DIPARTIMENTO DI SCIENZE ECOLOGICHE E BIOLOGICHE - DEB

DOTTORATO DI RICERCA IN

ECOLOGIA E GESTIONE SOSTENIBILE DELLE RISORSE AMBIENTALI - XXX CICLO

CURRICULUM: AMBIENTE, ADATTAMENTO E SALUTE

MONITORING AND BIOMONITORING TOOLS FOR HUMAN HEALTH RISK ASSESSMENT AND PUBLIC HEALTH POLICIES

(s.s.d. MED/42)

Tesi di dottorato di:

Dott.ssa Arianna Antonucci

Coordinatore del corso

Prof.ssa Roberta Cimmaruta

Tutor

Prof. Matteo Vitali

ANNO ACCADEMICO 2016 – 2017

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CHAPTER 1

INTRODUCTION TO THE RESEARCH: ENVIRONMENTAL POLLUTION AS ECOLOGICAL – HUMAN HEALTH RISK FACTOR AND ASSESSMENT OF THE AVAILABLE SURVEY TOOLS

Contaminants in the environment: a risk for human and environmental health

Harmful chemicals emitted in environmental matrices have been considerably increasing in the last 60 years both in developed and developing countries (Argent et al., 2004), leading to a severe increase of the number and typology of chemicals to which populations are exposed. This exposure mainly occurs through air, water, food and other consumer products. It is continuous over time, generally at low doses, and typically involves a mixture of contaminants (Carpenter et al., 2002; Kordas et al., 2007).

As a direct consequence, human adverse health effects due to the exposure to environmental contaminants are likewise increasing all over the world (Myers and Patz, 2009; Myers et al., 2013). Furthermore, the interactions between different chemical components in a mixture often result in a stronger (synergistic, potentiated) combined effect than the additive effect that would be expected from the action of each individual compound (Altenburger et al., 2013; Cedergreen et al., 2014; Rodea-Palomares et al., 2015; Lentz et al., 2015).

Although programs of risk assessment have been performing since many years, to date they are still relevant because of the many new challenges arising. Besides, additional detailed studies and frequent upgrades are needed to characterize the nature and magnitude of environmental and human health risk derived from the exposure to chemical contaminants. In order to meet this need, over the years comprehensive risk assessment guidance were draft by the Environmental Protection Agency of United States, which released its first risk guidelines in 1986 (USEPA, 1986) and several supplements in following years (USEPA, 1990a-2000-2003). However, the development and improvement of risk assessment procedures is a relevant issue for many authorities world-wide, and many activities are on-going (IPCS, 2009; Commission of the European Union, 2009-2012).

As regard to pollutant mixtures, in the last few years and in a in rapid succession, the International Agency for Research on Cancer (IARC) classified as carcinogenic to humans (group 1) some smog components (benzo[a]pyrene, IARC 2012; diesel emissions, IARC 2014), the whole outdoor air pollution (the mix of traffic combustions, heating and industrial emissions) and the particulate matter (PM) in outdoor air pollution (IARC monographs, 2016), based on "sufficient evidence of carcinogenicity in humans and experimental animals and strong support by mechanistic studies". More recently, IARC has classified also the welding fumes as carcinogen to human (forthcoming). Another well-known example of complex mixture with many different toxic components, likely to act through multiple pathways in causing disease and classified as known human carcinogen (group 1) is tobacco smoke (tobacco smoking, tobacco smokeless and

tobacco smoke secondhand, IARC 2012). Furthermore, tobacco smoke is one of the few examples of well-documented synergistic actions of environmental agents in humans (Valavanidis et al., 2009). There are strong evidences that smoking have synergistic effects when acting together with other environmental factors, enhancing the role of smoking as a causal factor of several diseases. For example, the combined effects of smoking and radon may contribute to cause lung cancer (National Research Council, 1998).

All these examples underline the importance of clean environment for public health, and relevant scientific and political consequences. It is now widely accepted that preventive measures for human health should include environmental protection (*health-environmental risk*) (WHO, 2006; Pattanayak et al., 2017). In this context, the assessment and management of environmental matrices quality has proven to be one of the most relevant strategies for preventing the risks for human diseases derived from exposure to environmental contaminants. Thus, the proper assessment of the risks must be conducted with a global approach, evaluating both the threats for human health and the risks for the environment and an integrated risks management, controlling and protecting both human and environmental heath (USEPA, 1990b). Indeed, since many years, the European Community has moved in this direction; for example, the air quality Directives (Council Directive 99/30/EEC - 2000/69/EC - 2002/3/EC) were subsequently implemented, and reviewed by the Clean Air for Europe Program (CAFE Program), released in 2001 to identify the key air quality objectives and the measures to be taken to reach these goals. Its main strategies consisted of integrated policy recommendations to protect against "significant negative effects of air pollution on human health and the environment". Since 2002, a fourth Directive (Directive 2004/107/EC) on heavy metals in ambient air and the new consolidated framework Directive (Directive 2008/50/EC) on ambient air quality and cleaner air for Europe were adopted. Italy has implemented the Directives by the LD 152/2006 (Legislative Decree No. 152) "Norms Concerning the Environment" that groups in a single legislative act the environmental laws previously contained in several decrees.

Environmental and human health risk assessment process: the need for an integrated approach

The evaluation of human health risks related to exposures to environmental contaminants is a key process increasingly important both for assessing the pollutants impact on the exposed populations and for allowing the evaluation of the sanitary measures required to protect human health. In general, the process for the assessment of human health risks associated to the presence of contaminants in the environment proceeds according to four classic activities (Figure 1) (IPCS/WHO, 2010).

