs are possibly responsible for the observed differences in urine Sb concentrations from pregnant women living in urban areas. The occurrence of this metal in the atmosphere has been attributed to traffic activity as consequence of its use in brake linings. The associations of Sb content in urine of pregnant women with seasonality, physical activity and traffic intensity near their homes is consistent with some dependence of the intake of this metal from atmospheric sources. These associations suggest that despite the estimated dietary inputs of this metal are somewhat higher than the estimated inhalation intake, the atmospheric inputs of Sb may be significant for the overall incorporation of this metal in populations of modern urban areas, e.g. in pregnant women.

Cu is also used in brake linings but the high predominance of inputs of this essential metal from dietary components make unlikely the significance of the atmospheric urban inputs in the overall human intake. This is consistent with the lack of statistical significance of the observed differences in Cu urine concentrations when grouped according to atmospheric pollution indicators.

5th case study – Temporal trends in concentrations and total serum burdens of organochlorine compounds from birth until adolescence in Menorca and Flix and the role of breastfeeding

Introduction

Organochlorine compounds (OCs) include a wide range of chemicals, such as polychlorinated biphenyls (PCBs), dioxins, dichlorodiphenyldichloroethylene (DDE) or hexachlorobenzene (HCB). They are lipophilic synthetic chemicals and belong to the family of persistent organic pollutants (POPs) because they persist in the environment for years and bioaccumulate through the food chain in human and animal fatty tissues (Carpenter 2011). Through the placenta, humans start being exposed to OCs during prenatal life. After birth, in the first months or years of life, mothers can transfer a certain amount of these compounds through breastfeeding because of the lipophilic properties of POPs (LaKind et al. 2004; Ribas-Fito et al. 2005). After breastfeeding, the child continues being exposed to OCs through diet, which currently is the most important source of exposure in general population (Llop et al. 2010; Vrijheid et al. 2010). Because of their persistency and the health effects associated to OCs exposure, including neurotoxic, immunotoxic and endocrine and reproductive health effects, as well as cancer, the production and use of most OCs are currently banned in the majority of countries, which has led to a general reduction of the levels in the environment and human tissues (Carpenter 2011). In Spain, these compounds were banned between the early 70's and the late 80's (Ribas-Fito et al. 2005). However, HCB has been unintentionally produced until now as a subproduct of industrial processes. This is the case of the chloro-alkali plant in the village of Flix, area of Ribera d'Ebre, Catalonia, Spain; in this village of around 5000 inhabitants, air and human serum samples collected in the early 90's had the highest levels of HCB ever reported worldwide (Grimalt et al. 1994; Sala et al. 1999, 2001), which made this case unique and particularly relevant at an international level (Herrero et al. 1999; Ballester et al. 2000; Ozalla et al. 2002; Ribas-Fito et al. 2003c; Sunyer et al. 2008). Furthermore, because of the high levels of HCB found in the area, a birth cohort study was set up in 1997 with the aim to study the health effects of prenatal and postnatal exposure to environmental pollutants, particularly OCs and mercury, in children (Guxens et al. 2012). In this same year, another birth cohort was set up in the island of Menorca, Spain, with the aim to study the effects of early life exposure to air-borne irritants and allergens on allergy and asthma (Guxens et al. 2012). In both sites OCs exposure was evaluated at birth and at the age of 4 years (Sala et al. 2001; Carrizo et al. 2007b, 2008) as well as the health effects associated (Ribas-Fito et al. 2002, 2003a, 2003b, 2006a, 2006b, 2007a, 2007b). The inclusion of both birth cohorts in a common study was of interest because of the different exposure scenarios in each site: whereas Ribera d'Ebre was an industrial site with a high production of OCs, Menorca had a rural environment without industrials sites manufacturing this type of chemicals. Thus,

children from the latter constituted an example of the background exposure to these pollutants in western countries (Carrizo et al. 2007b). In 2012, when children were around 14 years of age, a new follow-up with a common protocol was performed in both study areas and POPs were analyzed in serum samples of participant adolescents.

Summary of existing environmental and biomonitoring data – gaps identification

To our knowledge, no other birth cohort study has assessed exposure to OCs at different ages from birth until adolescence and in two different settings with contrasted sources of exposure and levels of OCs. Thus, the aims of the present study are to assess 1) the current concentrations of OC exposure in adolescents of both birth cohort studies, 2) the temporal trends of OC concentrations and total serum burdens from birth until adolescence and 3) the influence of breastfeeding in these temporal trends. Because OC exposure concentrations at birth and at the age of 4 years and differences between the two birth cohorts were already assessed and discussed in a previous study (Carrizo et al. 2007b), in the present study we will focus on exposure concentrations at the age of 14 years and on the exposure temporal trends from birth up to adolescence.

Biomonitoring data

Sampling and analytical protocol

Study populations

A total of 102 singleton children born in the main hospital of the Ribera d'Ebre between 1997 and 1999 were included in the Ribera d'Ebre birth cohort study. OCs were measured at three different follow-ups: at birth (cord blood, N=73), at the age of 4 years (serum, N=58, years 2001-2003) and at the age of 14 years (serum, N=36, year 2012). Additionally, at the age of 14 years, schoolmates of children of the original cohort were invited to participate in the study (all children attended the same high school in the village of Flix); 15 accepted to participate and to provide serum samples.

Between 1997 and 1998 the Menorca birth cohort study recruited all women presenting for antenatal care. In total, 482 children were enrolled and OCs were measured at three different follow-ups: at birth (cord blood, N=405, years 1997-1998), at the age of 4 years of children (serum, N=285, years 2001-2002) and at age 14 years (serum, N=43, year 2012).

Exposure assessment

OCs in serum samples were analyzed by gas chromatography (GC) with electron capture detection and GC coupled to chemical ionization negative-ion mass spectrometry. All the analyses were carried out in the Department of Environmental Chemistry (IDAEA-CSIC). Details of the methodology have been reported elsewhere (Ribas-Fito et al. 2003b; Carrizo et al. 2006, 2007b). The limits of detection (LOD) and quantification (LOQ) ranged between

0.01 ng/mL and 0.05 ng/mL depending on the year of analysis and the compound analyzed. Compounds measured in all samples were pentachlorobenzene (PeCB), four isomers of hexachlorocyclohexanes (α-HCH, β-HCH, δ-HCH, γ-HCH), HCB, dichlorodiphenyltrichloroethane (4,4'-DDT), and its main metabolite 4,4'-DDE, and seven PCB congeners (28, 52, 101, 118, 138, 153 and 180), which were summed into one single exposure variable (Σ7PCBs). The protocol and instruments used were the same in the three follow-ups, and the method performed satisfactorily in repeated international intercalibration exercises within the Arctic Monitoring and Assessment Program (Centre de Toxicologie. Institut National de Santé Publique du Québec, 2002).

At the age of 14 years, 23 children from Ribera d'Ebre (3 not belonging to the original cohort) and 43 from the Menorca birth cohort had information on total lipid serum levels. This information was used to calculate OC concentrations in ng/g lipid [based on the equation of (Phillips et al. 1989)] and compare these exposure concentrations with those of previous studies including adolescent population. However, because total lipid data was only available for some of the participants at the age of 14 years, the main results of the present study are based on non-lipid adjusted OC concentrations (ng/mL).

To calculate the total serum burden of OCs (ng) at each age, we estimated the total blood volume (mL) of each child and multiplied it by the concentrations of each OC (ng/mL). The total blood volume was estimated using the body weight and the gender of the child according to different references consulted (Booth; Green; Linderkamp et al. 1977; Stephen 2011). At birth (cord blood) we estimated 85 mL of blood per kg of body weight, at age 4 years we estimated 75 mL per kg of body weight, and at age 14 years we estimated 65 mL per kg of body weight for girls and 70 mL per kg of body weight for boys.

Breastfeeding definition

At the age of 1 and 2 years of the child mothers were asked whether they had breastfed their child. In the present study, we classified children as breastfed children (any breastfeeding, independently of the duration) and non-breastfed children.

Data analysis

OCs exposure concentrations (ng/mL) at each follow-up were calculated for each study population separately (median and the 25th and 75th percentiles). Children of Ribera d'Ebre not belonging to the original cohort were treated separately in order to compare OC concentrations with those of the original cohort. We compared the total serum burden of OCs (median and the 25th and 75th percentiles) between birth cohort studies and between breastfed and not breastfed children. Statistical significant differences ($p \leq 0.05$) were tested with the Kruskal-Wallis test.

As changes in the amount of adipose tissue in the body can lead to significant changes in the serum OC concentrations (Porta et al. 2012), we conducted a sensitivity analysis including only children with a healthy weight at both the ages of 4 and 14 years. To classify children, we first calculated their body mass index (BMI) and used the male and female CDC growth charts to determine whether a child was underweight, healthy weight, overweight or obese (Center for Disease Control and Prevention (CDC) 2000; Kuczmarski et al. 2002).

Results

Exposure concentrations

In children of Ribera d'Ebre, out of all OC compounds measured, HCB was the compound with the highest concentration at birth (median=1.13 ng/mL), at age 4 years (0.98 ng/mL) and at age 14 years (0.16 ng/mL). At 14 years the sum of the seven PCB congeners (Σ7PCBs) was 0.20 ng/mL, being PCB153 the congener present at higher concentrations (Table 30). Concentrations of 4,4'-DDE and β-HCH in adolescents were of 0.12 ng/mL and 0.02 ng/mL, respectively (Table 30). At 14 years there were no significant differences in OC concentrations between children of the original cohort and their schoolmates joining the study in 2012, except for PeCB, δ-HCH and DDT (p-value for differences between study populations ≤0.05), but very few children had detectable concentrations and in general these were very low in both study populations. In the Menorca birth cohort, 4,4'-DDE was the compound with the highest concentrations at birth (median=1.03 ng/mL), at age 4 years (0.81 ng/mL) and at age 14 years (0.33 ng/mL). In this birth cohort, the concentration of Σ7PCBs measured at the age of 14 years was 0.31 ng/mL, being PCB153 the congener present at higher concentrations (Table 30). Concentrations of HCB and β-HCH in the adolescents were 0.04 ng/mL and 0.02 ng/mL, respectively (Table 30).

At the age of 14 years, concentrations of all OCs significantly differed between the birth cohorts of Ribera d'Ebre and Menorca (p≤0.05; Figure 45) except PCB153, for which no differences were observed. Children of Ribera d'Ebre had higher concentrations of HCB and children of Menorca had higher 4,4'-DDE and Σ7PCBs concentrations. These results were the same with the inclusion or exclusion of children from Ribera d'Ebre not belonging to the original cohort (data not shown). In both study sites, temporal trends in concentrations from birth up to adolescence showed decreasing concentrations of all OCs (Table 30 and Figure 45); only concentrations of Σ7PCBs in adolescents of the Ribera d'Ebre increased a little compared to concentrations at birth.

OC's total serum burdens

We observed that in both study populations the total serum burdens of all OCs increased from birth to 14 years, with the exception of β-HCH in both birth cohorts and HCB in the Menorca birth cohort, which showed an opposite pattern (Table 30 and Figure 46). The most important

increases were observed at the age of 4 years, moment in which the total serum burdens of all OCs reached up to six times the total serum burdens at birth. In Ribera d'Ebre the Σ7PCBs burden at the age of 4 years (1047.6 ng) was 27.6 times higher than at birth (38.1 ng) (Table 30 and Figure 46). In this birth cohort, the most important total serum burdens at the age of 14 years were those of Σ7PCBs (703.9 ng), followed by HCB (484.1 ng) and 4,4'-DDE (410.1 ng). In the Menorca birth cohort, the highest total serum burdens at 14 years were those of 4,4'-DDE (1342.1 ng), followed by Σ7PCBs (1117.6 ng) and, much lower HCB (149.4 ng) (Table 30 and Figure 46). While for 4,4'-DDE, Σ7PCBs and HCB there were statistically significant differences between the two birth cohorts, no differences were observed for β-HCH (Table 30 and Figure 46).

The role of breastfeeding

The total serum burdens of all OCs were increased with increasing age both in breastfed and non-breastfed children, however, the increase was more accentuated among the first (Figure 47). At the age of 4 years there were statistically significant total serum burden differences between breastfeeders and non-breastfeeders for all OC compounds (Figure 47). At the age of 14 years, the total serum burdens of β-HCH and HCB did not differ between breastfeeders and non-breastfeeders. Total serum burdens for 4,4'-DDE and Σ7PCBs were higher among breastfeeders (1076.5 ng and 1070.2 ng, respectively) than among non-breastfeeders (433.5 ng and 622.3 ng) at this age (Figure 47).

Table 30. Characteristics of the study populations [original birth cohort of Ribera d'Ebre and birth cohort of Menorca].

	Ribera d'Ebre (Initial N=102)		Menorca (Initial N=482)	
	N		N	
Age of the child (years) at each visit (mean, min-max)				
Visit 4 years	74	4.2 (3.4, 5.3)	422	4.4 (3.4, 4.8)
Visit 14 years	35	13.6 (11, 14.5)	327	14.6 (14.2, 15.7)
Female (%)	102	54.9	482	48.6
Breastfeeding (%)	91	75.8	482	82.4
OC concentrations (ng/mL) (median, 25th-75th)				

β-HCH				
Cord blood	70	0.54 (0.03, 1.07)	405	0.16 (0.16, 0.52)
Serum visit 4 years	58	0.31 (0.19, 0.48)	285	0.20 (0.11, 0.30)
Serum visit 14 years	36	0.02 (0.01, 0.04)	43	0.02 (0.01, 0.03)
HCB				
Cord blood	70	1.13 (0.80, 1.69)	405	0.68 (0.40, 1.02)
Serum visit 4 years	58	0.98 (0.66, 1.48)	285	0.31 (0.19, 0.50)
Serum visit 14 years	36	0.16 (0.10, 0.25)	43	0.04 (0.02, 0.06)
4,4'-DDE				
Cord blood	70	0.86 (0.50, 1.68)	405	1.03 (0.57, 1.94)
Serum visit 4 years	58	0.75 (0.38, 1.32)	285	0.81 (0.44, 1.77)
Serum visit 14 years	36	0.12 (0.09, 0.19)	43	0.33 (0.23, 0.58)
Σ_7PCBs				
Cord blood	70	0.14 (0.10, 0.47)	405	0.71 (0.53, 0.97)
Serum visit 4 years	58	0.81 (0.20, 1.21)	285	0.82 (0.58, 1.23)
Serum visit 14 years	36	0.20 (0.12, 0.28)	43	0.31 (0.24, 0.42)
OC total serum burdens (ng)[a]				
(median, 25th-75th)				
β-HCH				
Cord blood	70	152.0 (7.2, 290.4)	405	52.2 (42.7, 120.7)
Serum visit 4 years	49	460.0 (289.8, 667.0)	178	279.9 (165.0, 429.5)
Serum visit 14 years	31	53.7 (40.7, 133.0)	43	52.4 (28.1, 102.1)
HCB				
Cord blood	70	312.9 (219.6, 486.6)	405	182.7 (120.1, 273.5)
Serum visit 4 years	49	1439.3 (943.1, 2755.6)	178	414.0 (271.6, 706.1)

Serum visit 14 years	31	484.1 (229.6, 1065.3)	43	149.4 (75.1, 232.5)
4,4'-DDE				
Cord blood	70	232.3 (122.3, 434.0)	405	275.1 (161.9, 503.1)
Serum visit 4 years	49	1050.0 (642.2, 1632.0)	178	1291.0 (645.6, 2646.3)
Serum visit 14 years	31	410.1 (301.8, 687.0)	43	1342.1 (791.8, 2519.0)
Σ_7PCBs				
Cord blood	70	38.1 (28.4, 150.7)	405	191.2 (145.5, 266.5)
Serum visit 4 years	49	1047.6 (305.7, 1647.4)	178	1201.1 (828.1, 1750.8)
Serum visit 14 years	31	703.9 (503.8, 1070.3)	43	1117.6 (807.2, 1628.1)

[a]Estimated by multiplying the concentrations of OCs (ng/mL) by the total blood volume (mL) at each age, which was estimated from the weight of the child.

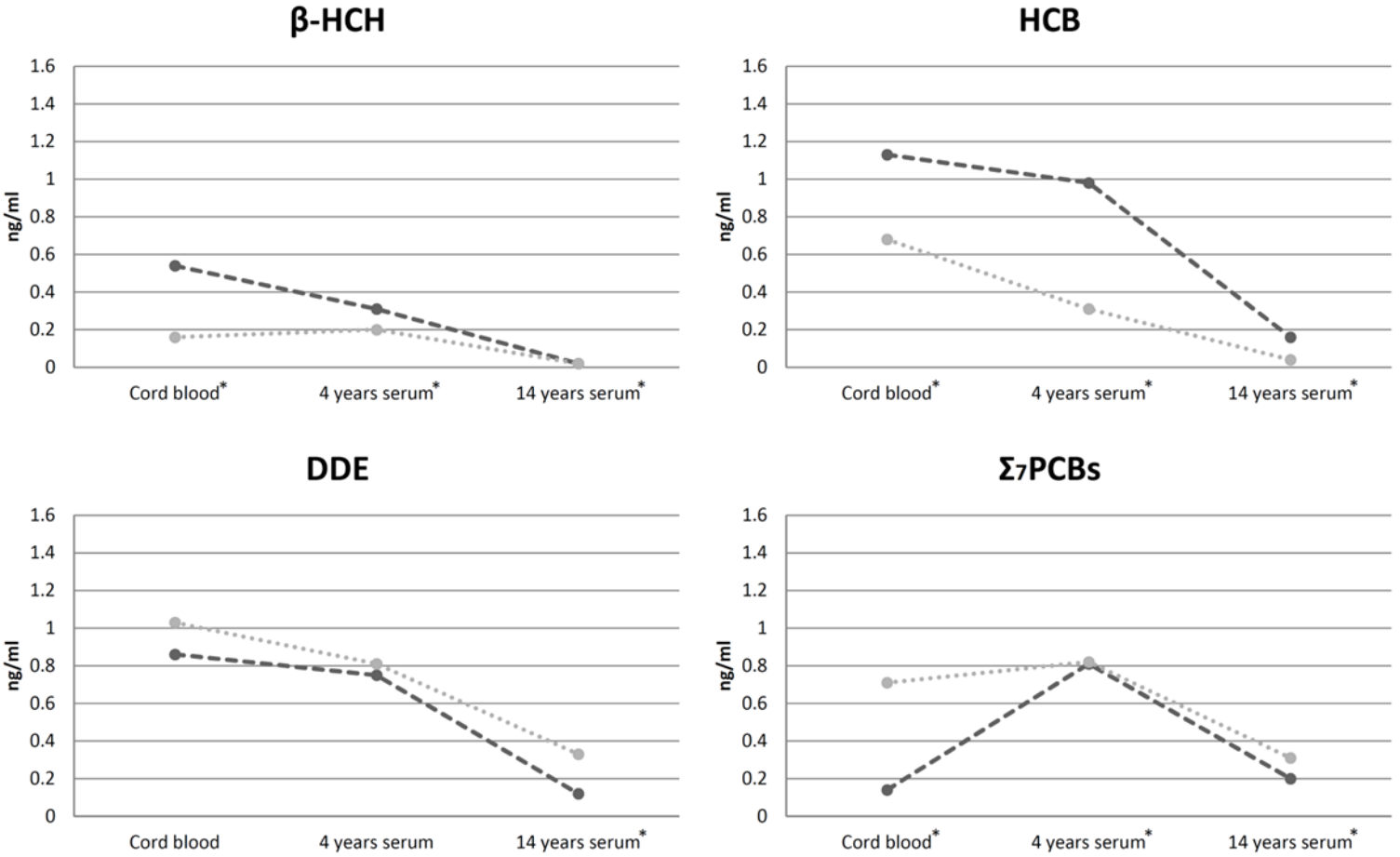


Figure 45: Evolution of serum concentrations (ng/mL, 50th percentile) of β-HCH, HCB, 4,4'-DDE and Σ7PCBs in the birth cohorts of Ribera d'Ebre (dark line) and Menorca (light line).