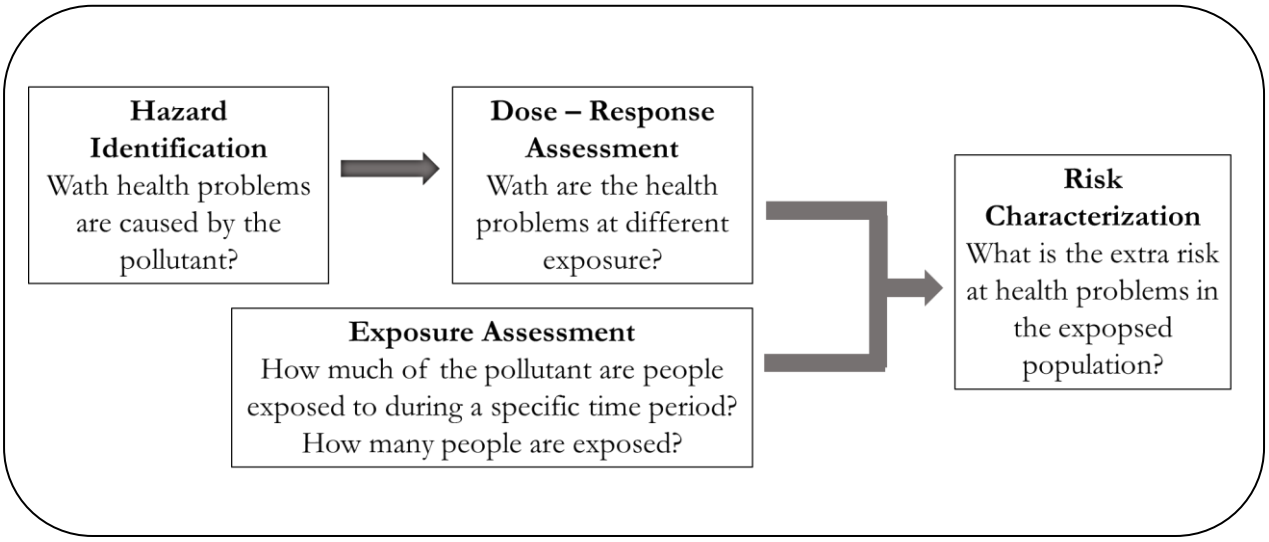

Figure 1. The four steps of risk assessment process.

The first phase qualitatively describes the effects that may occur in humans, providing the preliminary identification of contaminants that may pose health hazards, the recognition of potentially hazardous substances and the examination of their diffusion modes across the different environmental matrices (*hazard identification*). In the second phase (*hazard characterization*), a quantitative relationship between dose and effect in humans is established by the evaluation of the damage resulting from the exposure to different doses of a pollutant (dose-response analysis). The third phase (*exposure assessment*) draws the characterization of population exposure identifying and defining the exposures that occur, or are anticipated to occur, in human populations. The last phase describes the estimated risk to human health from the anticipated environmental exposure (*risk characterization*). In this final step, a prediction of probabilistic effects on the population in terms of adverse health events is generally obtained by calculating the ratio between the acute and chronic dose and the expected chemicals concentrations in the different environmental compartments. According to this explanation, the above diagram (figure 1) summarizes the sequential steps making up the risk assessment process.

Exposure assessment as a key step of the risk assessment process: methodologies and tools

The process of Environmental and Human Health Risk Assessment requires, in most cases, the carrying out of programs of exposure assessment. These evaluations are strongly recommended (WHO, 2005a) because they increase the knowledge to what extent, how, and when individuals and communities are exposed, and are essential for supporting the prevention measures to be adopted. Exposure estimation, needed to investigate the link between diseases and presence of

toxic agents in the environment, represents a critical step in the risk analysis and involves complex activities that commonly use several methodological approaches. In order of increasing accuracy, they include: the collection of information through questionnaires and activity diaries; the measurement of pollutant levels and their distribution (environmental monitoring, EM) both in environmental matrices (air, water, soil, food, sediments, etc) and in the breathable zone of the studied subjects (personal sampling, e.g. by means of portable air sampling devices); the collection of biological samples (biological monitoring, BM) for analytical determinations of the pollutant levels, their metabolites or specific biological effect parameters (Angerer et al., 2007). The first two approaches just allow an estimate of the exposure by theoretical assumptions and modeling techniques; BM instead, permits the identification of the actual and systemic exposure to pollutants because it evaluates the amount of the xenobiotic actually absorbed into the body and reflects the individual differences in absorption, metabolism and excretion.

Exposure assessment tools: a focus on environmental monitoring

In recent years, the increased levels of pollutants detected in all environmental matrices have determined the need of initiatives and legislative actions for controlling and preventing environmental pollution. Many measures (i.e., Italian LDs: 31/2001 concerning the quality of water intended for human consumptions; 152/2006 on environmental regulations; 155/2010 on ambient air quality and cleaner air for Europe) have been adopted in parallel with the increasing number of scientific evidences and of social concern in this field, as the legislation represents the main instrument for environmental protection. On the other hand, environmental monitoring is an essential activity for estimating the exposure both at individual and population levels, and for assessing human and environmental risks related to pollution. Anyway, the exposure results from complicated relationships and interactions between environmental and human systems, adding complexity to the assessment process. Traditionally, the approaches to quantify human exposure have relied on pollutant concentrations from fixed air quality network sites, which provide a large quantity of data for a wide range of pollutants, although for one point in space. New developments in sensor technology (e.g. portable and wearable monitors) now enable us to monitor personal exposure to air pollutants directly while people are moving through their activity spaces and varying concentration fields.

The need for economical and real time monitoring of environmental pollutants has encouraged the technological progress in monitoring systems design and the researches aimed to develop new more robust and suitable methodologies for new monitoring tools and their analytical determination. The main challenges of the employed systems should be the ability to monitor the increasing number of analytes of environmental relevance as quickly and as cheaply as possible

and the possibility of allowing on-site field monitoring. In this respect, plants and lichens have demonstrated great potentiality in recent years and, thus, they arise as proposed analytical tools for effective monitoring in environmental pollution control programs.

Exposure assessment tools: a focus on human biological monitoring

As explained by the International Program of Chemical Safety (IPCS, 1993), the biological monitoring indicators, referred as biomarkers, may be used to assess both the exposure and effects of chemicals and the susceptibility of individuals. Specifically, a biomarker of *exposure* is the concentration of pollutant absorbed or its active metabolite/s, in biological specimens (marker of internal dose), or accumulated in an organ, cell or molecule (marker of effective dose). This type of biomarker is used to evaluate the occurrence and extent of exposure of individuals or populations or specific target structures to a particular substance, providing an efficient and cost effective means of relating internal dose with external exposures. Its most useful requirement is the ability for assessing individual exposure to chemicals by all different routes (inhalation, dermal contact and ingestion). In human biomonitoring (HBM) of dose, blood and urine are by far the most approved and used matrices, even if hair and faeces are also used. In particular, urine samples are readily obtained, easy to collect in large volumes, available with non-invasive procedure and functional for being used in extended monitoring campaigns. Biochemical alterations or physiological disorders, which document adverse health effects caused by external exposure and adsorption of a chemical, are referred as biomarkers of *effect*. They provide a linkage between exposure and effects, contributing to the definition of dose-response relationships. Biomarkers of effects include early as well as clinical effects (e.g., 8-hydroxyguanine and 8-hydroxy-2'-deoxyguanosine are monitored as urinary markers of oxidative stress induced by Cr, As, Ni, and Cd - Yoshioka et al., 2008; 8-oxo-7,8-dihydro-2'-deoxyguanosine(8-oxodGuo), 8-oxo-7,8-dihydroguanosine(8-oxoGuo), and 8-oxo-7,8-dihydroguanine (8-oxoGua) are used as urinary biomarkers of DNA oxidation associated with children exposure to benzene - Andreoli et al., 2012). Finally, the biomarkers of *susceptibility* are biochemical characteristics expressing the individual sensitivity to an external challenge; these biological markers help to explain the variability degree of the inter-individual response upon exposure to similar pollutant levels. Biomarkers of susceptibility indicate individuals with increased sensitivity of target molecules or metabolism causing increased target dose (Nordberg, 2010). In routinely applications, BM is complementary to EM and is used also in occupational health to verify the compliance with safety standards (Mutti, 1999; Senzolo et al., 2001; Manno et al., 2010). Over the last decades, in the United States and European countries, BM has been consolidating as an important tool of occupational hygiene and medical health surveillance. Biological Exposure Indices (BEI) have

been defined for many substances and more recently biological equivalent exposure limits for unmodified volatile organic compounds (VOCs) in urine have been starting to be proposed for few substances (Ghittori et al., 2002). When compared to EM, biological approach is closer to the target organ dose and provides additional information to guarantee greater precision in risk estimates and in dose-response relationships. This further information include the assessment of the integrated total uptake of the chemical by different routes of absorption, an estimate of current, recent or past exposure, the degree of metabolism, the early biological effects and the ability of an organism to tolerate and respond to chemical aggression.

Exposure assessment: a focus on air pollution exposure and effects

In recent decades, although the progressive enhancements of air quality in numerous countries, air pollutants levels are still at relatively high levels, so that environmental air quality problems have been and continue to be one of the most common health risk factors for the population (Curtis et al., 2005; Cohen et al., 2005). Air pollution (group 1, IARC 2016) is a mixture of solid particles and gases in the air. The driving forces of air pollution produce more than one class of pollutants and include natural sources as well as human activities, such as energy consumption, urbanization, economic development, transportation, motorization and increase of urban population (Chen and Kan, 2008). Chemicals detected in air samples assemble a wide array of compounds, which differ in their chemical-physical properties. Among these, the most common and harmful air pollutants include inorganic elements [e.g., arsenic (As) and heavy metals such as cadmium (Cd), nickel (Ni) copper (Cu), mercury (Hg), and lead (Pb)], fine and ultrafine particulate matter (PM), carbon monoxide, ozone, nitrogen and sulphur oxides, benzene and other constituents of fossil fuels, polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs and PCDFs), and polychlorinated biphenyls (PCBs). The great abundance of different air contaminants, other than the adverse health effects observed in a wide range of air pollution levels and the large number of people at risk, make the exposure to air pollutants a problem of great concern. Many studies, aimed to evaluate the linkage between exposure to air pollutants and adverse health effects, showed that air contaminants include substances of toxicological interest, able to affect mainly the respiratory and cardiovascular systems, but also immunological, neurological and reproductive systems, of large sections of the population with different impacts on human health (USEPA, 2017). Besides, some of them are considered probable or possible carcinogenic, able to induce hematological problems and cancer (Folinsbee, 1992; Kampa et al., 2008; Rai, 2015; Ali and Haruna, 2015). The health risk factor associated with air pollution, both outdoor and indoor, although being a well-known issue, studied for many years, is still the main subject of several researches due to some