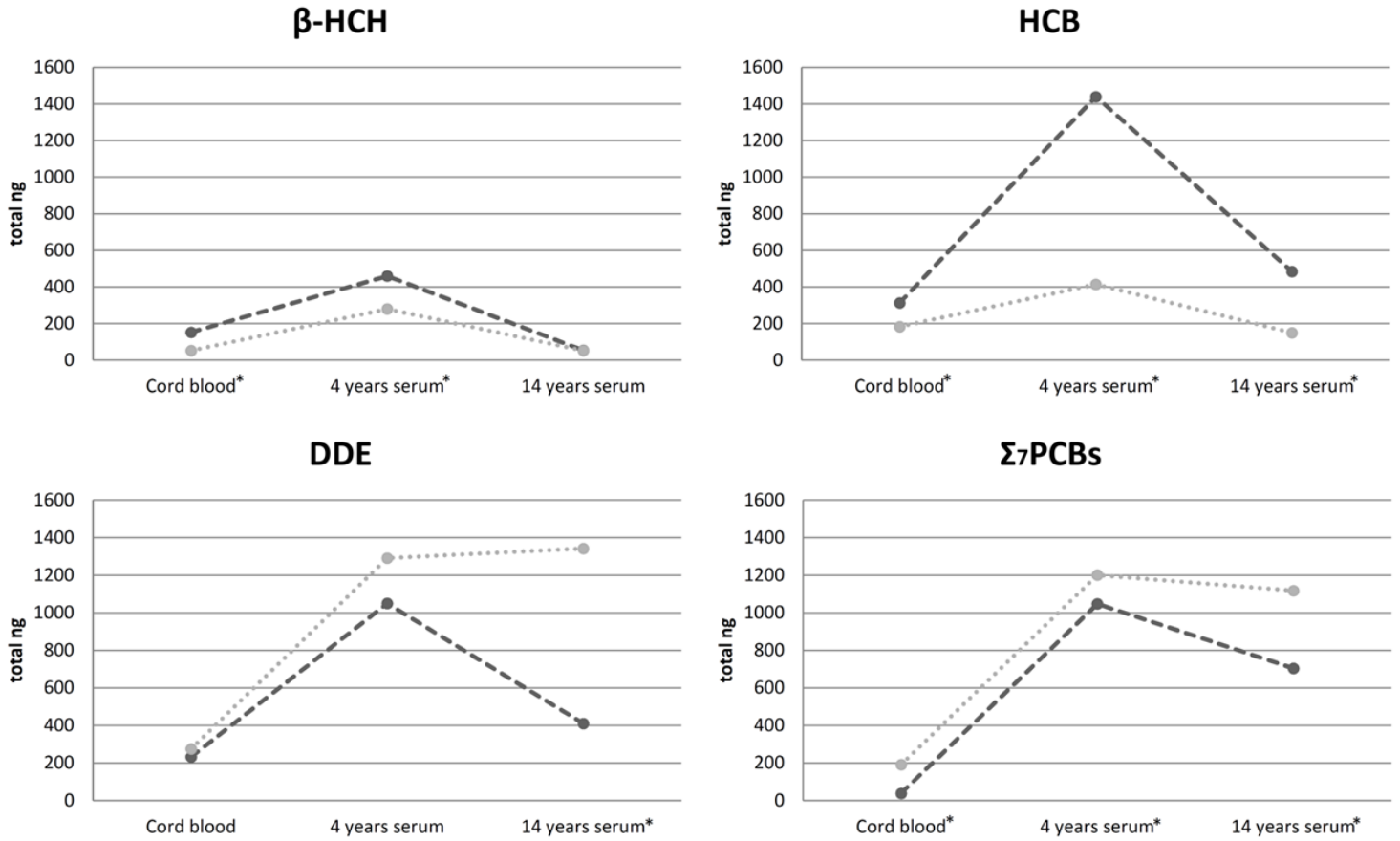


Figure 46: Evolution of total serum burden (ng, 50th percentile) of β-HCH, HCB, 4,4'-DDE and Σ7PCBs in the birth cohorts of Ribera d'Ebre (dark line) and Menorca (light line).

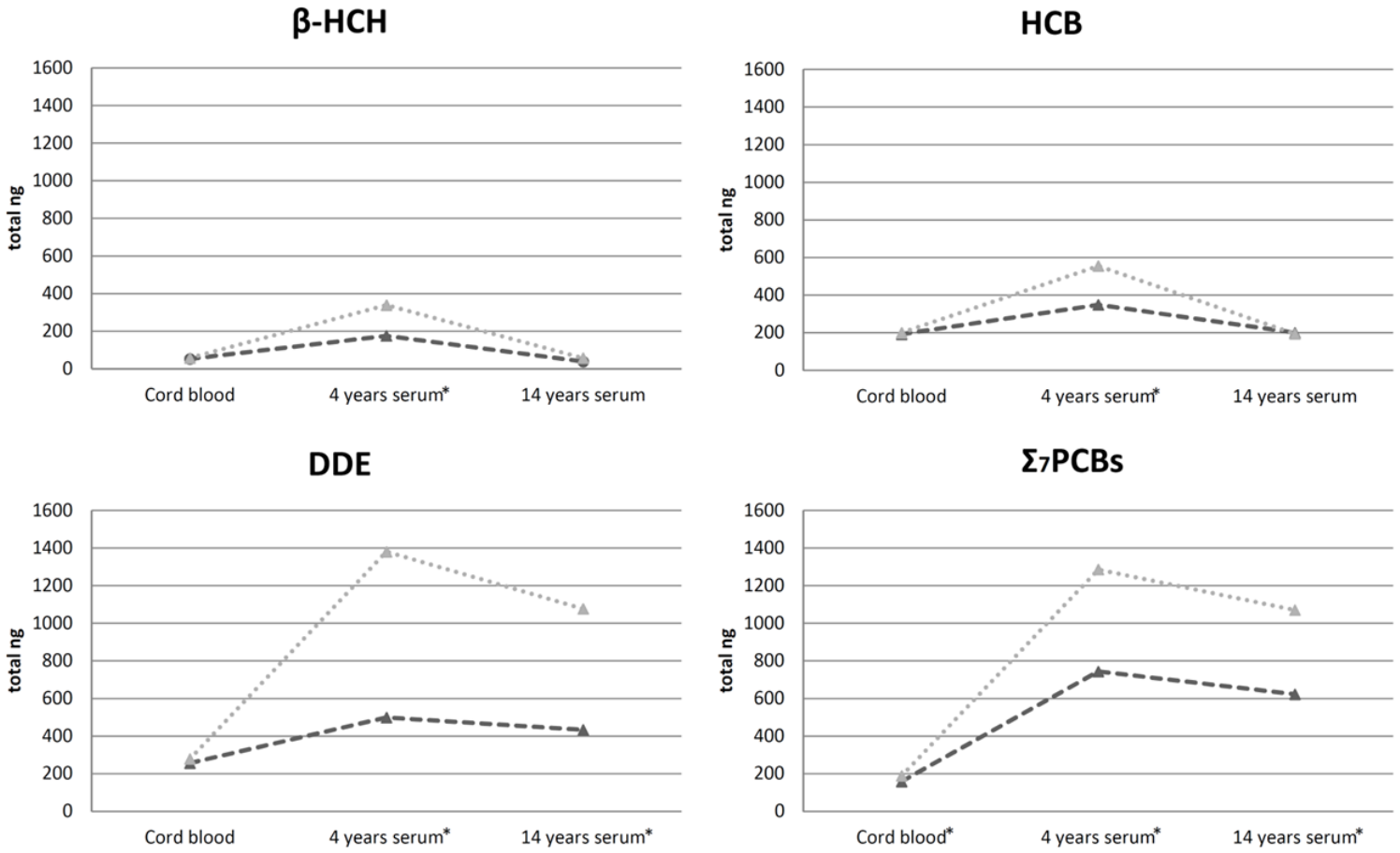


Figure 47: Evolution of total serum burden (ng, 50th percentile) of β-HCH, HCB, 4,4'-DDE and Σ7PCBs in breastfeeders (light line) and non-breastfeeders (dark line).

Table 31: Median OC concentrations (in ng/g lipid) found in serum of adolescents from different countries.

Location	Year of OCs assessment	n	Age	HCB	β-HCH	DDT	DDE	PCB118	PCB138	PCB153	PCB180	ΣPCBs[a]	Reference
Ribera d'Ebre	2012	20[b]	11-15										
ng/mL				[0.18]	[0.01]	[0.003]	[0.12]	[0.01]	[0.04]	[0.11]	[0.04]	[0.22]	
ng/g lipid				42.0	3.0	0.6	27.1	1.4	9.0	24.5	8.5	46.5	
Menorca	2012	43	14-16										
ng/mL				[0.04]	[0.02]	[0.03]	[0.33]	[0.05]	[0.06]	[0.12]	[0.06]	[0.31]	
ng/g lipid				7.4	3.0	7.1	65.7	9.9	12.4	22.1	12.3	63.4	
Bangladesh[c]	2008	30	7-16	NA	32	326	1327	NA	8.5	7.1	3.2	NA	Linderholm et al. (2001)
Germany[d]	2003-2006	1079	7-14	[0.10]	[0.01]	NA	[0.18]	NA	[0.09]	[0.12]	[0.06]	[0.28]	Schulz et al. (2009)
Germany[d]	2003-2004	15	11-20	[0.15]	NA	NA	[0.34]	[0.02]	[0.12]	[0.15]	[0.10]	[0.40]	Schettgen et al. (2011)
Belgium	2003-2004	1679	14-15	21	NA	NA	94	NA	NA	NA	NA	66	Schroijen et al. (2008)
US	2003-2004	598	12-19	13.3	NA	ND	94	NA	4.6[e]	5.4	3.0	NA	CDC (2009)
Nicaragua[f]	2002	37	11-15	NA	NA	12	993	39	67	86	33	278	Cuadra et al. (2006)
US	2001-2002	758	12-19	13.3	NA	ND	113	NA	ND[e]	ND	ND	NA	CDC (2009)
US	1999-2000	686	12-19	13.3	NA	ND	108	NA	ND[e]	ND	ND	NA	CDC (2009)
Canada (Inuit)[g]	1995-2000	271	10-17	(0.03)	NA	NA	(0.35)	(0.06)	(0.07)[e]	(0.08)	(0.04)	NA	Newman et al. (2009)

Brasil[d,e,h]	NR	103	10-19	[2.5]	[1.3]	[0.3]	NA	NA	NA	NA	NA	NA	Santos et al. (2003)

NA: Information not available (compound not analyzed or result not reported); ND: Not detected.
[a]Sum of all congeners analyzed in each study.
[b]Only 20 children from the Ribera d'Ebre birth cohort had information on total lipids. Thus, results are presented for these 20 children only.
[c]Medians for the whole population were not available in the cited paper. The presented values have been calculated based on the weighted mean of the OC's median of the different groups reported.
[d]Values expressed in [ng/ml].
[e]The reported value is the sum of PCB138 with other PCB congeners or all HCH congeners (in the case of β-HCH).
[f]Medians for the whole population were not available in the cited paper. The presented values have been calculated based on the weighted mean of the OC's median of the different groups reported (we excluded two groups of children working in a waste-disposal site).
[g]Values expressed in parts per billion (ppb).
[h]Mean reported instead of the median.

Sensitivity analyses

Two sensitivity analyses were performed. In one of them the general results were compared with those from the children providing complete exposure information at all three ages (n = 61). In the other, these general results were compared with the subset of children from which total blood volume at the three ages was available in addition (n = 45). The observed trends did not change. The only differences involved an intensification of the concentration maxima at the age of 4 years for 4,4'-DDE and HCB in the Menorca and Ribera d'Ebre cohorts, respectively. For instance, the median total serum burdens of 4,4'-DDE in Menorca at age 4 years reached up to 2040 ng in the sensitivity analysis. We also obtained similar results when we only included children with a healthy weight at both the ages of 4 and 14 years in the analyses (N=146 with complete information).

The results of the present campaign show an important decrease in concentrations (ng/mL) of OCs from birth up to adolescence in two Spanish birth cohorts. Total serum burden calculations show, however, that the reduction in OC concentrations was mainly due to the increase in dilution of these compounds in the total blood volume with age. Indeed, at the age of 14 years the total serum burdens of 4,4'-DDE and Σ7PCBs, especially in Menorca, were increased compared to total serum burdens at birth. In Ribera d'Ebre the total serum burden of HCB at age 14 years was also increased. These increases were more accentuated in breastfed children.

OC concentrations in adolescents

There are few studies assessing OCs exposure in adolescent subjects, and those available include study populations of a wide range of ages (between 7 and 20 years) and most of them were conducted more than ten years ago (Santos Filho et al. 2003; Cuadra et al. 2006; Schroijen et al. 2008; Centers for Disease Control and Prevention 2009; Newman et al. 2009; Schulz et al. 2009; Linderholm et al. 2011; Schettgen et al. 2011) (Table 31). Compared to adolescents of European and North-American countries (Germany, Belgium, the US and Canada), participants of Ribera d'Ebre and Menorca have similar OCs exposure concentrations, with some exceptions (Table 31). For instance, HCB concentrations in Ribera d'Ebre were higher than in adolescents of other westernized countries (Schroijen et al. 2008; Centers for Disease Control and Prevention 2009; Newman et al. 2009; Schulz et al. 2009; Schettgen et al. 2011). On the contrary, adolescents of the Menorca birth cohort showed the lowest HCB concentrations (Table 31). DDE concentrations in our study populations were similar to those reported in Germany (Schulz et al. 2009; Schettgen et al. 2011), but lower than those reported in Belgium or in the US (Schroijen et al. 2008; Centers for Disease Control and Prevention 2009). Finally, PCB concentrations were similar to those reported in European countries (Schroijen et al. 2008; Schulz et al. 2009; Schettgen et al. 2011) but

higher than those reported in the US (Centers for Disease Control and Prevention 2009) (Table 31). In any case, differences among study populations of westernized countries are generally minimal for all the compounds. Compared to low-/middle-income countries, the most remarkable difference is in relation to DDT and DDE concentrations, which are much higher in countries like Bangladesh, Nicaragua or Brasil (Santos Filho et al. 2003; Cuadra et al. 2006; Linderholm et al. 2011) (Table 31). This is mainly due to the fact that in these countries a more recent (and intensive) use of this insecticide has taken place for agricultural purposes or to control the malaria vector, a practice still used in countries with endemic malaria (Bouwman et al. 2011; Gascon et al. 2012).

Temporal trends of OC concentrations and total serum burdens

The overall decrease of OC concentrations in our study populations are in line with results obtained in previous studies focused on temporal trends of OC concentrations in adults of westernized countries (Ribas-Fito et al. 2003c; Carpenter 2011; Garí et al. 2014; Ryan and Rawn 2014). For instance, already between 1994 and 1999 concentrations of HCB in women of Ribera d'Ebre significantly decreased because of improvements in the incineration processes in the chlor-alkali plant (Ribas-Fito et al. 2003c). This might lead to the conclusion that the reductions in OC concentrations in adolescents of our study population are due to a reduction of the environmental background levels. However, when the total blood volume is taken into account and we calculate the total serum burden of these compounds at each age, we observe an increase of the total serum burdens of all OCs at age 4 years. Compared to birth, at age 14 years total serum burdens of 4,4'-DDE and Σ7PCBs remain increased, although there is a reduction compared to the total serum burdens at age 4 years. Thus, results indicate that there is not a reduction of OC exposure in our study populations. The big increase in total serum burdens from birth up to age 4 years is partly due to breastfeeding, as results in Figure 47 show. However, in non-breastfed children total serum burden increases also occurred, which indicates that breastfeeding is not the only exposure determinant and that diet, as already reported by previous studies in adults (Llop et al. 2010; Vrijheid et al. 2010), is probably the other main source of OCs exposure in our children. We see that from the age of 4 years up to the age of 14 years total serum burdens of all OCs reduced, probably because the effect of breastfeeding is not as strong as in the first 4 years of life and because in this period children eat less amount of food per kilogram of total serum weight than in the first 4 years of life (Nicklas and Johnson 2004), thus, the ingestion of OCs is also proportionally less. The fact that at age 14 years the total serum burdens of some OCs remained increased compared to the initial total serum burdens at birth could be explained by the influence of the environmental background levels of these compounds, as is the case of HCB in Ribera d'Ebre, 4,4'-DDE in Menorca (maybe due to a more intense use of DDT in this rural site in the past), and PCBs in both study populations. Increases in PCBs exposure between 1994 and

1999 were already reported in adult women in Ribera d'Ebre (Ribas-Fito et al. 2003c), something also observed for the total serum burdens of PCBs in the present study. It is remarkable that although in Ribera d'Ebre HCB serum burdens reached similar levels to those reported for 4,4'-DDE and Σ7PCBs at the age of 4 years, children were able to substantially reduce HCB exposure at the age of 4 years. However, reductions were less important for 4,4'-DDE and Σ7PCBs. This indicates that it is possible to slowly eliminate these compounds from our bodies unless there is a continuous or constant exposure to them (i.e. through diet).

Overall, results of the present study indicate that breastfeeding in the first 4 years of life and diet along the whole study period have an important influence on the total serum burden of OCs. Results also indicate that local environmental background levels of OCs is still a key factor in the final total serum burdens of the studied subjects. We can also conclude that, since birth, reductions in OC concentrations (ng/mL) were mainly derived from the dilution of OCs, associated to an increase in the total blood volume of children at the age of 4 and 14 years. Although these compounds bioaccumulate, OCs total serum burdens in the present study did not keep increasing in children between the 4th and the 14th year of life due to different factors: less influence of breastfeeding in this period, reductions in food intake per kilogram of body weight and/or reductions of the environmental exposures in this second period of study. Because OCs accumulate in fatty tissue, changes in body weight and body fat could have also influenced in the OCs concentrations over time (Porta et al. 2012). A sensitivity analysis using only the children with a healthy BMI at 4 and 14 years did not show any difference with respect to the concentration trends of the general comparison. Thus, the observed trends do not depend on the presence of individuals with underweight, overweight or obesity in the studied cohorts. In fact, in healthy weight children the percentage of fat remains constant along childhood (around 20%) whereas in obese children gradual changes of about 10% increase along 10 year periods are often observed (Knittle et al. 1979). The lack of dependence of the observed trends from these obese children supports that the concentration differences identified in the present study constitute a general feature of the OC accumulation in the first life years.