critical points not yet resolved, which make its estimation very difficult. First, human exposure to chemicals in the environment typically involves chemical mixtures (multi-chemical exposure) (Silins, 2011; Johns et al., 2012). For some combined exposures, the adverse health effects may increase more than what would be expected from simply adding the effects of each single component (Ikeda, 1995; Viau, 2002; Billionnet et al. 2012) and they can often be observed even when the pollution level is below the level indicated by air quality guidelines. Second, there has been an increased acknowledgment of the need of taking into account all routes of uptake such as inhalation, ingestion and dermal contact (multi-route exposure). Third, concentrations of air pollutants may undergo significant temporal and spatial changes. The levels of many pollutants, for example, show daily and seasonal differences (Fuselli et al. 2002; Paciencia et al., 2015), but also vary significantly according to the considered environments; for instance, pollutants arising from motor vehicle traffic, such as particulate air pollutants, can be higher in indoor rather that in outdoor air (WHO, 2010; Chen and Zhao, 2011). This evidence is even more significant considering that, globally, people spend the great part of their time in indoor environments (Jenkins et al., 1992; Hellweg et al., 2009).

Exposure assessment: a focus on children, a high-risk group of population

One of the main problems associated with the possibility of drawing exposure profiles for environmental pollutants is the influence of various factors, such as age, sex, lifestyles, occupation, socio-economic factors on the estimation of exposure. Moreover, individuals differ widely in genetic predisposition and physiological response to pollutants. These variables make it impossible to generalize the results of environmental pollution studies. The subgroups of people that have been recognized as particularly vulnerable to the effects of air pollutants include patients with diseases, such as cardiovascular and pulmonary diseases, elderly person, pregnant women and children (Salvi, 2007). Also workers in some industries could be at a higher risk due to their increased biological sensitivities and different exposure patterns. Among these susceptible subjects, children may have greater exposure than adults to air pollutants due their own characteristics. Children may react especially sensitively to influences from environment and have greater difficulty than adults in avoiding some types of pollution. In particular, they have higher respiratory, higher metabolic rate and absorption rates than adults, which would increase their exposure to atmospheric contaminants. They are more usual to mouth breathing than adults, bypassing the filtering effect of the nose and then inhaling higher levels of pollutants than adults. A particular health risk also results from the intake of high quantities of pollutants as related to the child's body weight because the ratio of height to body surface is clearly different from that of adults. With respect to their weight, they ingest greater amounts of water and foods

than adults. besides, their immune systems and developing organs are still immature (American Academy of Pediatrics, 2004). But also the typical children's behavior (hand-to-mouth contact, crawling, playing on the floor, digging in sand and uptake of dirt during outdoor games) plays an important role in making children a group with a high risk for health damage caused by environmental exposure. Moreover, it has been clearly demonstrated that socio-economic factors such as social environment, sports activities, parents smoking and diet habits may have an influence on children's health and then they must be taken into account when studying children's exposure. Specifically, they are exposed to an high degree of risks that arise from the use of consumer products or from building materials and furnishings, other than tobacco smoke, since children spend a great deal of time indoors (up to 90% of their time). On the other hand, household environment is the main source of exposure to passive smoking for children. Passive smoking, containing over 5,000 compounds, including many substances that are toxics for human health or carcinogens (e.g. benzene; IARC, 1982), is a significant risk factor for health, especially for children (US PHS 2006). As an example, benzene concentration present in tobacco smoke is sufficient to account for half of the estimated cases of acute myeloid leukaemia (IARC, 2004). Much research has been conducted to assess the links between youth benzene exposure, passive smoking exposure and the risk of lymphohematopoietic cancer in childhood; however this research has yielded contradictory findings (Chang, 2009). On the other hand, considering the prolonged latency of the disease and the early initiation of exposure, the possibility of cancers in adulthood after environmental tobacco smoke (ETS) exposure during childhood cannot be excluded. The World Health Organization Task Force for the environmental health of the children said that "children are not little adults" but they should be considered "unique", highlighting the importance of paying more attention to hazards of air pollution for children's health. Therefore, specific studied are needed to estimate the risk of adverse effect on children health resulting from air pollutants exposure (Anderson et al. 2000; Chance, 2001; WHO, 2005b). Despite of the great number of publications concerning air pollution and relative adverse outcomes on human health, both for occupational settings and for adults not professionally exposed, dedicated studies on pediatric exposure to air contaminants and relative health impacts are still scarce compared to the needs for scientific evidence and dedicated researches to explore this issue are required. In particular, techniques and methods need to be further developed to fill data gaps and increase the knowledge on harmful exposure estimation. With the intention of filling this gap, the WHO has recently reserved a document to the children's health ("call for action" - Global Plan of Action for Children's Health and the Environment 2010 - 2015), whose overall aim is to create a safe environment for children to grow in good health and to contribute to their global, both social and economic, development. To achieve this goal, the WHO has

identified five target study areas. The first field of work, defined "data collection and analysis", consists of a strategy that includes in its action plan the promotion of periodic biomonitoring programs in children's body fluids (WHO, 2009).

Main objectives of my research program

The general aim of my research program was to develop new useful tools to significantly improve Health and Environmental Risk Assessment (HERA) studies. Specific aims were to improve the methodologies needed by the environmental epidemiology; in particular, my project works were focused on the development, validation and application "in field" of new, easiness and low-cost analytical tools useful to:

- perform health risk assessment studies specifically dedicated to children and their exposure to chemicals (single contaminants and/or mixtures);
- carry out environmental monitoring researches by the use of transplanted and native lichens, well-known biomonitors and bioaccumulators of mixtures, but still under evaluation.

Summary of my research program

During my PhD studies I took part to some study projects, synthetized in the following paragraphs. The results of some steps of the projects were already elaborated and published, while the others are still in progress (subsequent chapters).

1. Human biomonitoring study among Italian schoolchildren

I participated to two human biomonitoring studies (respectively conducted in 2014 and 2017) performed among large samples of children living in central Italy. In the following two paragraph, the methodologies and some results were briefly described.

1.1 Urinary biomonitoring study: academic year 2014-2015

The aims of the study presented here derived from some critical points emerged from previous researches conducted by the same research group (Protano et al., 2010-2012a 2012b-2012c-2014; Andreoli et al. 2015). These points became the research agenda and encourage to continue the biomonitoring study among Italian schoolchildren:

- urinary unmodified benzene was demonstrated a good tracer for atmospheric pollution during pediatric age, but it is strongly influenced by ETS exposure. Are there other substances useful to trace exposure profile among children?

- Evaluation of exposure and "very early" effects deriving from exposure to environmental contaminants is the recommended approach in order to simultaneously trace exposure profile, references values and possible threats for children's health. Oxidative stress is one of the "very early" effect and our previous work demonstrated that extracellular oxidized guanine derivatives in urine samples are useful for this purpose. Are there other substances useful to trace "very early" effect profile among children?
- One of the main problems in human biomonitoring study is the time of sampling collection, due to the possibility of changes in target substances or their metabolites in biological fluids during the course of the day.

On the basis of the emerged research questions, at the beginning of 2014 the group started a new project on children exposure to environmental contaminants. In particular, we planned and realized a new biomonitoring study on schoolchildren, aged between 5 and 11 years, attending at primary schools located in different sites of central Italy. The cross-sectional study was carried out according to the steps described below.

Ethical issues: all the activities of the present study presented some ethical concerns; thus, before starting the study, an official proposal was submitted and approved by the local Ethical Committee (Policlinico Umberto I – "La Sapienza" University of Rome; protocol code 2894).

Literature review on biomonitoring research and elaboration of some analytical data resulted from previous monitoring studies (academic years 2007-2009): before the other activities of the projects, an update of the literature was performed in order to collect new information about the epidemiological methodologies and the analytical strategies. The update evidenced the need for further studies both in term of epidemiological evidences and in term of analytical tools useful for conducting biomonitoring studies. Besides, some of data resulted from analytical determination on urinary samples of previous monitoring study were re-elaborated in order to study in depth the simultaneous co-exposure to some carcinogens during paediatric age (already published and presented in the **chapter 2**).

Study area selection: according to the urbanization degree, we selected two areas with different environmental characteristics, classified as urban and rural sites. Site selection was based on urbanization characteristics (i.e. population density, green area density and motorization rate) obtained from national databases (i.e. National Institute of Statistics, Italian Automobile Club). In each investigated area, some district primary schools were

recruited; in particular, a school in the center of Rome (urban site) and some schools located in rural areas of Rieti province (rural sites), central of Italy, for a total of 619 children.

Development and optimization of the analytical method for VOCs: a multiresidue method was developed and optimized, as described in the second article. The method was used for quantifying the ten selected VOCs (benzene, toluene, ethylbenzene, o,m,p-xylenes, methyl *tert*-butyl ether, ethyl *tert*-butyl ether, diisopropyl ether and *tert*-amyl methyl ether) in urinary samples collected from children exposed to different levels of the monitored compounds according to living environments and ETS exposure (already published and presented in the **chapter 3**).

Development and optimization of the analytical method for other substances: other analytical methods were developed and validated for the determination, in the same urine samples, of a set of trace inorganic elements, cotinine (gold standard biomarkers of ETS exposure), S-phenilmercapturic acid and trans,trans-muconic acid (two metabolites of benzene), creatinine (for the normalization of urinary concentrations) and a set of extracellular oxidized and methylated guanine derivatives (very early effect biomarkers). The method for trace inorganic elements was developed by the use of inductively coupled plasma mass spectrometry technique in collaboration with a research group of the Chemistry Department of "La Sapienza" University; the methods for the other monitored chemicals were developed by the use of liquid chromatography–tandem mass spectrometry technique in collaboration with the research group of the Industrial Toxicology of Parma University.

Elaboration of a questionnaire "ad hoc": a questionnaire was designed specifically to investigate the possible factors associated with VOCs exposure in childhood. Questions were elaborated in order to collect information on the socio-demographic characteristics of the children and their families, the activities of the participant during the sampling day, household characteristics, and information about cohabitants' smoking habits and precautions taken by at-home smokers.

Monitoring campaign: all of the students attending each school and their parents received information about the goals and plans of the research and were invited to take part in the cross-sectional study. Then, formation meetings for all children and their parents on the modalities to compile the questionnaire and to collect and store urine sample were performed on just before sampling day. Informed consent and personal data processing authorization was signed by both parents of each participant and by older children (9- to

11-y old). All data obtained from questionnaires and biological samples were treated in an aggregate way only for scientific purposes.