Strengths and limitations

This is the first prospective study assessing the temporal trends of concentrations and total serum burdens of OCs from birth until adolescence (a study period of 14 years) and which also compares two sites with very different exposure scenarios. Based on previous studies (Llop et al. 2010; Vrijheid et al. 2010), in the present work we hypothesize that diet is one of the most influencing factors in the final OCs total serum burdens in Ribera d'Ebre and Menorca children. Unfortunately, we did not have accurate and complete diet information to assess the role of diet and particularly of different food items (fish, meat, dairy products or vegetables) in both study populations.

One aspect to be considered in this comparison of OC concentrations at different at ages is the lower lipid content of cord blood than venous serum (Aylward et al. 2014; Vizcaino et al. 2014). The average lipid contents in the cord blood and 4-year old venous blood serum of the children from the cohort of Menorca are 2.6 and 8.2 g/L, respectively (Carrizo et al., 2007a), and the average lipid concentrations of the 14 year-old serum is 5.2 g/L. No data is available for the cord blood serum in children of the Ribera d'Ebre cohort. Normalization of the cord blood serum OC concentrations from the Menorcan children to the average venous lipids can be done by multiplication by a factor of 2.58, (8.2+5.2)/(2*2.6). Thus, for comparative purposes the cord blood concentration values of Table 30 and Figure 45 should be transformed into 0.41, 1.8, 2.7 and 1.8 ng/mL for β-HCH, HCB, 4,4'-DDE and Σ7PCBs, respectively. These values are higher than those observed at 4 and 14 years of age, indicating that in qualitative terms the concentrations of OC compounds constitute a stronger potential chemical insult in the prenatal than in the postnatal period.

Likewise, normalization of the cord blood body burden serum amounts (Table 30; Figure 46) to venous lipids provides amounts of 130, 470, 700 and 490 ng for β-HCH, HCB, 4,4'-DDE and Σ7PCBs, respectively. These values obviously increase the total body burden serum of the prenatal vs the postnatal period. However, with the exception of HCB, the postnatal burden is again higher than the prenatal burden. In this respect, it has to be outlined that whereas the burdens of β-HCH and HCB tend to decrease with age, those of 4,4'-DDE and Σ7PCBs at 4 and 14 years of age are nearly constant and higher than in the prenatal period even after lipid correction. These results are consistent with a substantial intake of 4,4'-DDE and Σ7PCBs in the first 14 years of age.

Another limiting aspect of the present study is that the study population was small at the age of 14 years and that there was a small number of subjects with complete information on OCs exposure and total blood volume. However, and as shown, the results were robust and consistent in the two sensitivity analyses performed. It is important to note that in Ribera d'Ebre contact was lost with the families participating in the cohort since the follow-up in 2001-2003. This lack of contact could explain our capacity to encourage them to participate in the study of 2012. This is an important difference with the Menorca birth cohort, where additional follow-ups between 2001-2003 and 2012 have been performed. We limited OCs analysis to 43 children due to budget limitations but general participation in the follow-up was much higher (N=345). Finally, as OC levels were assessed at all follow-ups in the same laboratory and using the same protocol and instruments, differences in levels cannot be explained by differences in laboratory techniques.

Conclusions

Even after decades of banning OCs production and use, current young generations in westernized countries are still bioaccumulating these compounds, with higher total serum burdens at adolescent ages than at birth for 4,4'-DDE and Σ7PCBs. However, our results show that, in qualitative terms, the concentrations of OC in utero constitute a much strong dose, particularly after normalization to serum lipid content, in the prenatal than in the postnatal period.

In the present work, breastfeeding, diet and point-source secondary emissions had different degrees of influence in the final total serum burdens in each studied area. Given the bioaccumulation of OCs and their potential health effects, especial attention should be paid in the control of secondary emissions of these pollutants in the environment and in the control of food production and contamination. In countries with endemic malaria it is important to work towards effective alternatives to the use of DDT.

6th case study – Field campaign for the study of the impact of chemical pollution into humans in response to stakeholder requests after examination of CROME results (Flix, Catalonia, Spain)

Introduction

A large number of major chronic human diseases are likely to result from the combination of environmental exposures to chemical stressors and human genetics. Today the limiting factor of knowledge of health risks is the low level of awareness of environmental exposures. The concept of individual exposome, representing all environmental contributors to disease received by an individual during a lifetime, is useful to assess the environmental stresses in a time-integrated perspective. This concept is needed to better understand the underlying mechanisms of environment-health/disease associations.

Chlor-alkali plants (CAPs) provide examples of exposure to both organohlorine solvents and synthesis by-products such as hexachlorobenzene (HCB). The exposome concept requires the assessment of the impact of these industrial installations into the surrounding populations.

The city of Flix has a CAP neaby (< 1 km). Past activities involved the discharge of about 500 000 tons of industrial residues into the River Ebro, at the shore of the CAP. Remediation of this spill involved the construction of a double wall of sheet pilings to isolate the dumped material from erosion of the Ebro waters, removal of the dumped mud, thermal treatment of the materials rich in organic solvents and disposal in a landfill. These activities could potentially involve major emissions of organochlorine compounds into the air of the village, and the City Hall, in view of previous work in the area (see 5th Case Study), requested help from IDAEA for the monitoring and toxicity assessment of these emissions.

Summary of existing environmental and biomonitoring data – gaps identification

Air sampling for the control of organochlorine solvents and synthetic by-products in the City of Flix and surroundings was implemented in order to identify possible risk situations generated by the inhabitants of Flix. Initially, sampling was performed by staff from IDAEA-CSIC and later by personnel of the City Hall of Flix.

The decision criterion for sample collection was based on the detection of odors in the village. Therefore, the samples taken likely correspond to maximum levels of contamination impact, not average levels.

Estimate of the risks associated to these concentrations observed in episodes of strong odors were performed. These estimates should be interpreted as a limit and not as representing the real situation, which probably corresponds to lower values.

The compounds that showed concentrations closer to the levels of risk to humans were carbon tetrachloride, trichloroethylene, perchloroethylene, hexachlorobutadiene and hexachlorobenzene.

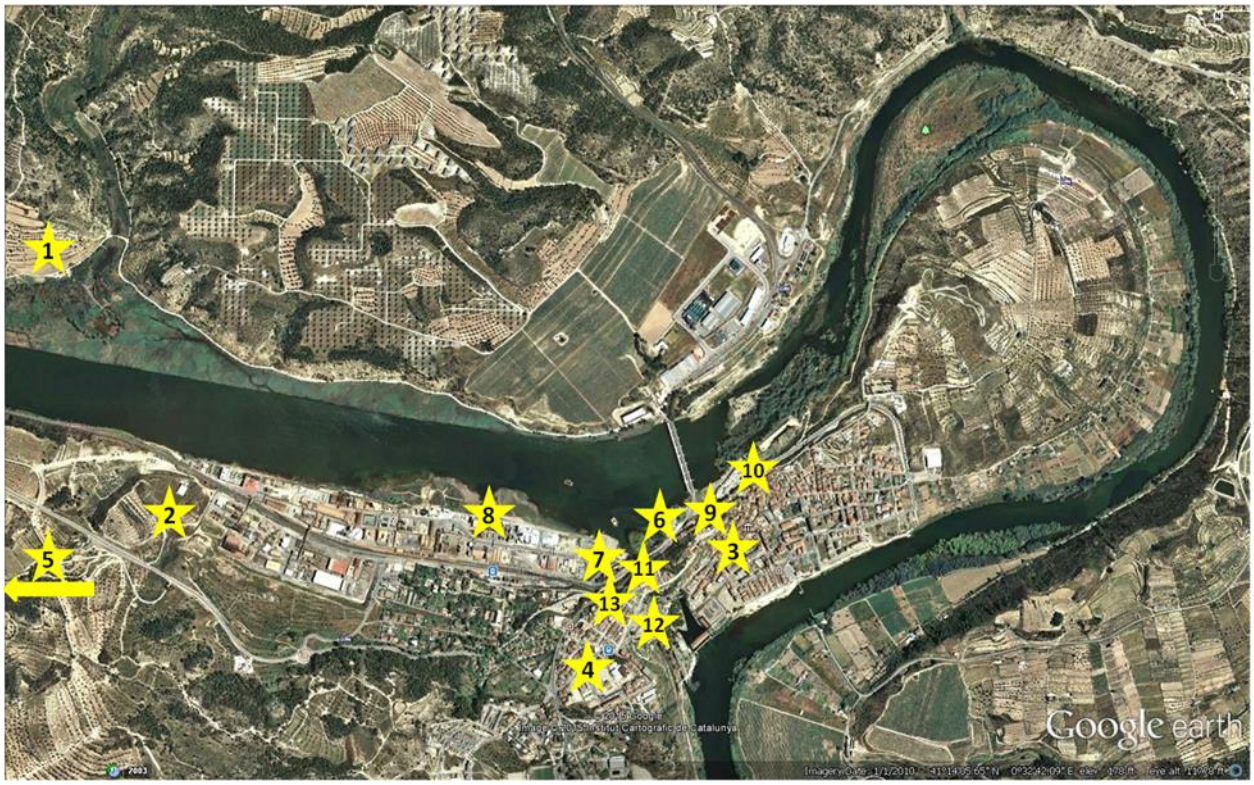
Additional data collected

Environmental data

Sampling and analysis protocol

Sampling

The location of the collected samples is shown in Figure 48. These sites were chosen based on notices of odors from the villagers. In October and November 2013 four samplings were carried out by members of IDEA-CSIC (Table 32). In this sampling campaigns representative samples of the air from the town of Flix were collected. Additional samples for assessment of the influence of the decontamination process were also collected. In 2014 and 2015 sampling was carried out by staff members of the municipality of Flix who were trained by IDEA-CSIC for this purpose. In this case, the samplings were based on notices of high smell by neighbors. The sampling periods are indicated in Table 33and in Table 34.



Site 1: Mas del Director.
Site 2: Water treatment plant
Site 3: Església square
Site 4: Schools.
Site 5: Riba-roja d'Ebre
Site 6: Chemistry hill
Site 7: Chemistry bridge
Site 8: Zone of mud removal
Site 9: Continental
Site 10: Costa del Graner
Site 11: Molí street
Site 12: Kinder garden
Site 13: Casetes Park

Figure 48: Sampling sites of the air monitoring campaigns.

The VOCs present in the air were concentrated in stainless steel (89 mm long x 6.4 mm external diameter) cartridges packed with a multiadsobent bed composed of 180 mg of Carbotrap, Carbotrap C and Carbotrap X. Sampling was performed using a suction pump (pump Universal Deluxe SKC) at a flow of 40 ml/min. Hexachlorobenzene was sampled with cartridges packed with 200 mg of Tenax TA. The pump was calibrated with a digital meter (510L Defender, SKC) at the beginning and the end of each sampling. The average flow was taken for estimating the air sampled volume. At the end of each sampling, the cartridges were capped on both ends and they were stored at 4°C inside a sealed box free of VOCs.

Table 32: Samples collected in 2013.

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
Oct. 2	12:05-12:40	19:35-20:05	17:10-17:40	13:35-14:05	18:45-19:15	-	-	-
Oct. 16	15:15-15:47	08:37-09:08	16:15-16:45	10:40-11:13	12:10-12:40	14:20-14:50	17:10-17:41	-
	-	09:15-09:50	-	-	-	-	-	-

Oct. 17	11:20-11:50	12:20-12:54	09:00-09:30	09:53-10:23	13:00-13:30	10:40-11:10	-	-
Nov. 8	-	12:55-13:10	-	-	-	-	09:40-10:10	12:15-12:40

Table 33: Samples collected in 2014.

	Site 1	Site 3	Site 4	Site 9	Site 10	Site 11	Site 12
Aug. 19	-	-	09:35-10:10	-	-	-	-
Aug. 20	-	-	-	-	12:20-12:39	-	-
Sept. 17	-	-	-	12:14-12:34	-	-	-
Sept. 24	-	-	-	-	-	18:12-18:55	-
Oct. 3	-	09:08-10:08	-	-	-	-	-
Oct. 8	-	-	12:45-13:45	-	-	-	-
Oct. 16	-	-	10:38-12:31	-	-	-	-
Oct. 30	09:19-10:56	-	-	-	-	-	-
Nov. 6	-	12:42-14:18	-	-	-	-	-
Nov. 11	-	-	13:33-15:11	-	-	-	-
Dec. 10	-	12:17-14:18	-	-	-	-	-
Dec. 16	-	16:33-18:15	-	-	-	-	-
Dec. 23	-	11:34-13:22	-	-	-	-	-
Dec. 24	-	-	-	-	-	-	18:10-21:03
Dec. 29	-	13:26-14:52	-	-	-	-	-

Table 34: Samples collected in 2015.

	Site 1	Site 3	Site 12	Site 13
Jan. 7	-	10:00-12:12	-	-
Jan. 14	-	-	12:22-14:45	-
Feb. 2	-	16:27-18:32	-	-
Feb. 10	11:10-13:52	-	-	-
Feb. 11	-	17:00-18:24	-	09:02-10:30
Feb. 25	-	09:25-10:48	-	-

The VOCs were desorbed in an automatic thermal desorber coupled to a gas chromatograph-mass spectrometer. The compounds quantified with this method were: trichlorofluoromethane (CFC-11), 1,1-dichloroethene, trans-1,2-dichloroethene, 1,1-dichloroethane, cis-1,2-dichloroethene, 2,2-dichloropropane, bromochloromethane, chloroform, 1,1,1-trichloroethane, 1,2-dichloroethane, 1,1-dichloropropene, carbon tetrachloride, trichloroethylene, 1,2-dichloropropane, dibromomethane, bromodichloromethane, trans-1,3-dichloropropene, cis-

1,3-dichloropropene, 1,1,2-trichloroethane, 1,3-dichloropropane, dibromochloromethane, tetrachloroethylene, 1,2-dibromoethane, chlorobenzene, 1,1,1,2-tetrachloroethane, bromoform, 1,1,2,2-tetrachloroethane, 1,2,3-trichloropropane, bromobenzene, 2-chlorotoluene, 4-chlorotoluene and hexachlorobenzene.

Results

Carbon tetrachloride shows a wide concentration range between 0.1 and 10 μg/m^3. The concentrations of this compound generally follow those of trichloroethylene and tetrachloroethylene. This suggests that these three compounds have a similar origin, probably mobilization emissions from sludge. The highest concentrations are found in the Església Square and in a second level in Molí Street and the Chemistry bridge.

In the 90s, similar concentrations to those found at present were observed, October 1992, but those found between November 1996 and October 1997 were much higher. In October 1992, concentrations of 2-7.6 μg/m^3 in Església Square, 4-9 μg/m^3 in the schools and 6.1-13.2 μg/m^3 in the car parking of the factory were found. Between November 1996 and October 1997, 1.5-20 μg/m^3 in the Església Square, nd-6.6 μg/m^3 in the Schools, 1.2-52 μg/m^3 in the car parking of the factory were found.

The samples studied show that the most abundant compounds are trichloroethylene and perchloroethylene. These compounds have been observed over a range of concentrations ranging between 0.3 and 140 μg/m^3 in the first case and between 0.1 and 100 μg/m^3 in the second (Figure 49 to Figure 58).

The concentrations of these compounds are highly variable both geographically and temporarily. This means that pollution events respond to specific issues and changes in weather conditions instead of continuous emissions from constant sources into the atmosphere.

In general, the sampling station in the Església Square is where highest concentrations are observed (site 3). Also high concentrations have been observed in some samplings in the Casetes Park, Chemistry bridge, Continental, Molí Street and the Mas del Director. The highest concentrations were observed on 6 November 2014 (Figure 55). Other days of high concentrations were 3rd October 2014 (Figure 54), 30th October 2014 (Figure 54), 10th December 2014 (Figure 56), 2nd February 2015 (Figure 58) and 11th February 2015 (Figure 58). According to these concentrations we can conclude that cold periods in the fall and winter highest concentrations were observed.

Comparison with other measured concentrations in the past shows that the current concentrations have been important in some cases. For example, the trichloroethylene concentrations measured in October 1992 vary from 0.5-2 μg/m^3 in some cases in the Església

square to 0.2-2.5 μg/m^3 in the schools and 1.2-3.6 μg/m^3 the car parking of the factory. Between the months of November 1996 and October 1997 they vary between not detected (nd) and 2.5 μg/m^3 in the Església square, nd and 2.7 μg/m^3 in the schools, nd and 10 μg/m^3 in the car parking of the Factory, nd and 4.6 μg/m^3 in the Mas del Director. Now, the concentrations are in the range between 0 and 140 μg/m^3.

The tetrachloroethylene concentrations during November 1992 varied between 3.3-7 μg/m^3 in the Església square, 2.2-7.5 μg/m^3 in the schools and 4.9-10.4 μg/m^3 in the car parking of the Factory. Between the months of November 1996 and October 1997 they ranged from 1.5-20 μg/m^3 in the Església square, nd-6.6 μg/m^3 in the schools, 1.2-52 μg/m^3 in the parking lot of the Factory and nd-18 μg/m^3 in the Mas del Director. Now, the concentrations are found in the range of 0.1 to 100 μg/m^3.