For each participant two urine samples were collected: the last at the end of the day (just before going to sleep) and the first at the morning of day after. The choice of the double collection respect to the single sample was done for investigating possible differences in urinary excretion of the monitored substances. Besides, since there are lack of reference ranges of urinary concentrations of contaminants for pediatric ages, it is essential to have data for different moment of the day. Urine were collected in a benzene-free polypropylene bottle with hermetic closure, and immediately stored in the refrigerator at 4°C. In the morning, the samples were placed into a polystyrene cooler containing an ice pack and delivered together with the compiled questionnaire and the signed informed consent and personal data processing authorization to the research team.

In total, 435 children took part to our research, 247 children attended a school in the center of Rome (urban site), 188 children attended some schools located in rural areas of Rieti province, central of Italy (Poggio Nativo, Casali di Poggio Nativo, Toffia, Frasso Sabino; rural sites).

Collection of the information from questionnaires: information from questionnaires were coded and entered into a database specifically created for the research. Based on the two most important factors emerging from information collected in the questionnaires (urbanization of residence area and exposure to ETS), children were classified in four groups, defined as follows: children residing in areas of high urbanization and not exposed to passive smoking by cohabitants (group 1); children residing in areas of high urbanization and exposed to passive smoking by cohabitants (group 2); children residing in rural areas with low urbanization and not exposed to passive smoking by cohabitants (group 3); children residing in rural areas with low urbanization rates and exposed to passive smoking by cohabitant (group 4). All children were considered to be exposed to ETS if they lived in households where at least one person was a smoker (notice that the responses of the questionnaires about the smoking habits of cohabitants smokers were validated using urinary cotinine concentrations).

Analytical determination of VOCs: Timetable, methods and analytical procedures were the same as described in the second article. Briefly, a 20-mL glass vial, previously added with 3 g of NaCl, was filled with an aliquot of 14-mL of each spot urine sample, promptly closed with a rubber lid with a polyperfluoroethylene lining, and crimped with an aluminum seal for VOCs determination. All samples were coded, frozen at -20°C until analysis and then

analyzed. A total of 876 urine samples (two samples – evening and next morning – for each child) were analyzed by means of headspace solid phase micro extraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS).

Statistical elaborations: Statistical analyses were carried out using SPSS software (version 14.0 for Windows, Chicago, Illinois, USA; version 20 IBM Corp., Armonk, NY, USA). An accurate report on the results of this monitoring campaign have not yet been published as we found a special high concentration levels for one target analyte, among those investigated, in children living in specific sites. The possible explanations of the unusual exposure for these subjects are currently under further investigations. For this purpose, aliquots of the urine samples were send to the laboratory of the SIPOMICS-Metabolomics Platform, Universitat Rovita i Virgili, Tarragona, Spain for some analytical determination by the use of the metabolomics approach.

1.2 Children biomonitoring study: academic year 2017-2018

The findings of the previous human monitoring study was presented to various academic and non-academic policy makers, arousing the interest of the Sabina Universitas and ALCLI "Giorgio e Silvia" ONLUS No Profit Association, which is particularly interested to the health programs dedicated to the population living in Rieti province. The research funding allowed us to perform a new biomonitoring study on schoolchildren living in the Rieti province. In particular, children were recruited in some schools district located in different scenarios (rural, industrial, urban setting of the same provinces). The study was conducted in June of 2017 and performed with the same methodology of the previous studies (2007-2009 and 2014). One of the main novelty of the present study was the assessment of the potential changes in urine excretion of the contaminants related to the season of the years. Indeed, previous studies (according to other ones in the same field) were performed in winter season. The present study was planned in order to perform two monitoring campaign: summer (performed in June 2017) and winter campaign (planned for 2018). In total, 378 children, aged between 5 and 11 years, were included in the study. Samples coded and stored in our laboratory are currently under analyses.

2. Environmental monitoring studies by using transplanted or native lichens

I participated to two environmental monitoring studies performed with the aim to assess the air quality of some different outdoor and indoor scenarios. The first study was performed with the aim of evaluating the ability of transplanted lichens to biomonitor and

bioaccumulate a great number of airborne contaminants in indoor settings (already published and presented in the **chapter 4**).

The second one was performed with the aim of assessing the ability of native lichens as biomonitors tools for several chemicals in some different selected areas of central Italy (manuscript draft presented in the **chapter 5**).

CHAPTER 2

EXPOSURE TO INDIVIDUAL AND MULTIPLE CARCINOGENIC METALS DURING PAEDIATRIC AGE: AN EXPERIENCE FROM AN ITALIAN URBAN SCENARIO

Protano C, Astolfi ML, Canepari S, Andreoli R, Mutti A, Valeriani F, Romano Spica V, ***Antonucci A***, Mattei V, Martellucci S, Vitali M. *Exposure to individual and multiple carcinogenic metals during paediatric age: an experience from an Italian urban scenario*. Ann Ig. 29 (2017); 494-503. DOI: 10.7416/ai.2017.2180.