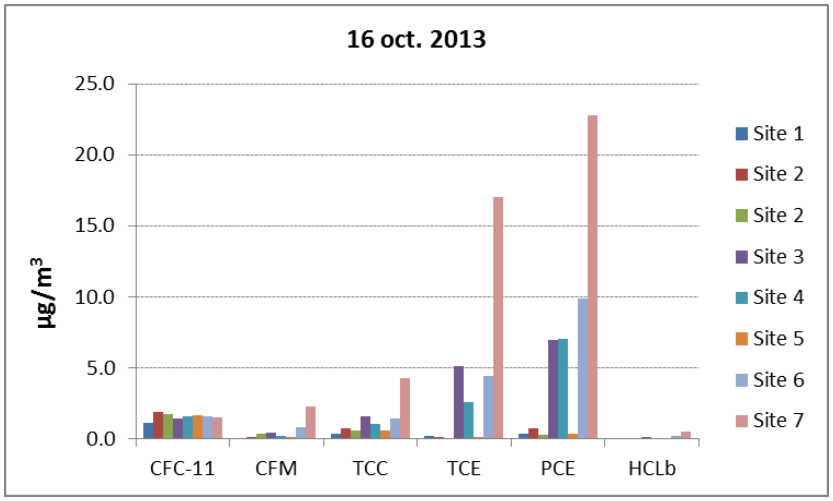


Figure 49: Concentrations of the main VOCs

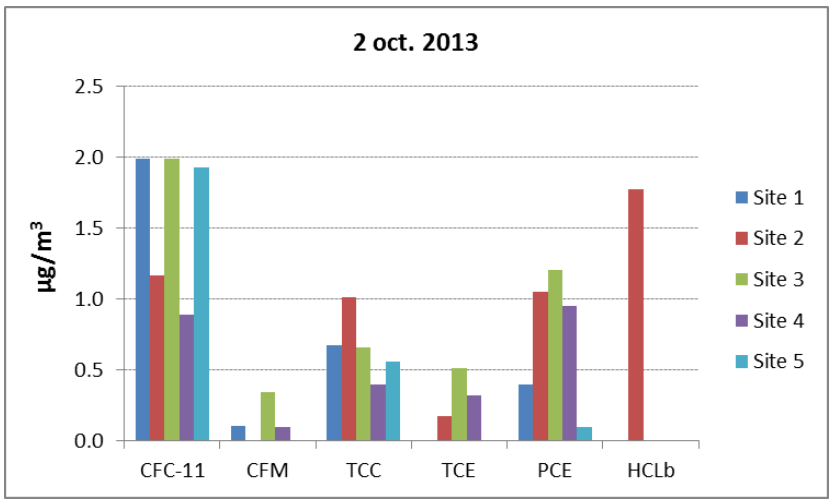


Figure 50: Concentrations of the main VOCs

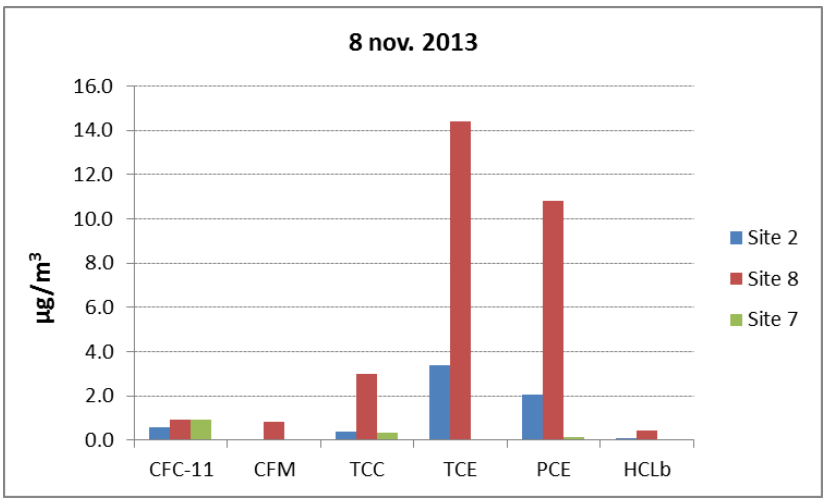


Figure 51: Concentrations of the main VOCs

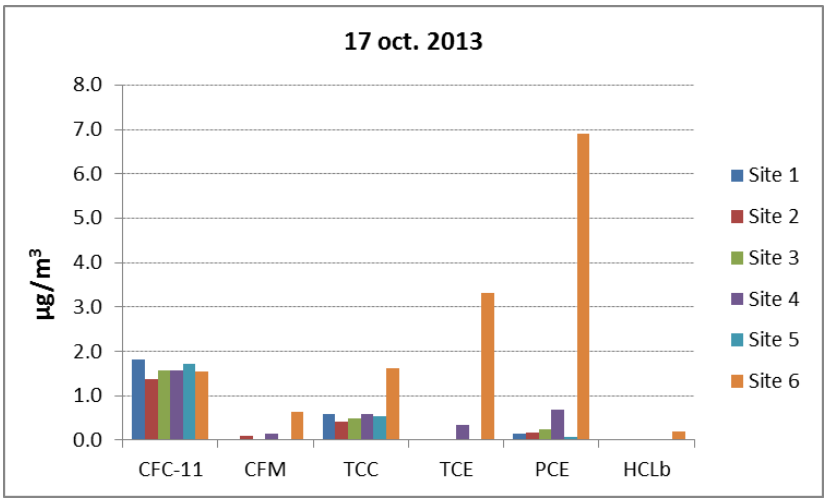


Figure 52: Concentrations of the main VOCs

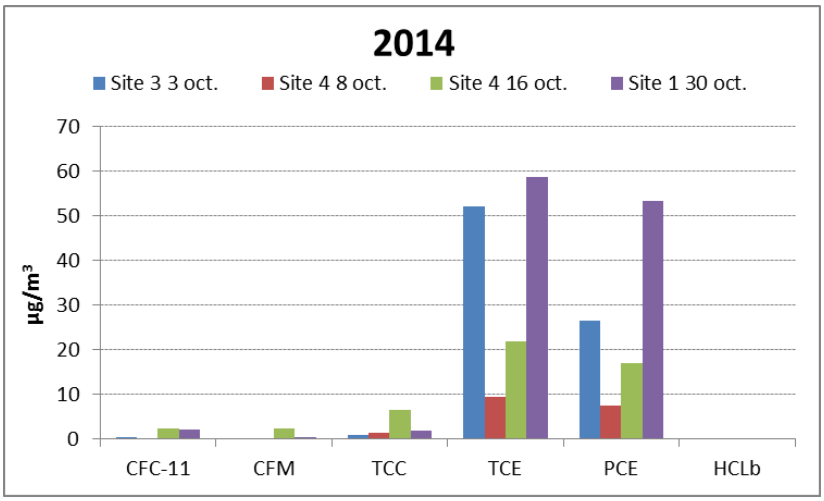


Figure 53: Concentrations of the main VOCs

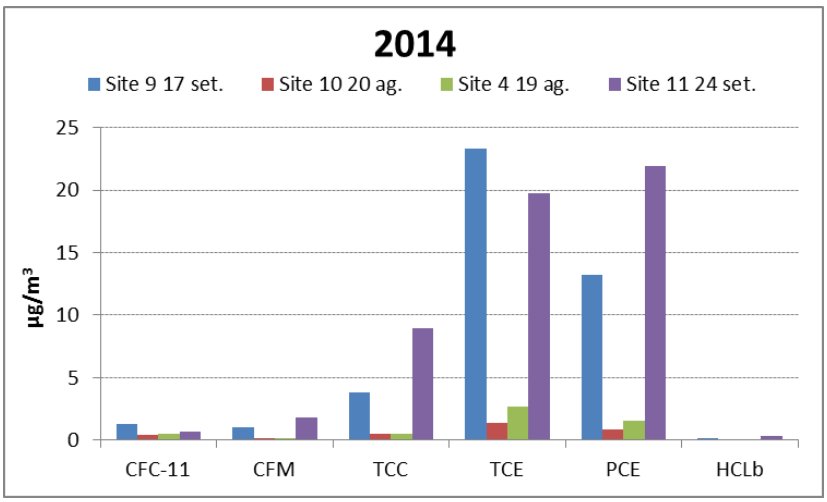


Figure 54: Concentrations of the main VOCs

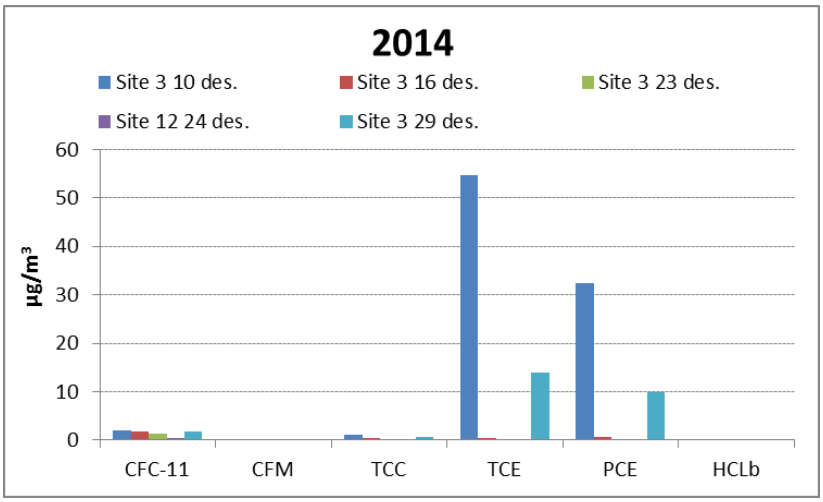


Figure 55: Concentrations of the main VOCs

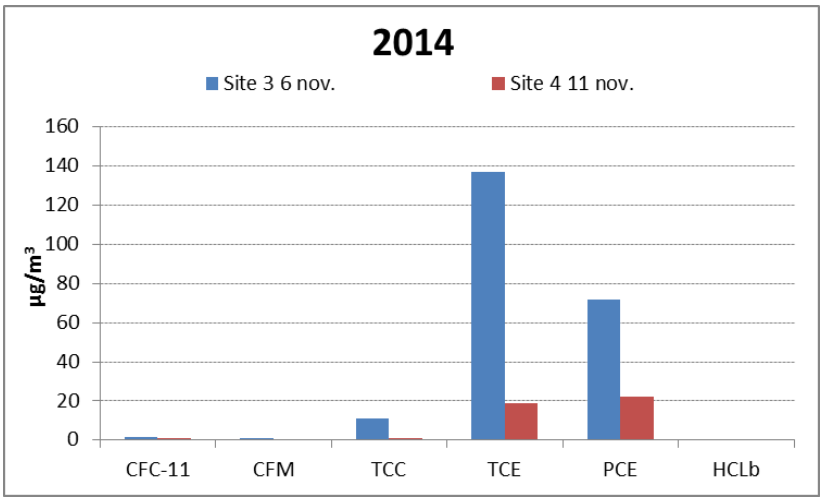


Figure 56: Concentrations of the main VOCs

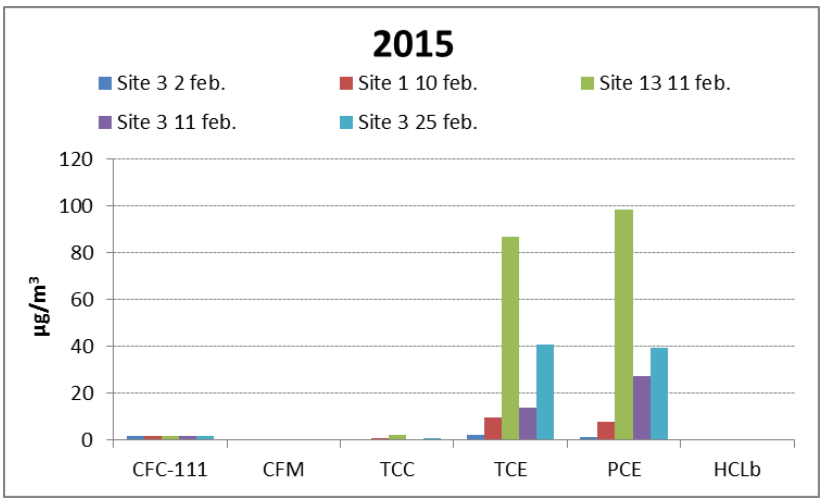


Figure 57: Concentrations of the main VOCs

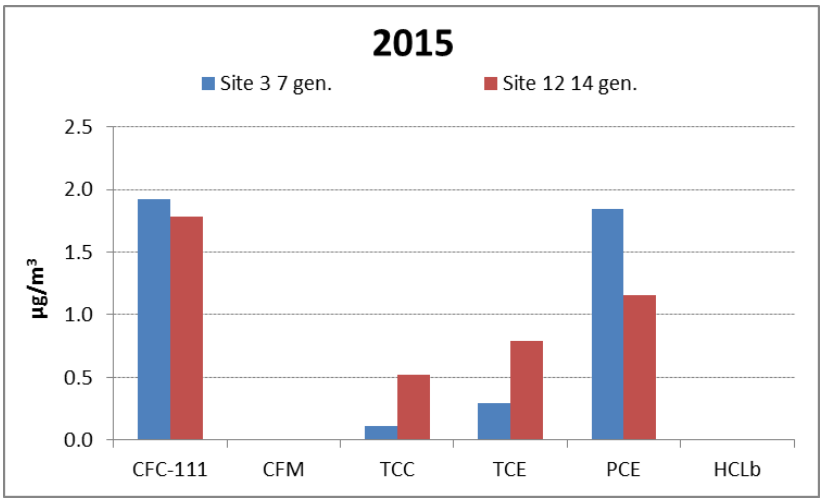


Figure 58: Concentrations of the main VOCs

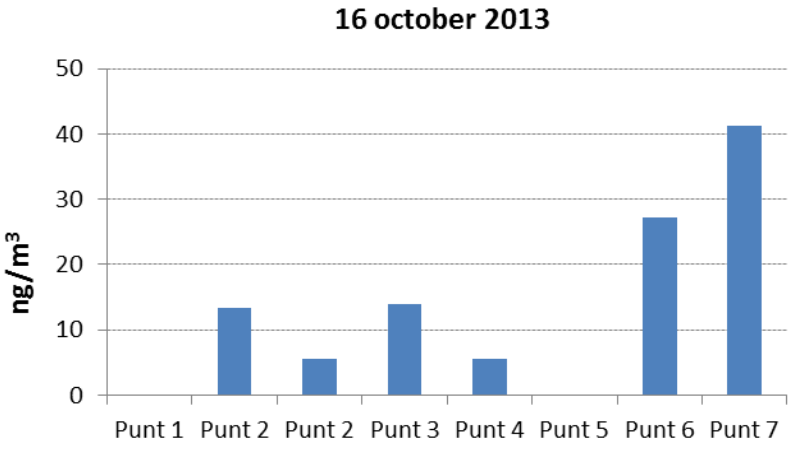


Figure 59: Concentrations of hexachlorobenzene.

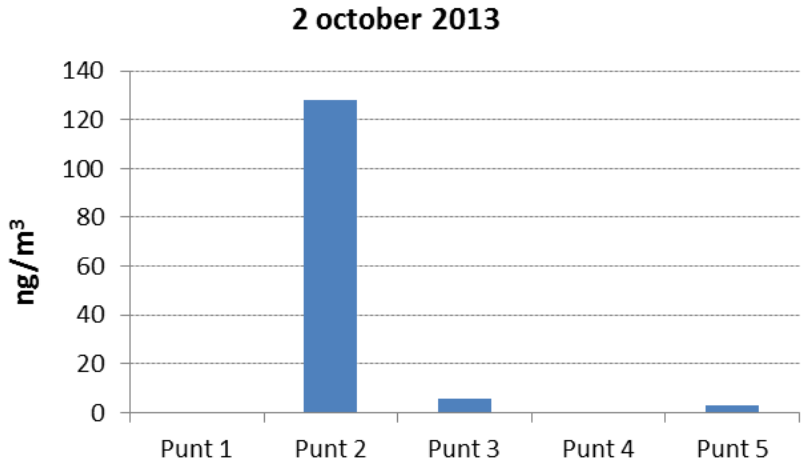


Figure 60: Concentrations of hexachlorobenzene.

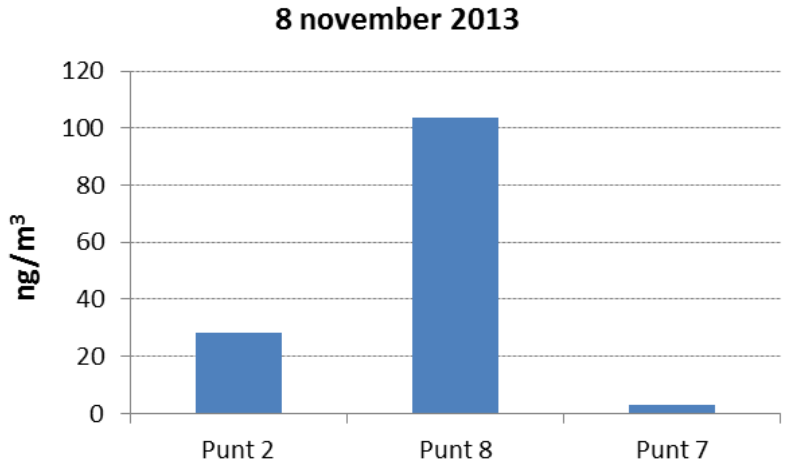


Figure 61: Concentrations of hexachlorobenzene.

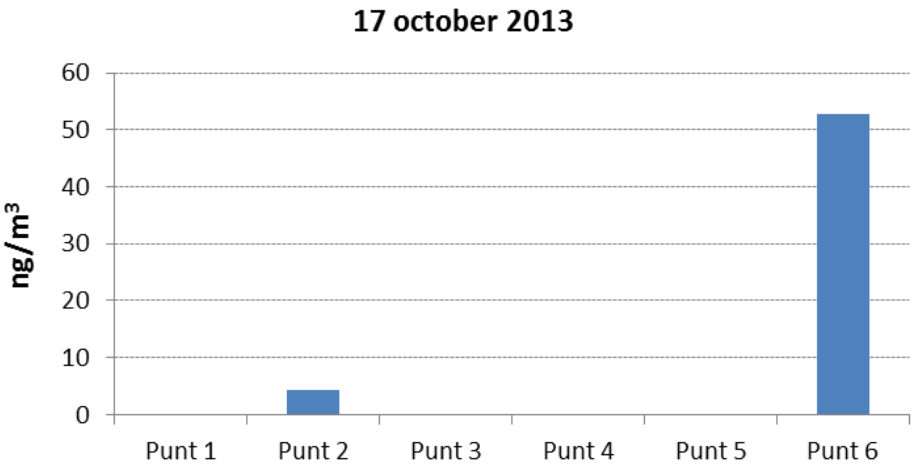


Figure 62: Concentrations of hexachlorobenzene.

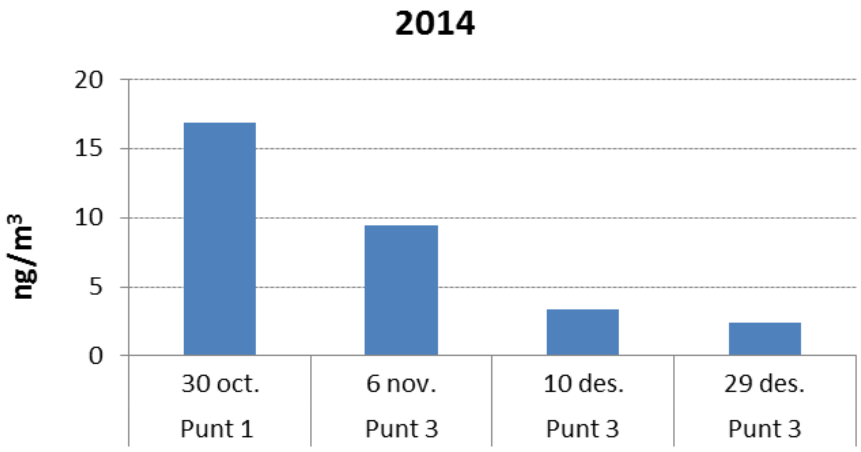


Figure 63: Concentrations of hexachlorobenzene.