Framework of the present study of my research program

Human exposure to metals present in the environment

Metals constitute significant potential threats to human health in both occupational and the general environments, as it is increasingly evident their correlation with the carcinogenic effects (Pellerin and Booker, 2000; Dorman et al., 2000; Coderre and Srivastava, 2004; Kapaj et al., 2006; Swaminathan et al., 2007; Vather, 2008; Sharma et al., 2014). Metals that occur naturally (e.g., arsenic, cadmium, chromium, nickel, lead, mercury, etc) are found to be ubiquitous elements, detected at low concentrations in virtually all environmental settings. However, anthropogenic activities contribute significantly to environmental contamination. Some of them are further biotransformed in the environment into very toxic organic derivates that have been causing serious poisonings (for example, methylmercury derivate occurring in seafood is very toxic to neurological development in utero at very low levels of exposure) (Sanfeliu et al., 2003; Dopp et al., 2004; Sarkar, 2005).

The metals to which people are exposed derive from both natural sources and human activities. Because metals form a natural part of our environment, human exposure may occur as a result of the natural erosion of metal-containing minerals (Hamilton-Taylor and Price, 1983; Smedley and Kinniburgh, 2002; Nath et al., 2008). Otherwise, the exposure may derive from human activities (e.g., mining, smelting, fossil-fuel combustion, tobacco smoking) and industrial applications (e.g., metal processing in refineries, coal burning in power plants, petroleum combustion, nuclear power stations and high tension lines, plastics, textiles, microelectronics, wood preservation and paper processing plants) (Arruti et al., 2010; Sträter et al., 2010). Exposure to metals mainly occurs through polluted air, food and drinking-water (Marshall et al., 2007), as after being dissolved in water and having contaminated the soil, metals become available for uptake by vegetation and consumption by animals; in addition, vegetables can be polluted by metal particles that they fix from the air (Reimann et al., 2001). Natural phenomena such as weathering and volcanic eruptions have also been reported to significantly contribute to heavy metal pollution (Nriagu, 1989; Fergusson, 1990; Bradl, 2000; He et al., 2005). Metals are also contained in several usual consumer goods: cooking utensils, containers, soldering, alcoholic beverages and tobacco. Since different sources may expose people to the same metals by different routes, for most metals it is impossible to define one single exposure route. The highest metals exposure often occurs within contaminated areas or the workplaces, where the most important routes of exposure generally are inhalation of metals dusts or fumes (tiny particles generated by combustion) as well as involuntary ingestion. Several studies are known for assessing the

exposure of people occupationally exposed to metals or people resident in polluted environments (Langård and Vigander, 1983; Aragon-Piña et al., 2000; Benedetti et al., 2001; Guertin, 2005; OSHA, 2006). However, heavy metals are also considered as trace elements because of their presence in trace concentrations (from ppb range to less than 10ppm) in various environmental matrices (Kabata-Pendia, 2001). The research during recent decades has shown that systemic damage and carcinogenic effects can result even from low-level exposure to metals, involving general population (Satarug and Moore, 2004).

Several metals such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn) are essential nutrients that are required for various biochemical and physiological functions, so that an inadequate supply of these micro-nutrients results in a variety of deficiency diseases or syndromes (WHO/FAO/IAEA, 1996). However, for some of them, there is a very narrow range of concentrations between beneficial and toxic effects caused by an excessive amount of such elements intake (Chang et al., 1996; Tchounwou et al., 2008). Other metals, for example aluminium (Al), antinomy (Sb), arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), chromium (Cr-VI), lead (Pb), mercury (Hg), uranium (U) and vanadium (V), have no known nutritional or beneficial effects on human health and are considered as non-essential metals. Among metals, As, Cd, Cr (VI), Pb, Be, Ni and Hg are classified as the priority metals that are of great public health significance, due to their high degree of toxicity and their ubiquitous presence in air, water, and soil. They are all systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure, including cardiovascular diseases, developmental abnormalities, neurologic and neurobehavioral disorders, diabetes, hearing loss, hematologic and immunologic disorders, and various types of cancer. The main pathways of exposure include ingestion, inhalation, and dermal contact. According to the United States Environmental Protection Agency (U.S. EPA), and the International Agency for Research on Cancer (IARC), these metals are also classified as either "known" or "probable" human carcinogens based on epidemiological and experimental studies showing an association between exposure and cancer incidence in humans and animals. Given the severity of adverse health effects caused by potential exposure to metals a primary issue should be to increase the understanding of the specific risks met by populations and how these risks can be avoided.

Exposure to multiple elements

When considering general environmental exposure to metals it should be emphasized that exposure to these toxic metals usually involves simultaneous exposure to a combination of metals rather than to just a single metal, as well as other environmental factors (Mitchell et al., 2011). In many areas of

metal pollution, chronic low dose exposure to multiple elements is a major public health concern. Many researchers documented the acute and chronic effects causing long term health problems in human populations attributed to the toxic metals, both individually and in combination with each other. In the last few years, scientific evidences on exposures to multiple metals and other environmental pollutants are increasing, even if they are typically related to specific sources or risk behaviors, such as living in very contaminated areas. When subjects are exposed to metals in combination, the metals may each have their own typical health effects, otherwise synergistic or antagonistic effects on each other. For example, ingestion of metals in combination may increase or decrease the absorption of the individual metals in the digestive tract. In other case some metals promote the excretion of other metals. Recent reports have pointed out that these toxic elements may interfere metabolically with nutritionally essential metals such as iron, calcium, copper, and zinc (Lopez et al., 2004; Abdulla and Chmielnicka, 1990). A review reassuming some individual studies focused on metals interactions reported that co-exposure to metal mixtures of arsenic, lead and cadmium produced more severe effects at both relatively high dose and low dose levels (Wang and Fowler, 2008). Another study on human co-exposure to cadmium and inorganic arsenic showed a more pronounced renal damage than exposure to each of the elements alone (Nordberg et al., 2005). For this reasons, when calculating cancer risk due to environmental contaminants, it is a primary need to consider not only the health risks due to individual contaminant, but also those risks due to combinations of contaminants. Hence, research is needed to further elucidate the molecular mechanisms and public health impact associated with human exposure to mixtures of toxic metals. Their toxic effects were found to be mediated by dose, duration of exposure and genetic factors. In particular, when assessing exposure to metals a relevant factor to take into account is that the harmful concentration level varies individually due to the differences in susceptibility. Thus, threshold values above which an adverse effect is observed will also differ for individuals.