The present hexachlorobutadiene concentrations range between below limit of quantification and 1.8 μg/m^3 (Figure 49 to Figure 58). In most samples this compound is below the limit of quantification (12:07 μg/m^3). The sample of 1.8 μg/m^3 corresponds to the wastewater treatment plant (2 October 2013). This compound has been found above 0.07 μg/m^3 in various locations, such as the Església Square (0.16 μg/m^3, 16 October 2013, 0.23 μg/m^3, 3 October 2014, 0.42 μg/m^3, 6 November 2014, 0.22 μg/m^3, 10 December 2014, 0.07 μg/m^3, 29 December 2014, 0.13 μg/m^3, 11 February 2015, 0.26 μg/m^3, 25 February 2015), schools (0.08 μg/m^3, 16 October 2013; 0.14 μg/m^3, 8 October 2014, 0.24 μg/m^3, 16 October 2014, 0.22 μg/m^3, 11 November 2014), Chemical hill (0.21 μg/m^3, 16 October 2013; 0.2 μg/m^3, 17 October 2013), Chemistry bridge (0.5 μg/m^3, 16 October 2013), Park of Casetes (0.40 μg/m^3, 11 February 2015), Continental (0.14 μg/m^3, 17 September 2014), Molí street (0.32 μg/m^3, 24 September 2014), the zone of sludge dredging (0.43 μg/m^3, 8 November 2013), Mas del Director (0.26 μg/m^3, 30 October 2014) and the water treatment plant (0.1 μg/m^3, 8 November 2013).

In October 1992, the concentrations of this compound varied between nd-0.3 μg/m^3 in the Església square, 0.1-0.8 μg/m^3 in the schools and 0.2-1.6 μg/m^3 in the car parking of the Factory. In the period between November 1996 and October 1997, the concentrations of this compound varied between nd-11 μg/m^3 in Església square, nd-5.6 μg/m^3 in the schools, nd-16 μg/m^3 in the parking of the Factory and nd-4.9 μg/m^3 in the Mas del Director. They were, therefore, in some cases higher than today.

The concentrations of hexachlorobenzene (Figure 59 to Figure 63) show the highest level in a sample next to the water treatment plant, on 2nd October 2013 (130 ng/m^3), coinciding with the maximum of hexachlorobutadiene. Hexachlorobenzene is found in concentrations above the threshold of 2 ng/m^3 in air samples taken in the Església square (5.8 ng/m^3, 2 October 2013; 14 ng/m^3, 16 October 2013; 9.4 ng/m^3, 6 November 2014; 3.4 ng/m^3, 10 December

2014; 2 ng/m^3, 16 December 2014; 0.7 ng/m^3, 23 December 2014; 2.4 ng/m^3, 29 December 2014) in Ribarroja (3.2 ng/m^3, 2 October 2013), also in the water treatment plant (5.5-13 ng/m^3, 16 October 2013; 4.4 ng/m^3, 17 October 2013; 28 ng/m^3, 8 November 2013) Hill Chemistry (27 ng/m^3, 16 October 2013; 53 ng/m^3, 17 October 2013), the Chemistry bridge (41 ng/m^3, 16 October 2013; 3 ng/m^3, 8 November 2013), the schools (5.6 ng/m^3, 16 October 2013), in the area of sludge dredging (100 ng/m^3, 8 November 2013) and Mas del Director (17 ng/m^3, 30 October 2014).

In the period between July 1989 and October 1992 the concentrations of this compound varied between 11-44 ng/m^3, and in the period between November 1996 and October 1997 they ranged between 6-255 ng/m^3 in Església square, nd-64 ng/m^3 in the schools, 62-1200 ng/m^3 in the car parking of the factory, nd-130 ng/m^3 in the Mas del Director. That is, although in some cases the concentrations of hexachlorobenzene during this period of waste removal have not been low, in the past the observed concentrations were much higher.

Toxicity by inhalation

Carbon tetrachloride

In this compound the level for diseases not related to cancer is estimated at 100 μg/m^3. Considering the level of one cancer per million people throughout life (70 years), the threshold is 0.17 μg/m^3. This compound is possibly carcinogenic to humans, Group 2B (International Agency for Research on Cancer, IARC, 2013). USEPA (Integrated Risk Information System of the Environmental Protection Agency of the United States, USEPA IRIS, 2012) defines it as probably carcinogenic to humans. The WHO sets 6.1 μg/m^3 as air quality reference value or tolerable concentration (World Health Organization, WHO, 2000).

Trichloroethylene

The IARC and the USEPA IRIS classified this compound as carcinogenic in humans, Group 1 (IARC, 2014) and Category A (USEPA IRIS, 2011). The WHO established a concentration threshold of 2.3 μg/m^3 for the risk of developing cancer throughout the life of 1 in 1,000,000 (WHO, 2010). The USEPA sets a concentration reference for chronic inhalation of 2 μg/m^3. This value probably poses no appreciable risk of adverse health effects throughout life, excluding cancer risk (IRIS USEPA, 2011). The reference value for neurotoxic effects is 500 μg/m^3.

Tetrachloroethylene

Both the IARC and the USEPA IRIS define tetrachloroethylene as probable carcinogenic to humans, Group 2A (IARC, 2013) and Group B1 (IRIS USEPA, 2012). According to the USEPA, the concentration threshold for chronic inhalation (RFC) is 40 μg/m^3, a value below which neurotoxic effects are probably not observed (IRIS USEPA, 2012). The air quality guideline of WHO established an annual concentration averaged of 250 μg/m^3 for a general

assessment of risks to health (WHO, 2010). The threshold of toxicity for cancer (one into one million) is 4 μg/m^3 (for constant exposure throughout life time). According to this standard, the concentration of 40 μg/m^3 corresponds to one cancer per hundred thousand inhabitants.

Hexachlorobutadiene

The concentration threshold for cancer throughout the life period of every million people is 0.05 μg/m^3 (EPA IRIS 1987). There is no set value concentration reference chronic inhalation from USEPA IRIS nor air quality guide from WHO. IARC classifies this compound in Group 3, as non-carcinogenic in humans (IARC 1987) and USEPA IRIS in Group C as possible carcinogen (EPA IRIS 1987).

Hexachlorobenzene

This compound has not any reference value for chronic inhalation from USEPA IRIS nor air quality guide from WHO. The threshold for one cancer per million people is 0002 μg/m^3 (EPA, 2000). The International Agency for Research on Cancer classifies this compound in Group 2B as possible carcinogenic to humans (IARC, 2015).

Table 35 compares these values with median values corresponding to the samplings performed in similar sites during the years 1996-97. It is observed that the medians of carbon tetrachloride and hexachlorobenzene in this earlier period, 2.2 μg/m^3 and 0.045 μg/m^3, respectively, are clearly than the current 0.63 μg/m^3 and 0.002 μg/m^3, respectively. In contrast, the median of perchloroethylene and hexachlorobutadiene are approximately the same in both periods (Table 35) and trichloroethylene is higher in the latter period, 2.4 μg/m^3 than in the period 1996-97, 0:37 μg/m^3.

Since the toxicological reference values correspond to chronic exposures, it was considered an eight hours lifetime exposure over the 24 daily hours. Table 35 compares the observed concentrations of exposure by inhalation with the toxicology threshold levels.

Table 35: Median concentrations of the samples collected in 2013-15 compared with the concentrations measured in the same sites in 1992, in the 1996-97 period and toxic thresholds.

Compound	Median 2013-15	Concentration of exposure	Median 1992	Median 1996-97	Threshold of non-carcinogenic effects (Rfc)	Threshold of carcinogenic effects [b]
Trichloroethylene	2.4[a]	0.8	1.5	0.37	2	0.2
Tetrachloroethylene	1.7	0.6	6.5	1.6	40	3.3
Carbon tetrachloride	0.63	0.21	6.7	2.2	100	0.17
Hexachlorobutadiene	<LD	<LD	0.3	<LD	-[c]	0.05
Hexachlorobenzene	0.006	0.0007	0.002	0.045	-	0.002

[a]Units in μg/m[3]. [b]One cancer in one million of people throughout life (70 years). [c]Not reported

As toxicology reference it has been decided to use the values set by the EPA IRIS (www.epa.gov/iris; 12 September 2014).

In all cases in which EPA IRIS has established threshold concentrations for not carcinogenic health risks, the exposure concentrations measured Flix are lower than these risks (trichloroethylene, perchloroethylene and carbon tetrachloride) (Table 35). In the case of hexachlorobutadiene and hexachlorobenzene there are no levels published by EPA IRIS recently. Concerning the toxicological risk thresholds for cancer, which are usually lower than those of non-carcinogenic effects (Table 35), the median exposure concentrations of these two compounds are also below the reference levels.

Regarding the threshold concentrations corresponding risk of cancer, perchloroethylene, hexachlorobenzene and the hexaclorobutadiene show a concentration corresponding to the threshold of cancer per million people throughout life (70 years), while trichloroethylene and carbon tetrachloride are below a threshold of cancer per hundred thousand people throughout life.

Conclusions

The observed chlorinated VOCs present in the air of the village correspond to emissions associated with the extraction and treatment of the sludge accumulated in the river. The estimates of chronic exposure to these compounds based on the data analyzed show concentration levels of trichloroethylene, perchloroethylene and carbon tetrachloride that are always below the values corresponding to non-carcinogenic effects.

In the case of hexachlorobutadiene and hexachlorobenzene there are no levels published by EPA IRIS recently but if the cancer toxicological risk thresholds are considered, the medians of these two compounds are also below the reference levels.

Regarding the threshold concentrations corresponding to cancer risk, perchloroethylene, hexachlorobenzene and hexaclorobutadiene show concentrations that are below the level of one cancer per million people throughout life (70 years), while trichlorethylene and carbon tetrachloride concentrations are below the level corresponding to one cancer per hundred thousand people throughout life.

7st case study – Ascertainment of ways of incorporation of mercury into humans (Menorca and Balearic Islands, Spain)

Introduction

Mercury is a recognized toxic pollutant of public health concern (WHO/IPCS, 1990). It is distributed globally by both natural processes and anthropogenic activities. Inorganic mercury may be transformed into organic forms (mainly methylmercury, MeHg) which may then accumulate and biomagnify through the food chain.

The developing foetus is particularly vulnerable to acute and chronic mercury exposures. As MeHg crosses the placenta it may accumulate and damage the developing central nervous system because it may get across the permeable blood-brain barrier (Davidson et al., 2004). Contamination episodes in Japan showed irreversible neurological damage upon exposure to this compound (Harada et al., 1995). Several studies have focussed on neurotoxicity and neurodevelopmental risk among children exposed to low or moderate mercury levels (Karagas et al., 2012) but the association is still inconclusive.

It is generally agreed that seafood consumption, either fish or mammals, is the main source of MeHg in humans (International Programme on Chemical Safety, 1991; Centers for Disease Control and Prevention, 2005; US Environmental Protection Agency, 2007; McDowell et al., 2004). Accordingly, infants from the populations of several islands have been studied for assessment of the accumulation patterns and neurodevelopmental effects, e.g. Faroe Islands (n= 917; Grandjean et al., 1997), Madeira Islands (n= 149; Murata et al., 1999), Seychelles Islands (n= 229; Myers et al., 2009).

The Mediterranean basin is a global store of mercury (Renzoni et al., 1998). Marine organisms living in this basin accumulate higher mercury concentrations than those from other seas. To the best of our knowledge, mercury accumulation in people on Mediterranean Islands have only been considered in three sites, Eastern Aegean Islands (n = 246, Gibicar et al., 2006), Sardinia (n = 22; Carta et al., 2003) and Menorca (n = 65; Montuori et al., 2006; Diez et al., 2009; Gari et al., 2013).

Menorca is a Balearic Island located in the northwestern Mediterranean Sea. Its resident population is about 94 000 inhabitants and their livelihoods depend on extensive farming and tourism, with little industry. The inhabitants also have easy access to fish and seafood, a portion of them being fishermen. The fresh fish and shellfish consumed in the island are of local origin, i.e. Mediterranean. This island is therefore an interesting site for the assessment of the exposure levels of mercury in children from general population living in a Mediterranean environment.

Studies in newborns and pre-school children in Catalonia, Valencia, Granada and Menorca

showed elevated exposure to mercury contamination which was related to high fish intake (Montuori et al., 2006; Ramon et al., 2008; Diez et al., 2009; Freire et al., 2010; Gari et al., 2013). However, direct assessment of the influence of diet into the mercury intake has not been addressed.

Summary of existing environmental and biomonitoring data – gaps identification

In the present case study, several campaigns have been performed for assessment of the contribution of fish to the incorporation of mercury in the diet of the inhabitants of Menorca. For this purpose, the main food items produced in Menorca were analysed for mercury concentration. In addition, samples of a large number of fish species captured in the waters nearby Menorca and consumed by the population were also examined.

Preliminary results of the study were shown to the Department of Health, Family and Social Welfare of the Autonomous Government of the Balearic Islands. This department also decided to perform sampling and mercury analysis of several fish species covering the whole waters of the Balearic Islands. Collaboration was established between this Department and IDAEA-CSIC in the context of CROME-LIFE for the analysis of the content of mercury and organochlorine compounds in human consumed fish species and food item products in general. The overall information generated has provided a detailed description of the main food items responsible for the incorporation of mercury into the Mediterranean populations.

Additional data collected

Environmental data

Sampling and analysis protocol

0.5 g of fish muscle or other food items were treated with HNO3 (Baker Instra) and H2O2 (Merck Suprapur) in a Teflon vessel (90ºC overnight). The digested sample was diluted with deionized water (Purelab Ultra). One procedural blank was included in each sample batch for possible contamination control. THg determination was performed using inductively coupled plasma mass spectrometry (Agilent 7500 CE) operating under standard conditions and using rhodium as internal standard. All samples were above detection limit.

Certified reference materials from the International Atomic Energy Agency, IAEA-085 and IAEA-086, with 23.2 µg/g and 0.57 µg/g total mercury concentrations, respectively (Heller-Zeisler et al., 1998), were used for validation and verification of the accuracy of the analytical method.

Results

A summary of the mercury and cadmium concentrations found in the food items of Menorca is shown in Table 36. In both cases, fish and seafood were the materials containing these

metals in highest concentrations, in the case of mercury more than ten times the concentrations in meat and about 8 times the concentrations in cheese. However, the most remarkable aspect of this metal is that the content in fish and seafood was above the EU legal regulation for human consumption.

Table 36: Average Hg and Cd (µg/g wet weight) in the main food items produced in the island of Minorca (n = 150)

	Hg		Cd	
Food type	**Maó**	**Ciutadella**	**Maó**	**Ciutadella**
Fish and seafood	**1.11**	**0.94**	0.05	0.03
Meat	0.10	0.01	0.01	0.01
Cheese	0.16		0.01	
Eggs	0.01	0.01	0.01	0.01
Fruits	0.01		0.01	
Vegetables	0.01		0.01	

In red, values above the EU threshold for human consumption

These results confirmed the previous observations based on accumulation of mercury in hair of 4 year old children and diet questionnaires (Gari et al., 2013). However, they showed additionally that this preferential intake of mercury associated to fish and seafood consumption involveed food items that do not fulfill the EU requirements for human ingestion.

Table 37: Mercury concentrations (µg/g wet weight) in fish species for human consumption captured in the waters of the Balearic Islands

			Market	Market	
		2014 N=160	Mao 2014 N=90	Ciutadella 2014 N=75	2015 N=290
Transparent goby	*Aphia minuta*				0.09
Shrimp	*Aristeus antennatus*		**2.35**	**1.85**	
Boops boops	*Boops boops*				0.21
European conger	*Conger conger*	**0.56**			**0.58**
Dolphin Fish	*Coryphaena hippurus*				0.09
Common dentex	*Dentex dentex*	**1.15**	**2.03**		**0.78**
White seabream	*Diplodus sargus*				0.34
European anchovy	*Engraulis encrasicolus*				0.09
Dusky Grouper	*Epinephelus marginatus*		**2.54**		**1.24**
giant moray	*Gymnothorax javanicus*	0.41	1.11		0.43
four-spot megrim	*Lepidorhombus boscii*				0.46
Squid	*Loligo vulgaris*		0.36	0.30	

angler/monkfish	*Lophius piscatorius*	0.97	0.62	0.27	0.63
European hake	*Merluccius merluccius*	0.42	**0.56**	**1.22**	0.27
Forkbeard	*Phycis phycis*		**1.36**	**1.02**	0.19
Small-spotted catshark	*Scyliorhinus canicula*		**1.07**	**3.77**	**0.78**
Mussel	*Mytilus sp*		0.07		
Scampi	*Nephrops norvegicus*		**0.77**	**0.74**	
Pagellus acarne	*Pagellus acarne*			0.21	
Common pandora	*Pagellus erythrinus*		0.16	0.50	0.35
Common seabream	Pagrus pagrus	0.30	**0.69**		0.31
Thornback ray	*Raja Clavata*		**2.12**	**1.68**	
European pilchard	*Sardina pilchardus*				0.09
Round sardinella	*Sardinella aurita*				0.11
black scorpionfish	*Scorpaena porcus*	0.15			0.16
red scorpionfish	*Scorpaena scrofa*	0.26	0.42	**1.39**	0.19
common pandora	Seriola dumerili	0.05			0.30
Comber	*Serranus cabrilla*				0.30
painted comber	*Serranus scriba*				0.28
European barracuda	Sphyraena sphyraena				**1.00**
Picarel	*Spicara smaris*				0.10
black seabream	Spondyliosoma cantharus		**0.98**	**0.76**	0.19
Atlantic horse mackerel	Trachurus trachurus				0.19
Shi drum	*Umbrina cirrosa*				0.12
pearly razorfish	*Xyrichtys novacula*				0.09
Peter's Fish	*Zeus faber*				0.34

In red, values above the EU threshold for human consumption

These observations led us to study which fish species commonly used for human consumption were responsible for these high mercury concentrations. Two types of samples were used for this approach, those collected in the markets in which fish was sold and those directly obtained from the fishermen.

The criterion for inclusion in the study was that the specimens had to be obtained in waters nearby the Balearic Islands. This requirement was checked for each specimen included in the study. The overall survey encompassed the analysis of 36 the fish and seafood species of highest local consumption, none of them was oily fish (Table 37).

The results of this extended study (Table 37) showed that seafood species of common human consumption obtained in Mediterranean waters, such as shrimps, have concentrations of mercury above the EU requirements. Moreover, other very common lean fish species such as common dentex, black seabream, European hake, forkbeard, small-spotted catshark and scampi also showed mercury concentrations well above the EU indications for human consumption. The identification of these species as mercury sources increase considerably the number of fish species that are responsible for the incorporation of mercury into the population, including four-year old children. These species do not belong to the group of oily

fish. Fish are therefore shown to be a critical food source for mercury intake. Their influence is much larger than previously assumed since it also encompasses an important number of highly consumed lean species which are not considered as possible mercury sources by the population.

Conclusions

This study showed that consumption of fish obtained locally in Mediterranean waters nearby the Balearic Islands was a main mechanism for the incorporation of mercury into the population living in Mediterranean areas. Specifically, this was the case of the inhabitant of Menorca.

Besides oily fish, many Mediterranean fish species were observed to contain mercury concentrations above the UE threshold for human consumption. The identification of this source will provide an important clue for the implementation of prophylactic measures to decrease the rate of human incorporation of this toxic pollutant through diet, particularly in the case of newborns. The public opinion must be informed of the new species that are also responsible for mercury incorporation into humans. This task will also be performed by the research partners and stakeholders participating into CROME-LIFE.

References

Abernathy C., Gomez-Caminero A, Howe P, Hughes M, Kenyon E, Lewis D, R.,, Moore M, Becking. R. Environmental health criteria for arsenic and arsenic compounds, 2001, pp. pp. i-xxviii+1-521.

Adachi K, Tainosho Y. Characterization of heavy metal particles embedded in tire dust. Environ Int 2004; 30: 1009-17.

Afridi HI, Kazi TG, Jamali MK, Kazi GH, Arain MB, Jalbani N, Shar GQ, Sarfaraz RA. Evaluation of toxic metals in biological samples (scalp hair, blood and urine) of steel mill workers by electrothermal atomic absorption spectrometry. Toxicology and Industrial Health 2006; 22: 381-393.

Afridi HI, Kazi TG, Kazi N, Baig JA, Jamali MK, Arain MB, Sarfraz RA, Sheikh HU, Kandhro GA, Shah AQ. Status of essential trace metals in biological samples of diabetic mother and their neonates. Arch Gynecol Obstet 2009; 280: 415-23.

Ahmed S, Mahabbat-e Khoda S, Rekha RS, Gardner RM, Ameer SS, Moore S, Ekstrom EC, Vahter M, Raqib R. Arsenic-associated oxidative stress, inflammation, and immune disruption in human placenta and cord blood. Environ Health Perspect 2011; 119: 258-64.

Al-Saleh I, Shinwari N, Mashhour A, Mohamed Gel D, Rabah A. Heavy metals (lead, cadmium and mercury) in maternal, cord blood and placenta of healthy women. Int J Hyg Environ Health 2011; 214: 79-101.

Alimonti A, Petrucci F, Krachler M, Bocca B, Caroli S. Reference values for chromium, nickel and vanadium in urine of youngsters from the urban area of Rome. J Environ Monit 2000; 2: 351-4.

Amato F, Pandolfi M, Moreno T, Furger M, Pey J, Alastuey A, Bukowiecki N, Prevot ASH, Baltensperger U, Querol X. Sources and variability of inhalable road dust particles in three European cities. Atmospheric Environment 2011; 45: 6777-6787.

Amato F, Pandolfi M, Viana M, Querol X, Alastuey A, Moreno T. Spatial and chemical patterns of PM10 in road dust deposited in urban environment. Atmospheric Environment 2009; 43: 1650-1659.

Andrew AS, Jewell DA, Mason RA, Whitfield ML, Moore JH, Karagas MR. Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a U.S. population. Environ Health Perspect 2008; 116: 524-31.

Apeagyei E, Bank MS, Spengler JD. Distribution of heavy metals in road dust along an urban-rural gradient in Massachusetts. Atmospheric Environment 2011; 45: 2310-2323.

Argos M, Kalra T, Pierce BL, Chen Y, Parvez F, Islam T, Ahmed A, Hasan R, Hasan K, Sarwar G, Levy D, Slavkovich V, Graziano JH, Rathouz PJ, Ahsan H. A Prospective Study of Arsenic Exposure From Drinking Water and Incidence of Skin Lesions in Bangladesh. American Journal of Epidemiology 2011; 174: 185-194.

Arnich N, Sirot V, Riviere G, Jean J, Noel L, Guerin T, Leblanc JC. Dietary exposure to trace elements and health risk assessment in the 2nd French Total Diet Study. Food Chem Toxicol 2012; 50: 2432-49.

ATSDR. Toxicological Profile for Arsenic, GA: Agency for Toxic Substances and Disease Registry, 2007.

Aylward L, Hays S, Kirman C, Marchitti S, Kenneke J, English C, Mattison D, Becker R. Relationships of chemical concentrations in maternal and cord blood: a review of

available data. Journal of Toxicology and Environmental Health, Part B 2014; 17: 175-203.

Aylward LL, Brunet RC, Starr TB, Carrier G, Delzell E, Cheng H, Beall C. Exposure reconstruction for the TCDD-exposed NIOSH cohort using a concentration- and age-dependent model of elimination. Risk Anal 2005; 25: 945-56.

Baker RD, Greer FR. Diagnosis and prevention of iron deficiency and iron-deficiency anemia in infants and young children (0-3 years of age). Pediatrics 2010; 126: 1040-50.

Ballester F, Sala M, Sunyer J, Grimalt J. Serum concentrations of hexachlorobenzene in family members of workers in an electrochemical factory. Scand J Work Environ Health 2000; 26: 67-70.

Banza CL, Nawrot TS, Haufroid V, Decree S, De Putter T, Smolders E, Kabyla BI, Luboya ON, Ilunga AN, Mutombo AM, Nemery B. High human exposure to cobalt and other metals in Katanga, a mining area of the Democratic Republic of Congo. Environ Res 2009; 109: 745-52.

Barany E, Bergdahl IA, Bratteby LE, Lundh T, Samuelson G, Skerfving S, Oskarsson A. Iron status influences trace element levels in human blood and serum. Environ Res 2005; 98: 215-23.

Belis CA, Karagulian F, Larsen BR, Hopke PK. Critical review and meta-analysis of ambient particulate matter source apportionment using receptor models in Europe. Atmospheric Environment 2013; 69: 94-108.

Belzile N, Chen Y-W, Filella M. Human Exposure to Antimony: I. Sources and Intake. Critical Reviews in Environmental Science and Technology 2011; 41: 1309-1373.

Bocca B, Forte G, Petrucci F, Senofonte O, Violante N, Alimonti A. Development of methods for the quantification of essential and toxic elements in human biomonitoring. Ann Ist Super Sanita 2005; 41: 165-70.

Bonithon-Kopp C, Huel G, Grasmick C, Sarmini H, Moreau T. Effects of pregnancy on the inter-individual variations in blood levels of lead, cadmium and mercury. Biol Res Pregnancy Perinatol 1986; 7: 37-42.

Bonvallot N., Harrison P., Loh M. Trichloroethylene. Geneva: World Health Organization, 2010.

Boos KS, Lintelmann J, Kettrup A. Coupled-column high-performance liquid chromatographic method for the determination of 1-hydroxypyrene in urine of subjects exposed to polycyclic aromatic hydrocarbons. J Chromatogr 1992; 600: 189-94.

Borak J, Hosgood HD. Seafood arsenic: Implications for human risk assessment. Regulatory Toxicology and Pharmacology 2007; 47: 204-212.

Bouwman H, Van den Berg H, Kylin H. DDT and malaria prevention: addressing the paradox. 2011.

Brantsaeter AL, Birgisdottir BE, Meltzer HM, Kvalem HE, Alexander J, Magnus P, Haugen M. Maternal seafood consumption and infant birth weight, length and head circumference in the Norwegian Mother and Child Cohort Study. Br J Nutr 2012; 107: 436-44.

Brantsaeter AL, Haugen M, Thomassen Y, Ellingsen DG, Ydersbond TA, Hagve TA, Alexander J, Meltzer HM. Exploration of biomarkers for total fish intake in pregnant Norwegian women. Public Health Nutr 2010; 13: 54-62.

Brender JD, Suarez L, Felkner M, Gilani Z, Stinchcomb D, Moody K, Henry J, Hendricks K. Maternal exposure to arsenic, cadmium, lead, and mercury and neural tube defects in offspring. Environ Res 2006; 101: 132-9.

Brochu P, Ducré-Robitaille J-F, Brodeur J. Physiological Daily Inhalation Rates for Free-Living Pregnant and Lactating Adolescents and Women Aged 11 to 55 Years, Using Data from Doubly Labeled Water Measurements for Use in Health Risk Assessment. Human and Ecological Risk Assessment: An International Journal 2006; 12: 702-735.

Brooke PJ, Evans WH. Determination of total inorganic arsenic in fish, shellfish and fish products. Analyst 1981; 106: 514-20.

Buchet JP, Lison D, Ruggeri M, Foa V, Elia G. Assessment of exposure to inorganic arsenic, a human carcinogen, due to the consumption of seafood. Arch Toxicol 1996; 70: 773-8.

Caldwell KL, Jones RL, Verdon CP, Jarrett JM, Caudill SP, Osterloh JD. Levels of urinary total and speciated arsenic in the US population: National Health and Nutrition Examination Survey 2003-2004. J Expo Sci Environ Epidemiol 2009; 19: 59-68.

Callan AC, Hinwood AL, Ramalingam M, Boyce M, Heyworth J, McCafferty P, Odland JO. Maternal exposure to metals--concentrations and predictors of exposure. Environ Res 2013; 126: 111-7.

Cameron BM, Neufeld LM. Estimating the prevalence of iron deficiency in the first two years of life: technical and measurement issues. Nutr Rev 2011; 69 Suppl 1: S49-56.

Campbell WW, Anderson RA. Effects of aerobic exercise and training on the trace minerals chromium, zinc and copper. Sports Med 1987; 4: 9-18.

Cancer IAfRo. IARC monographs on the evaluation of carcinogenic risks to humans: some drinking-water disinfectants and contaminants, including arsenic, 2004.

Carpenter DO. Health effects of persistent organic pollutants: the challenge for the Pacific Basin and for the world. Rev Environ Health 2011; 26: 61-9.

Carrizo D, Grimalt JO, Ribas-Fito N, Sunyer J, Torrent M. Influence of breastfeeding in the accumulation of polybromodiphenyl ethers during the first years of child growth. Environ Sci Technol 2007a; 41: 4907-12.

Carrizo D, Grimalt JO, Ribas-Fito N, Torrent M, Sunyer J. In utero and post-natal accumulation of organochlorine compounds in children under different environmental conditions. J Environ Monit 2007b; 9: 523-9.

Carta P, Flore C, Alinovi R, Ibba A, Tocco MG, Aru G, Carta R, Girei E, Mutti A, Lucchini R, Randaccio FS. Sub-clinical neurobehavioral abnormalities associated with low level of mercury exposure through fish consumption. Neurotoxicology 2003; 24: 617-23.

Casas i Sabata JMHRCL. Salinitat i contaminació en la conca del Llobregat: Rius Llobregat, Cardener i Anoia. Dovella [en línia] 2001: 27-32.

Caseiro A, Bauer H, Schmidl C, Pio CA, Puxbaum H. Wood burning impact on PM10 in three Austrian regions. Atmospheric Environment 2009; 43: 2186-2195.

Caserta D, Mantovani A, Ciardo F, Fazi A, Baldi M, Sessa MT, la Rocca C, Ronchi A, Moscarini M, Minoia C. Heavy metals in human amniotic fluid: a pilot study. Prenat Diagn 2011; 31: 792-6.

Castillo S, Moreno T, Querol X, Alastuey A, Cuevas E, Herrmann L, Mounkaila M, Gibbons W. Trace element variation in size-fractionated African desert dusts. Journal of Arid Environments 2008; 72: 1034-1045.

CDC. Growth charts, 2000.

CDC. Fourth National Report on Human Exposure to Environmental Chemicals Atlanta, GA, 2009.

Centre IVC. Heavy metals in European Mosses, Survey. United Nation Economic Commission for Europe Convention on long-range transboundary air pollution.Monitoring of atmospheric deposition of heavy metals, nitrogen and POPs in Europe using Bryophytes. . In: Manual M, editor. 2010 Survey, 2010.

Chen CJ, Wang SL, Chiou JM, Tseng CH, Chiou HY, Hsueh YM, Chen SY, Wu MM, Lai MS. Arsenic and diabetes and hypertension in human populations: a review. Toxicol Appl Pharmacol 2007; 222: 298-304.

Chen Y, Graziano JH, Parvez F, Liu M, Slavkovich V, Kalra T, Argos M, Islam T, Ahmed A, Rakibuz-Zaman M, Hasan R, Sarwar G, Levy D, van Geen A, Ahsan H. Arsenic exposure from drinking water and mortality from cardiovascular disease in Bangladesh: prospective cohort study. BMJ 2011; 342.

Christensen JM. Human exposure to toxic metals: factors influencing interpretation of biomonitoring results. Science of The Total Environment 1995; 166: 89-135.

Christensen JM, Poulsen OM, Thomsen M. A short-term cross-over study on oral administration of soluble and insoluble cobalt compounds: sex differences in biological levels. International Archives of Occupational and Environmental Health 1993; 65: 233-240.

Cole P, Trichopoulos D, Pastides H, Starr T, Mandel JS. Dioxin and cancer: a critical review. Regul Toxicol Pharmacol 2003; 38: 378-88.

Concha G, Vogler G, Lezcano D, Nermell B, Vahter M. Exposure to inorganic arsenic metabolites during early human development. Toxicol Sci 1998; 44: 185-90.

Control CfD, Prevention. Third national report on human exposure to environmental chemicals: Executive summary. Third national report on human exposure to environmental chemicals: executive summary. NCEH, 2005.

Control CfD, Prevention. Fourth national report on human exposure to environmental chemicals. Atlanta, Ga, USA, 2009.

Costopoulou D, Vassiliadou I, Papadopoulos A, Makropoulos V, Leondiadis L. Levels of dioxins, furans and PCBs in human serum and milk of people living in Greece. Chemosphere 2006; 65: 1462-1469.

Cuadra SN, Linderholm L, Athanasiadou M, Jakobsson K. Persistent organochlorine pollutants in children working at a waste-disposal site and in young females with high fish consumption in Managua, Nicaragua. Ambio 2006; 35: 109-16.

Davidson PW, Myers GJ, Weiss B. Mercury exposure and child development outcomes. Pediatrics 2004; 113: 1023-1029.

Diez S, Delgado S, Aguilera I, Astray J, Perez-Gomez B, Torrent M, Sunyer J, Bayona JM. Prenatal and early childhood exposure to mercury and methylmercury in Spain, a high-fish-consumer country. Arch Environ Contam Toxicol 2009; 56: 615-22.

Domingo JL, Perello G, Gine Bordonaba J. Dietary intake of metals by the population of Tarragona County (Catalonia, Spain): results from a duplicate diet study. Biol Trace Elem Res 2012; 146: 420-5.

Dong Z, Yuan G, Hu J. Uncertainty analysis in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) cancer dose-response for three occupational cohorts. Environ Int 2016; 88: 53-9.

Economou-Eliopoulos M, Megremi I, Vasilatos C. Factors controlling the heterogeneous distribution of Cr(VI) in soil, plants and groundwater: Evidence from the Assopos basin, Greece. Chemie der Erde - Geochemistry 2011; 71: 39-52.

Edmonds JS, Francesconi KA. Arsenic in seafoods: Human health aspects and regulations. Marine Pollution Bulletin 1993; 26: 665-674.

Emond C, DeVito M, Warner M, Eskenazi B, Mocarelli P, Birnbaum LS. An assessment of dioxin exposure across gestation and lactation using a PBPK model and new data from Seveso. Environ Int 2016; 92-93: 23-32.

Emond C, Michalek JE, Birnbaum LS, DeVito MJ. Comparison of the Use of a Physiologically Based Pharmacokinetic Model and a Classical Pharmacokinetic Model for Dioxin Exposure Assessments. Environmental Health Perspectives 2005; 113: 1666-1668.

EPA. Hexachlorobenzene, 2000.

EPA. National Primary Drinking Water Regulations. In: 816-F-09-004 E, editor, 2009.

Esteban M, Castano A. Non-invasive matrices in human biomonitoring: a review. Environ Int 2009; 35: 438-49.

Farmer TD, Shaw PJ, Williams ID. Destined for indecision? A critical analysis of waste management practices in England from 1996 to 2013. Waste Manag 2015; 39: 266-76.

Fernando S, Jobst KJ, Taguchi VY, Helm PA, Reiner EJ, McCarry BE. Identification of the halogenated compounds resulting from the 1997 Plastimet Inc. fire in Hamilton, Ontario, using comprehensive two-dimensional gas chromatography and (ultra)high resolution mass spectrometry. Environ Sci Technol 2014; 48: 10656-63.

Feunekes FD, Jongeneelen FJ, vd Laan H, Schoonhof FH. Uptake of polycyclic aromatic hydrocarbons among trainers in a fire-fighting training facility. Am Ind Hyg Assoc J 1997; 58: 23-8.

Filella M, Belzile N, Chen Y-W. Human Exposure to Antimony. III. Contents in Some Human Excreted Biofluids (Urine, Milk, Saliva). Critical Reviews in Environmental Science and Technology 2013a; 43: 162-214.

Filella M, Belzile N, Chen Y-W. Human Exposure to Antimony. IV. Contents in Human Blood. Critical Reviews in Environmental Science and Technology 2013b; 43: 2071-2105.

Finley BL, Monnot AD, Paustenbach DJ, Gaffney SH. Derivation of a chronic oral reference dose for cobalt. Regul Toxicol Pharmacol 2012; 64: 491-503.

Flanagan PR, Haist J, Valberg LS. Comparative effects of iron deficiency induced by bleeding and a low-iron diet on the intestinal absorptive interactions of iron, cobalt, manganese, zinc, lead and cadmium. J Nutr 1980; 110: 1754-63.

Fontcuberta M, Calderon J, Villalbi JR, Centrich F, Portana S, Espelt A, Duran J, Nebot M. Total and inorganic arsenic in marketed food and associated health risks for the Catalan (Spain) population. J Agric Food Chem 2011; 59: 10013-22.

Forbes JR, Gros P. Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane. Blood 2003; 102: 1884-92.

Fort M, Cosin-Tomas M, Grimalt JO, Querol X, Casas M, Sunyer J. Assessment of exposure to trace metals in a cohort of pregnant women from an urban center by urine analysis in the first and third trimesters of pregnancy. Environ Sci Pollut Res Int 2014; 21: 9234-41.

Freire C, Ramos R, Lopez-Espinosa M-J, Díez S, Vioque J, Ballester F, Fernández M-F. Hair mercury levels, fish consumption, and cognitive development in preschool children from Granada, Spain. Environmental research 2010; 110: 96-104.

Gal J, Hursthouse A, Tatner P, Stewart F, Welton R. Cobalt and secondary poisoning in the terrestrial food chain: data review and research gaps to support risk assessment. Environ Int 2008; 34: 821-38.

Gardner RM, Nermell B, Kippler M, Grander M, Li L, Ekstrom EC, Rahman A, Lonnerdal B, Hoque AM, Vahter M. Arsenic methylation efficiency increases during the first trimester of pregnancy independent of folate status. Reprod Toxicol 2011; 31: 210-8.

Garg BD, Cadle SH, Mulawa PA, Groblicki PJ, Laroo C, Parr GA. Brake Wear Particulate Matter Emissions. Environmental Science & Technology 2000; 34: 4463-4469.

Garí M, Bosch C, Grimalt JO, Sunyer J. Impacts of atmospheric chlor-alkali factory emissions in surrounding populations. Environment international 2014; 65: 1-8.

Gari M, Grimalt JO, Torrent M, Sunyer J. Influence of socio-demographic and diet determinants on the levels of mercury in preschool children from a Mediterranean island. Environ Pollut 2013; 182: 291-8.

Garrick MD, Dolan KG, Horbinski C, Ghio AJ, Higgins D, Porubcin M, Moore EG, Hainsworth LN, Umbreit JN, Conrad ME, Feng L, Lis A, Roth JA, Singleton S, Garrick LM. DMT1: a mammalian transporter for multiple metals. Biometals 2003; 16: 41-54.

Garrick MD, Singleton ST, Vargas F, Kuo HC, Zhao L, Knopfel M, Davidson T, Costa M, Paradkar P, Roth JA, Garrick LM. DMT1: which metals does it transport? Biol Res 2006; 39: 79-85.

Gascon M, Vrijheid M, Martínez D, Ballester F, Basterrechea M, Blarduni E, Esplugues A, Vizcaino E, Grimalt JO, Morales E. Pre-natal exposure to dichlorodiphenyldichloroethylene and infant lower respiratory tract infections and wheeze. European Respiratory Journal 2012; 39: 1188-1196.

Genuis SJ, Birkholz D, Rodushkin I, Beesoon S. Blood, urine, and sweat (BUS) study: monitoring and elimination of bioaccumulated toxic elements. Arch Environ Contam Toxicol 2011; 61: 344-57.

Gibicar D, Horvat M, Nakou S, Sarafidou J, Yager J. Pilot study of intrauterine exposure to methylmercury in Eastern Aegean islands, Greece. Sci Total Environ 2006; 367: 586-95.

Goonewardene M, Shehata M, Hamad A. Anaemia in pregnancy. Best Pract Res Clin Obstet Gynaecol 2012; 26: 3-24.

Grandjean P, Landrigan PJ. Developmental neurotoxicity of industrial chemicals. Lancet 2006; 368: 2167-78.

Grandjean P, Weihe P, White RF, Debes F, Araki S, Yokoyama K, Murata K, Sørensen N, Dahl R, Jørgensen PJ. Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. Neurotoxicology and teratology 1997; 19: 417-428.

Green A. How much blood is too much: Guideline 2004.

Grimalt JO, Sunyer J, Moreno V, Amaral OC, Sala M, Rosell A, Anto JM, Albaiges J. Risk excess of soft-tissue sarcoma and thyroid cancer in a community exposed to airborne organochlorinated compound mixtures with a high hexachlorobenzene content. Int J Cancer 1994; 56: 200-3.

Gulson BL, Mizon KJ, Palmer JM, Korsch MJ, Taylor AJ, Mahaffey KR. Blood lead changes during pregnancy and postpartum with calcium supplementation. Environ Health Perspect 2004; 112: 1499-507.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 1997; 388: 482-488.

Guxens M, Ballester F, Espada M, Fernandez MF, Grimalt JO, Ibarluzea J, Olea N, Rebagliato M, Tardon A, Torrent M, Vioque J, Vrijheid M, Sunyer J. Cohort Profile: the INMA--INfancia y Medio Ambiente--(Environment and Childhood) Project. Int J Epidemiol 2012; 41: 930-40.

Hamadani JD, Tofail F, Nermell B, Gardner R, Shiraji S, Bottai M, Arifeen SE, Huda SN, Vahter M. Critical windows of exposure for arsenic-associated impairment of cognitive function in pre-school girls and boys: a population-based cohort study. Int J Epidemiol 2011; 40: 1593-604.

Harada M. Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. Critical reviews in toxicology 1995; 25: 1-24.

Harp MJ, Scoular FI. Cobalt Metabolism of Young College Women on Self-Selected Diets. The Journal of Nutrition 1952; 47: 67-72.

Heitland P, Köster HD. Biomonitoring of 30 trace elements in urine of children and adults by ICP-MS. Clinica Chimica Acta 2006; 365: 310-318.

Heller-Zeisler SF, Parr RM, Zeisler R. Certification of two human hair reference materials issued by the International Atomic Energy Agency. Fresenius' Journal of Analytical Chemistry 1998; 360: 419-422.

Hernandez M, Schuhmacher M, Fernandez JD, Domingo JL, Llobet JM. Urinary cadmium levels during pregnancy and postpartum. A longitudinal study. Biol Trace Elem Res 1996; 53: 205-12.

Herrero C, Ozalla D, Sala M, Otero R, Santiago-Silva M, Lecha M, To-Figueras J, Deulofeu R, Mascaro JM, Grimalt J, Sunyer J. Urinary porphyrin excretion in a human population highly exposed to hexachlorobenzene. Arch Dermatol 1999; 135: 400-4.

Hjortenkrans DST, Bergbäck BG, Häggerud AV. Metal Emissions from Brake Linings and Tires: Case Studies of Stockholm, Sweden 1995/1998 and 2005. Environmental Science & Technology 2007; 41: 5224-5230.

Hopenhayn C, Bush HM, Bingcang A, Hertz-Picciotto I. Association between arsenic exposure from drinking water and anemia during pregnancy. J Occup Environ Med 2006; 48: 635-43.

Hopenhayn C, Huang B, Christian J, Peralta C, Ferreccio C, Atallah R, Kalman D. Profile of urinary arsenic metabolites during pregnancy. Environ Health Perspect 2003; 111: 1888-91.

Horvat M., Mazej D., Snoj Tratnik J., Šlejkovec Z., Jagodic M., Fajon V., Pavlin M., Stajnko A., Krsnik M., Prezelj M., Skitek M. HBM in Slovenia Monitoring Kemikalij v Organizmih 2011-2014. , 2015.

Howie SR. Blood sample volumes in child health research: review of safe limits. Bull World Health Organ 2011; 89: 46-53.

Hsueh YM, Hsu MK, Chiou HY, Yang MH, Huang CC, Chen CJ. Urinary arsenic speciation in subjects with or without restriction from seafood dietary intake. Toxicol Lett 2002; 133: 83-91.

Huang HM, Leung PL, Sun DZ, Zhu MG. Hair and serum calcium, iron, copper, and zinc levels during normal pregnancy at three trimesters. Biol Trace Elem Res 1999; 69: 111-20.

Hytten F. Blood volume changes in normal pregnancy. Clin Haematol 1985; 14: 601-12.

IARC. HEXACHLOROBUTADIENE, 1987.
IARC. Some Non-heterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Exposures. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 92. Lyon: International Agency for Research on Cancer, 2010.
IARC. Agents Classified by the IARC Monographs. 1-118, 2013a.
IARC. TETRACHLOROETHYLENE, 2013b.
IARC. IARC Monographs on the evaluation of carcinogenic risks to humans. 106, Lyon, France, 2014.
IARC. HEXACHLOROBENZENE, 2015.
Iavicoli I, Caroli S, Alimonti A, Petrucci F, Carelli G. Biomonitoring of a worker population exposed to low antimony trioxide levels. J Trace Elem Med Biol 2002; 16: 33-9.
IOM. Cobalt. Institute of Medicine, Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothetic Acid, Biotin, and Choline. . In: Press NA, editor, Washington D.C., 1998, pp. 306-356.
IRIS U. Trichloroethylene, 2011.
Izquierdo Alvarez S, Castanon SG, Ruata ML, Aragues EF, Terraz PB, Irazabal YG, Gonzalez EG, Rodriguez BG. Updating of normal levels of copper, zinc and selenium in serum of pregnant women. J Trace Elem Med Biol 2007; 21 Suppl 1: 49-52.
Jarup L. Hazards of heavy metal contamination. Br Med Bull 2003; 68: 167-82.
Julvez J, Grandjean P. Genetic susceptibility to methylmercury developmental neurotoxicity matters. Frontiers in Genetics 2013; 4: 278.
Kanduc M, Trulsson M, Naji A, Burak Y, Forsman J, Podgornik R. Weak- and strong-coupling electrostatic interactions between asymmetrically charged planar surfaces. Phys Rev E Stat Nonlin Soft Matter Phys 2008; 78: 061105.
Karagas MR, Choi AL, Oken E, Horvat M, Schoeny R, Kamai E, Cowell W, Grandjean P, Korrick S. Evidence on the human health effects of low-level methylmercury exposure. Environmental health perspectives 2012; 120: 799.
Kerger BD, Leung H-W, Scott P, Paustenbach DJ, Needham LL, Patterson DG, Gerthoux PM, Mocarelli P. Age- and Concentration-Dependent Elimination Half-Life of 2,3,7,8-Tetrachlorodibenzo-p-dioxin in Seveso Children. Environmental Health Perspectives 2006; 114: 1596-1602.
Kilinc M, Coskun A, Bilge F, Imrek SS, Atli Y. Serum reference levels of selenium, zinc and copper in healthy pregnant women at a prenatal screening program in southeastern Mediterranean region of Turkey. J Trace Elem Med Biol 2010; 24: 152-6.
Kim JH, Gibb, H.J., Howe, P.D., . Cobalt and inorganic cobalt compounds, 2006.
King JC. Physiology of pregnancy and nutrient metabolism. Am J Clin Nutr 2000; 71: 1218s-25s.
Kippler M, Goessler W, Nermell B, Ekstrom EC, Lonnerdal B, El Arifeen S, Vahter M. Factors influencing intestinal cadmium uptake in pregnant Bangladeshi women--a prospective cohort study. Environ Res 2009; 109: 914-21.
Knittle J, Timmers K, Ginsberg-Fellner F, Brown R, Katz D. The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size. Journal of Clinical Investigation 1979; 63: 239.
Komaromy-Hiller G, Ash KO, Costa R, Howerton K. Comparison of representative ranges based on U.S. patient population and literature reference intervals for urinary trace elements. Clinica Chimica Acta 2000; 296: 71-90.

Kovacs L, Zamboni CB, Nunes LAS, Lourenço TF, Macedo DV. Concentrations of ions and metals in blood of amateur and elite runners using NAA. Journal of Radioanalytical and Nuclear Chemistry 2013; 297: 393-398.

Krachler M, Radner H, Irgolic KJ. Microwave digestion methods for the determination of trace elements in brain and liver samples by inductively coupled plasma mass spectrometry. Anal Bioanal Chem 1996; 355: 120-8.

Kuczmarski RJ, Ogden CL, Guo SS, Grummer-Strawn LM, Flegal KM, Mei Z, Wei R, Curtin LR, Roche AF, Johnson CL. 2000 CDC Growth Charts for the United States: methods and development. Vital Health Stat 11 2002: 1-190.

Kwok RK, Mendola P, Liu ZY, Savitz DA, Heiss G, Ling HL, Xia Y, Lobdell D, Zeng D, Thorp JM, Jr., Creason JP, Mumford JL. Drinking water arsenic exposure and blood pressure in healthy women of reproductive age in Inner Mongolia, China. Toxicol Appl Pharmacol 2007; 222: 337-43.

LaKind JS, Amina Wilkins A, Berlin CM, Jr. Environmental chemicals in human milk: a review of levels, infant exposures and health, and guidance for future research. Toxicol Appl Pharmacol 2004; 198: 184-208.

Lauwers LF, Roelants A, Rosseel PM, Heyndrickx B, Baute L. Oral antimony intoxications in man. Crit Care Med 1990; 18: 324-6.

Lauwerys R, Lison D. Health risks associated with cobalt exposure--an overview. Sci Total Environ 1994; 150: 1-6.

Lee J, Prokopec SD, Watson JD, Sun RX, Pohjanvirta R, Boutros PC. Male and female mice show significant differences in hepatic transcriptomic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. BMC Genomics 2015; 16: 625.

Linderholm L, Jakobsson K, Lundh T, Zamir R, Shoeb M, Nahar N, Bergman Å. Environmental exposure to POPs and heavy metals in urban children from Dhaka, Bangladesh. Journal of Environmental Monitoring 2011; 13: 2728-2734.

Linderkamp O, Versmold HT, Riegel KP, Betke K. Estimation and prediction of blood volume in infants and children. Eur J Pediatr 1977; 125: 227-34.

Link B, Gabrio T, Piechotowski I, Zöllner I, Schwenk M. Baden-Wuerttemberg Environmental Health Survey (BW-EHS) from 1996 to 2003: Toxic metals in blood and urine of children. International Journal of Hygiene and Environmental Health 2007; 210: 357-371.

Linos A, Petralias A, Christophi CA, Christoforidou E, Kouroutou P, Stoltidis M, Veloudaki A, Tzala E, Makris KC, Karagas MR. Oral ingestion of hexavalent chromium through drinking water and cancer mortality in an industrial area of Greece - An ecological study. Environmental Health: A Global Access Science Source 2011; 10.

Lippi G, Franchini M, Guidi GC. Blood doping by cobalt. Should we measure cobalt in athletes? Journal of Occupational Medicine and Toxicology (London, England) 2006; 1: 18-18.

Liu J, Yang H, Shi H, Shen C, Zhou W, Dai Q, Jiang Y. Blood copper, zinc, calcium, and magnesium levels during different duration of pregnancy in Chinese. Biol Trace Elem Res 2010; 135: 31-7.

Liu J, Yu L, Coppin JF, Tokar EJ, Diwan BA, Waalkes MP. Fetal arsenic exposure appears to facilitate endocrine disruption by postnatal diethylstilbestrol in neonatal mouse adrenal. Chem Biol Interact 2009; 182: 253-8.

Llop S, Ballester F, Vizcaino E, Murcia M, Lopez-Espinosa MJ, Rebagliato M, Vioque J, Marco A, Grimalt JO. Concentrations and determinants of organochlorine levels among pregnant women in Eastern Spain. Sci Total Environ 2010; 408: 5758-67.

Lucier GW, Portier CJ, Gallo MA. Receptor mechanisms and dose-response models for the effects of dioxins. Environ Health Perspect 1993; 101: 36-44.

Ludersdorf R, Fuchs A, Mayer P, Skulsuksai G, Schacke G. Biological assessment of exposure to antimony and lead in the glass-producing industry. Int Arch Occup Environ Health 1987; 59: 469-74.

Lunde G. Separation and analysis of organic-bound and inorganic arsenic in marine organisms. J Sci Food Agric 1973; 24: 1021-7.

Mackenzie B, Garrick MD. Iron Imports. II. Iron uptake at the apical membrane in the intestine. Am J Physiol Gastrointest Liver Physiol 2005; 289: G981-6.

Mason KE. A conspectus of research on copper metabolism and requirements of man. J Nutr 1979; 109: 1979-2066.

McDowell MA, Dillon CF, Osterloh J, Bolger PM, Pellizzari E, Fernando R, de Oca RM, Schober SE, Sinks T, Jones RL. Hair mercury levels in US children and women of childbearing age: reference range data from NHANES 1999-2000. Environmental health perspectives 2004: 1165-1171.

Meltzer HM, Brantsaeter AL, Borch-Iohnsen B, Ellingsen DG, Alexander J, Thomassen Y, Stigum H, Ydersbond TA. Low iron stores are related to higher blood concentrations of manganese, cobalt and cadmium in non-smoking, Norwegian women in the HUNT 2 study. Environ Res 2010; 110: 497-504.

Meltzer HM, Maage A, Ydersbond TA, Haug E, Glattre E, Holm H. Fish arsenic may influence human blood arsenic, selenium, and T4:T3 ratio. Biological Trace Element Research 2002; 90: 83-98.

Messiha FS. Maternal cesium chloride ingestion and the newborn. Neurosci Biobehav Rev 1988; 12: 209-13.

Miao Q, Bouchard M, Chen D, Rosenberg MW, Aronson KJ. Commuting behaviors and exposure to air pollution in Montreal, Canada. Science of the Total Environment 2015; 508: 193-198.

Michalek JE, Tripathi RC. Pharmacokinetics of TCDD in veterans of Operation Ranch Hand: 15-year follow-up. J Toxicol Environ Health A 1999; 57: 369-78.

Miklavčič A, Casetta A, Snoj Tratnik J, Mazej D, Krsnik M, Mariuz M, Sofianou K, Špirić Z, Barbone F, Horvat M. Mercury, arsenic and selenium exposure levels in relation to fish consumption in the Mediterranean area. Environmental Research 2013; 120: 7-17.

Miklavčič A, Stibilj V, Heath E, Polak T, Tratnik JS, Klavž J, Mazej D, Horvat M. Mercury, selenium, PCBs and fatty acids in fresh and canned fish available on the Slovenian market. Food Chemistry 2011; 124: 711-720.

Milbrath MO, Wenger Y, Chang CW, Emond C, Garabrant D, Gillespie BW, Jolliet O. Apparent half-lives of dioxins, furans, and polychlorinated biphenyls as a function of age, body fat, smoking status, and breast-feeding. Environ Health Perspect 2009; 117: 417-25.

Milman N. Iron and pregnancy--a delicate balance. Ann Hematol 2006; 85: 559-65.

Minguillón MC, Campos AA, Cárdenas B, Blanco S, Molina LT, Querol X. Mass concentration, composition and sources of fine and coarse particulate matter in Tijuana, Mexico, during Cal-Mex campaign. Atmospheric Environment 2014; 88: 320-329.

Minguillon MC, Rivas I, Aguilera I, Alastuey A, Moreno T, Amato F, Sunyer J, Querol X. Within-city contrasts in PM composition and sources and their relationship with nitrogen oxides. J Environ Monit 2012; 14: 2718-28.

Minoia C, Sabbioni E, Apostoli P, Pietra R, Pozzoli L, Gallorini M, Nicolaou G, Alessio L, Capodaglio E. Trace element reference values in tissues from inhabitants of the European community. I. A study of 46 elements in urine, blood and serum of Italian subjects. Sci Total Environ 1990; 95: 89-105.

Montuori P, Jover E, Díez S, Ribas-Fitó N, Sunyer J, Triassi M, Bayona JM. Mercury speciation in the hair of pre-school children living near a chlor-alkali plant. Science of the total environment 2006; 369: 51-58.

Moon CS, Paik JM, Choi CS, Kim DH, Ikeda M. Lead and cadmium levels in daily foods, blood and urine in children and their mothers in Korea. Int Arch Occup Environ Health 2003; 76: 282-8.

Muñoz O, Devesa V, Suñer MA, Vélez D, Montoro R, Urieta I, Macho ML, Jalón M. Total and Inorganic Arsenic in Fresh and Processed Fish Products. Journal of Agricultural and Food Chemistry 2000; 48: 4369-4376.

Murata K, Weihe P, Renzoni A, Debes F, Vasconcelos R, Zino F, Araki S, JØrgensen PJ, White RF, Grandjean P. Delayed evoked potentials in children exposed to methylmercury from seafood. Neurotoxicology and teratology 1999; 21: 343-348.

Myers GJ, Thurston SW, Pearson AT, Davidson PW, Cox C, Shamlaye CF, Cernichiari E, Clarkson TW. Postnatal exposure to methyl mercury from fish consumption: a review and new data from the Seychelles Child Development Study. Neurotoxicology 2009; 30: 338-349.

Navas-Acien A, Francesconi KA, Silbergeld EK, Guallar E. Seafood intake and urine concentrations of total arsenic, dimethylarsinate and arsenobetaine in the US population. Environ Res 2011; 111: 110-8.

Nemery B, Casier P, Roosels D, Lahaye D, Demedts M. Survey of cobalt exposure and respiratory health in diamond polishers. Am Rev Respir Dis 1992; 145: 610-6.

Newman J, Gallo MV, Schell LM, DeCaprio AP, Denham M, Deane GD. Analysis of PCB congeners related to cognitive functioning in adolescents. Neurotoxicology 2009; 30: 686-96.

NHANES. Fourth National Report on Human Exposure to Environmental Chemicals, 2009.

Nichols GM, Bacon BR. Hereditary hemochromatosis: pathogenesis and clinical features of a common disease. Am J Gastroenterol 1989; 84: 851-62.

Nicklas T, Johnson R. Position of the American Dietetic Association: dietary guidance for healthy children ages 2 to 11 years. Journal of the American Dietetic Association 2004; 104: 660-677.

Nieboer E, Thomassen Y, Romanova N, Nikonov A, Odland JO, Chaschin V. Multi-component assessment of worker exposures in a copper refinery. Part 2. Biological exposure indices for copper, nickel and cobalt. J Environ Monit 2007; 9: 695-700.

Nohara K, Ao K, Miyamoto Y, Ito T, Suzuki T, Toyoshiba H, Tohyama C. Comparison of the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced CYP1A1 gene expression profile in lymphocytes from mice, rats, and humans: most potent induction in humans. Toxicology 2006; 225: 204-13.

Ohashi F, Fukui Y, Takada S, Moriguchi J, Ezaki T, Ikeda M. Reference values for cobalt, copper, manganese, and nickel in urine among women of the general population in Japan. Int Arch Occup Environ Health 2006; 80: 117-26.

Organization WH. Methylmercury, environmental health criteria. WHO, Geneva 1990; 101.

Ostro B, Tobias A, Querol X, Alastuey A, Amato F, Pey J, Perez N, Sunyer J. The effects of particulate matter sources on daily mortality: a case-crossover study of Barcelona, Spain. Environ Health Perspect 2011; 119: 1781-7.

Ozalla D, Herrero C, Ribas-Fitó N, To-Figueras J, Toll A, Sala M, Grimalt J, Basagaña X, Lecha M, Sunyer J. Evaluation of urinary porphyrin excretion in neonates born to mothers exposed to airborne hexachlorobenzene. Environmental Health Perspectives 2002; 110: 205-209.

Parajuli RP, Fujiwara T, Umezaki M, Watanabe C. Association of cord blood levels of lead, arsenic, and zinc with neurodevelopmental indicators in newborns: a birth cohort study in Chitwan Valley, Nepal. Environ Res 2013; 121: 45-51.

Paschal DC, Ting BG, Morrow JC, Pirkle JL, Jackson RJ, Sampson EJ, Miller DT, Caldwell KL. Trace metals in urine of United States residents: reference range concentrations. Environ Res 1998; 76: 53-9.

Pastorelli AA, Baldini M, Stacchini P, Baldini G, Morelli S, Sagratella E, Zaza S, Ciardullo S. Human exposure to lead, cadmium and mercury through fish and seafood product consumption in Italy: a pilot evaluation. Food Additives & Contaminants: Part A 2012; 29: 1913-1921.

Paustenbach DJ, Tvermoes BE, Unice KM, Finley BL, Kerger BD. A review of the health hazards posed by cobalt. Critical Reviews in Toxicology 2013a; 43: 316-362.

Paustenbach DJ, Tvermoes BE, Unice KM, Finley BL, Kerger BD. A review of the health hazards posed by cobalt. Crit Rev Toxicol 2013b; 43: 316-62.

Perez L, Medina-Ramón M, Künzli N, Alastuey A, Pey J, Pérez N, Garcia R, Tobias A, Querol X, Sunyer J. Size Fractionate Particulate Matter, Vehicle Traffic, and Case-Specific Daily Mortality in Barcelona, Spain. Environmental Science & Technology 2009; 43: 4707-4714.

Perrone MG, Larsen BR, Ferrero L, Sangiorgi G, De Gennaro G, Udisti R, Zangrando R, Gambaro A, Bolzacchini E. Sources of high PM2.5 concentrations in Milan, Northern Italy: Molecular marker data and CMB modelling. Science of the Total Environment 2012; 414: 343-355.

Pey J, Pérez N, Querol X, Alastuey A, Cusack M, Reche C. Intense winter atmospheric pollution episodes affecting the Western Mediterranean. Science of The Total Environment 2010a; 408: 1951-1959.

Pey J, Querol X, Alastuey A. Discriminating the regional and urban contributions in the North-Western Mediterranean: PM levels and composition. Atmospheric Environment 2010b; 44: 1587-1596.

Phillips DL, Pirkle JL, Burse VW, Bernert JT, Jr., Henderson LO, Needham LL. Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Arch Environ Contam Toxicol 1989; 18: 495-500.

PHIME. Public health impact of long-term, low-level mixed element exposure in susceptible population strata. Project no. FOOD-CT-2006-016253.Thematic priority: Priority 5,Food Quality and Safety, 2006.

Plaat F., L. A. Hypoxia in pregnancy. Fet Mat Med Rev 2012; 23: 71-96.

Pope CA, 3rd, Dockery DW. Health effects of fine particulate air pollution: lines that connect. J Air Waste Manag Assoc 2006; 56: 709-42.

Porta M, Gasull M, Puigdomènech E, Garí M, de Basea MB, Guillén M, López T, Bigas E, Pumarega J, Llebaria X. Distribution of blood concentrations of persistent organic

pollutants in a representative sample of the population of Catalonia. Environment international 2010; 36: 655-664.

Porta M, Lopez T, Gasull M, Rodriguez-Sanz M, Gari M, Pumarega J, Borrell C, Grimalt JO. Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Barcelona in 2006, and comparison with levels in 2002. Sci Total Environ 2012; 423: 151-61.

Prescott E, Netterstrom B, Faber J, Hegedus L, Suadicani P, Christensen JM. Effect of occupational exposure to cobalt blue dyes on the thyroid volume and function of female plate painters. Scand J Work Environ Health 1992; 18: 101-4.

Prokopec SD, Watson JD, Lee J, Pohjanvirta R, Boutros PC. Sex-related differences in murine hepatic transcriptional and proteomic responses to TCDD. Toxicol Appl Pharmacol 2015; 284: 188-96.

Rahman A, Vahter M, Smith AH, Nermell B, Yunus M, El Arifeen S, Persson LA, Ekstrom EC. Arsenic exposure during pregnancy and size at birth: a prospective cohort study in Bangladesh. Am J Epidemiol 2009; 169: 304-12.

Ramon R, Murcia M, Ballester F, Rebagliato M, Lacasana M, Vioque J, Llop S, Amurrio A, Aguinagalde X, Marco A, Leon G, Ibarluzea J, Ribas-Fito N. Prenatal exposure to mercury in a prospective mother-infant cohort study in a Mediterranean area, Valencia, Spain. Sci Total Environ 2008; 392: 69-78.

Renzoni A, Zino F, Franchi E. Mercury levels along the food chain and risk for exposed populations. Environmental Research 1998; 77: 68-72.

Ribas-Fito N, Cardo E, Sala M, Eulalia de Muga M, Mazon C, Verdu A, Kogevinas M, Grimalt JO, Sunyer J. Breastfeeding, exposure to organochlorine compounds, and neurodevelopment in infants. Pediatrics 2003; 111: e580-5.

Ribas-Fito N, Grimalt JO, Marco E, Sala M, Mazon C, Sunyer J. Breastfeeding and concentrations of HCB and p,p'-DDE at the age of 1 year. Environ Res 2005; 98: 8-13.

Ribas-Fitó N, Sala M, Cardo E, Mazón C, De Muga M, Verdú A, Marco E, Grimalt J, Sunyer J. Organochlorine compounds and concentrations of thyroid stimulating hormone in newborns. Occupational and environmental medicine 2003a; 60: 301-303.

Ribas-Fito N, Sala M, Cardo E, Mazon C, De Muga ME, Verdu A, Marco E, Grimalt JO, Sunyer J. Association of hexachlorobenzene and other organochlorine compounds with anthropometric measures at birth. Pediatr Res 2002; 52: 163-7.

Ribas-Fitó N, Sunyer J, Sala M, Grimalt JO. Cambios en las concentraciones de compuestos organoclorados en las mujeres de Flix, Tarragona. Gaceta Sanitaria 2003b; 17: 309-311.

Rodriguez E, Diaz C. Iron, copper and zinc levels in urine: relationship to various individual factors. J Trace Elem Med Biol 1995; 9: 200-9.

Rose M, Baxter M, Brereton N, Baskaran C. Dietary exposure to metals and other elements in the 2006 UK Total Diet Study and some trends over the last 30 years. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2010; 27: 1380-404.

Ryan JJ, Rawn DF. Polychlorinated dioxins, furans (PCDD/Fs), and polychlorinated biphenyls (PCBs) and their trends in Canadian human milk from 1992 to 2005. Chemosphere 2014; 102: 76-86.

Sala M, Ribas-Fito N, Cardo E, de Muga ME, Marco E, Mazon C, Verdu A, Grimalt JO, Sunyer J. Levels of hexachlorobenzene and other organochlorine compounds in cord blood: exposure across placenta. Chemosphere 2001; 43: 895-901.

Santos Filho E, Silva RdSe, Barretto HHC, Inomata ONK, Lemes VRR, Kussumi TA, Rocha SOB. Grau de exposição a praguicidas organoclorados em moradores de aterro a céu aberto. Revista de Saúde Pública 2003; 37: 515-522.

Saoudi A, Zeghnoun A, Bidondo ML, Garnier R, Cirimele V, Persoons R, Frery N. Urinary arsenic levels in the French adult population: the French National Nutrition and Health Study, 2006-2007. Sci Total Environ 2012; 433: 206-15.

Sarigiannis DA, Karakitsios SK, Kermenidou M, Nikolaki S. The effect of urban biomass combustion for space heating on PM exposure. AICHE 2013 Annual Meeting, San Fransisco, CA, 2013.

Scheepers PTJ, Heussen GAH, Peer PGM, Verbist K, Anzion R, Willems J. Characterisation of exposure to total and hexavalent chromium of welders using biological monitoring. Toxicology Letters 2008; 178: 185-190.

Schettgen T, Gube M, Alt A, Fromme H, Kraus T. Pilot study on the exposure of the German general population to non-dioxin-like and dioxin-like PCBs. Int J Hyg Environ Health 2011; 214: 319-25.

Schroijen C, Baeyens W, Schoeters G, Den Hond E, Koppen G, Bruckers L, Nelen V, Van De Mieroop E, Bilau M, Covaci A, Keune H, Loots I, Kleinjans J, Dhooge W, Van Larebeke N. Internal exposure to pollutants measured in blood and urine of Flemish adolescents in function of area of residence. Chemosphere 2008; 71: 1317-25.

Schuhmacher M, Domingo JL, Corbella J. Zinc and copper levels in serum and urine: relationship to biological, habitual and environmental factors. Sci Total Environ 1994; 148: 67-72.

Schulz C, Angerer J, Ewers U, Heudorf U, Wilhelm M. Revised and new reference values for environmental pollutants in urine or blood of children in Germany derived from the German environmental survey on children 2003-2006 (GerES IV). Int J Hyg Environ Health 2009; 212: 637-47.

Schulz C, Conrad A, Becker K, Kolossa-Gehring M, Seiwert M, Seifert B. Twenty years of the German Environmental Survey (GerES): human biomonitoring--temporal and spatial (West Germany/East Germany) differences in population exposure. Int J Hyg Environ Health 2007; 210: 271-97.

Seifert B, Becker K, Helm D, Krause C, Schulz C, Seiwert M. The German Environmental Survey 1990/1992 (GerES II): reference concentrations of selected environmental pollutants in blood, urine, hair, house dust, drinking water and indoor air. J Expo Anal Environ Epidemiol 2000; 10: 552-65.

Shirai S, Suzuki Y, Yoshinaga J, Mizumoto Y. Maternal exposure to low-level heavy metals during pregnancy and birth size. J Environ Sci Health A Tox Hazard Subst Environ Eng 2010; 45: 1468-74.

Simonsen LO, Harbak H, Bennekou P. Cobalt metabolism and toxicology--a brief update. Sci Total Environ 2012; 432: 210-5.

Sirot V, Guerin T, Volatier JL, Leblanc JC. Dietary exposure and biomarkers of arsenic in consumers of fish and shellfish from France. Sci Total Environ 2009; 407: 1875-85.

Sundar S, Chakravarty J. Antimony Toxicity. International Journal of Environmental Research and Public Health 2010; 7: 4267-4277.

Sunyer J, Alvarez-Pedrerol M, To-Figueras J, Ribas-Fito N, Grimalt JO, Herrero C. Urinary porphyrin excretion in children is associated with exposure to organochlorine compounds. Environ Health Perspect 2008; 116: 1407-10.

Swanson CA, King JC. Zinc and pregnancy outcome. Am J Clin Nutr 1987; 46: 763-71.

Swanson HI, Tullis K, Denison MS. Binding of transformed Ah receptor complex to a dioxin responsive transcriptional enhancer: evidence for two distinct heteromeric DNA-binding forms. Biochemistry 1993; 32: 12841-9.

Swennen B, Buchet JP, Stanescu D, Lison D, Lauwerys R. Epidemiological survey of workers exposed to cobalt oxides, cobalt salts, and cobalt metal. Br J Ind Med 1993; 50: 835-42.

Taiwo AM, Beddows DCS, Shi Z, Harrison RM. Mass and number size distributions of particulate matter components: Comparison of an industrial site and an urban background site. Science of The Total Environment 2014; 475: 29-38.

Thorpe A, Harrison RM. Sources and properties of non-exhaust particulate matter from road traffic: A review. Science of The Total Environment 2008; 400: 270-282.

Tokar EJ, Diwan BA, Waalkes MP. Arsenic exposure in utero and nonepidermal proliferative response in adulthood in Tg.AC mice. Int J Toxicol 2010; 29: 291-6.

Tollefson L. Use of epidemiology data to assess the cancer risk of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Regul Toxicol Pharmacol 1991; 13: 150-69.

Trdin A. Connection between mutations in the gene for apolipoprotein E with concentrations of mercury in the mothers and newborns. Master's thesis. 2015.

Unice KM, Kerger BD, Paustenbach DJ, Finley BL, Tvermoes BE. Refined biokinetic model for humans exposed to cobalt dietary supplements and other sources of systemic cobalt exposure. Chemico-Biological Interactions 2014; 216: 53-74.

Unice KM, Monnot AD, Gaffney SH, Tvermoes BE, Thuett KA, Paustenbach DJ, Finley BL. Inorganic cobalt supplementation: prediction of cobalt levels in whole blood and urine using a biokinetic model. Food Chem Toxicol 2012; 50: 2456-61.

US Environmental Protection Agency. Mercury. Human exposure. 2007.

USEPA I. Hexachlorobutadiene; CASRN 87-68-3, 1987.

USEPA I. Tetrachloroethylene, 2012.

Vahter M. Health effects of early life exposure to arsenic. Basic Clin Pharmacol Toxicol 2008; 102: 204-11.

Vahter M. Effects of arsenic on maternal and fetal health. Annu Rev Nutr 2009; 29: 381-99.

Vahter ME, Li L, Nermell B, Rahman A, El Arifeen S, Rahman M, Persson LA, Ekstrom EC. Arsenic exposure in pregnancy: a population-based study in Matlab, Bangladesh. J Health Popul Nutr 2006; 24: 236-45.

Vahter ME LL, Nermell B, Rahman A, El Arifeen S, Rahman M, Persson LÅ, Ekström EC Effects of arsenic on maternal and fetal health Journal of Health, Population and Nutrition 2006a; 24.

Van den Berg M, Birnbaum L, Bosveld AT, Brunström B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 1998; 106: 775-792.

Vasilatos C., Megremi I., Economou-Eliopoulos M., Mitsis I. Hexavalent chromium and other toxic elements in natural waters in the Thiva – Tanagra – Malakasa Basin, Greece, 2008.

Viau C, Bouchard M, Carrier G, Brunet R, Krishnan K. The toxicokinetics of pyrene and its metabolites in rats. Toxicol Lett 1999; 108: 201-7.

Vizcaino E, Grimalt JO, Fernández-Somoano A, Tardon A. Transport of persistent organic pollutants across the human placenta. Environment international 2014; 65: 107-115.

Vrijheid M, Martinez D, Aguilera I, Ballester F, Basterrechea M, Esplugues A, Guxens M, Larrañaga M, Lertxundi A, Mendez M. Socioeconomic status and exposure to multiple environmental pollutants during pregnancy: evidence for environmental inequity? Journal of epidemiology and community health 2010: jech. 2010.117408.

Vrijheid M, Martinez D, Aguilera I, Ballester F, Basterrechea M, Esplugues A, Guxens M, Larranaga M, Lertxundi A, Mendez M, Murcia M, Marina LS, Villanueva CM, Sunyer J. Socioeconomic status and exposure to multiple environmental pollutants during pregnancy: evidence for environmental inequity? J Epidemiol Community Health 2012; 66: 106-13.

Wåhlin P, Berkowicz R, Palmgren F. Characterisation of traffic-generated particulate matter in Copenhagen. Atmospheric Environment 2006; 40: 2151-2159.

Wapnir RA. Copper absorption and bioavailability. Am J Clin Nutr 1998; 67: 1054s-1060s.

Wells EM, Jarrett JM, Lin YH, Caldwell KL, Hibbeln JR, Apelberg BJ, Herbstman J, Halden RU, Witter FR, Goldman LR. Body burdens of mercury, lead, selenium and copper among Baltimore newborns. Environmental research 2011; 111: 411-417.

WHO-ICPS WHO-IPoCS. Methylmercury. Environmental health criteria 101. Geneva: World Health Organization 1990: 1-144.

WHO. CARBON TETRACHLORIDE. Environmental Health Criteria 208, Geneva, 2000.

WHO. Human biomonitoring: facts and figures. WHO Regional Office for Europe, Copenhagen, 2015.

Williams PN, Villada A, Deacon C, Raab A, Figuerola J, Green AJ, Feldmann J, Meharg AA. Greatly Enhanced Arsenic Shoot Assimilation in Rice Leads to Elevated Grain Levels Compared to Wheat and Barley. Environmental Science & Technology 2007; 41: 6854-6859.

Wiseman CLS, Zereini F. Characterizing metal(loid) solubility in airborne PM10, PM2.5 and PM1 in Frankfurt, Germany using simulated lung fluids. Atmospheric Environment 2014; 89: 282-289.

Wright RO, Baccarelli A. Metals and neurotoxicology. J Nutr 2007; 137: 2809-13.

Yokota K, Johyama Y, Kunitani Y, Michitsuji H, Yamada S. Urinary elimination of nickel and cobalt in relation to airborne nickel and cobalt exposures in a battery plant. Int Arch Occup Environ Health 2007; 80: 527-31.

Zhang JD, Li XL. [Chromium pollution of soil and water in Jinzhou]. Zhonghua Yu Fang Yi Xue Za Zhi 1987; 21: 262-4.

Zhang T, Claeys M, Cachier H, Dong S, Wang W, Maenhaut W, Liu X. Identification and estimation of the biomass burning contribution to Beijing aerosol using levoglucosan as a molecular marker. Atmospheric Environment 2008; 42: 7013-7021.

Zubero MB, Aurrekoetxea JJ, Ibarluzea JM, Arenaza MJ, Rodriguez C, Saenz JR. Heavy metal levels (Pb, Cd, Cr and Hg) in the adult general population near an urban solid waste incinerator. Sci Total Environ 2010; 408: 4468-74.