Children's exposure

Environmental risk exposure for children requires special attention as children are subjects at particular risk respect to adults, both in terms of exposure and effects. For example when estimating children's exposure to metals it must be taken into consideration the higher breathing rate, the greater consumption of water and food per body weight respect to adults, their rapid development and undeveloped ability to metabolize or excrete toxic metals, the large amount of time spent in their homes where the exposure occurs, but also the special conditions seen in young children, exposed orally via dust and dirt during play. In the case of some elements (lead or mercury, for example) clinical observations have noted that children are more susceptible than

adults to toxic metals exposure as they may develop adverse health effects before adults given the same level of exposure (Chakraborti et al., 2003; Kordas et al., 2010-2015). Many studies conducted by the National Health and Nutrition Examination Surveys (NHANES) have demonstrated that the large population of children continue to have elevated blood lead levels (> 10μg/dL) in contrast to the general decline in blood lead levels observed since the 1970s for the adult population (Pirkle et al., 1994). In fact, although the industrial use of lead has been significantly reduced from paints and ceramic products, caulking, and pipe solder (CDC, 1991), it has been reported that 25% of homes hosting more than one child younger than 6 years per household still had significant amounts of lead-contaminated deteriorated paint, dust, or soil (Jacobs et al., 2002). Because the largest source of lead poisoning in children comes from dust and chips from deteriorating lead paint on interior surfaces (Lanphear et al., 1998); for example, children who live in homes with deteriorating lead paint can achieve blood lead concentrations of 20μg/dL or greater (Charney et al., 1980; CDC, 2001). In addition the long life expectancy of children may allow time for developing delayed toxic effects due to early exposures (Bouchard et al., 2007; Magdo et al., 2007; Kordas et al., 2007; Rice and Barone, 2007; Wright and Baccarelli, 2007). Epidemiological studies involving children and metals have mostly focused on learning intellectual deficits rather than on more general health effects (Calderon et al., 2001; Rodrigues et al., 2016; Shamsudin et al., 2017). Many studies have shown a statistical relationship between exposure to some metals and decreased intellectual functions, whose non specific symptoms may be mood change, and decreased concentration and learning ability (Amodio-Cocchieri et al., 1996; Kaul et al., 1999; Tsai et al., 2003; Watanabe et al., 2003; do Nascimento et al., 2015). Instead, relatively little epidemiological research has focused on the health effects related to metal exposures in childhood. Besides, while scientific evidences on exposures to multiple metals and other environmental pollutants related to people professionally exposed or living in very contaminated areas are increased (Gebel et al., 1998; Benin et al., 1999), still little is known about children's exposure to multiple metals, both in term of exposure profile and possible negative outcomes. Thus, there is an urgent need to carry out epidemiological studies specifically dedicated to children and their exposure to environmental metals.

Biological monitoring

When evaluating exposure it should be taken into account inter- and intra- individual metabolic and physical workload differences in the total uptake of metals. The individual exposure may differ considerably because of differences in breathing pattern, for example as a result of a light or heavy workload, or because the breathing is through the nose rather than the mouth. Furthermore, as the exposure to metals may be due to environmental (water, air, soil, dust),

occupational, medicinal, and dietary sources the use of biomarkers is needed for comprehensive, multi-pathway assessments of exposure. For these reasons, in the last years, biological monitoring has been used to evaluate exposure and risk for various metals, by means analysis of tissue, or of biological indicator media such as blood, urine, faeces, hair or maternal milk (WHO, 1994). Since there are many simultaneous exposure routes and metals absorption often differs from predicted values, BM is often the only tool of estimating total exposure, showing how much of the metal has been taken up by the body. The measurement of metals in biological fluids is the primary means of quantifying biomarkers of exposure for metals by occupational health organizations such as the American Congress of Governmental Industrial Hygienists. Some time, when the mechanism of action is known, BM may be performed to make risk estimation. This form of monitoring can be also used to determine the total exposure from different environmental media, which is an advantage when evaluating the risk of systemic effects. Since many metals remain in the body for a considerable time after exposure, BM can provide information on exposure that occurred a considerable time previously. In long term epidemiological studies, samples then can be stored for future analyses. For many metals, urine is an important route of excretion and frequently the predominant one. To evaluate a metal concentration it is necessary to consider the degree of urine concentration. This can be done easily by specifying the concentration in relation to creatinine, which is excreted in the urine in fairly constant quantities. In light of these issues, it is clearly essential to promote studies for assessing the concurrent exposure to multiple environmental contaminants, in order to characterize at its best the risk for human health, especially for the most susceptible group of population such as children.

Our study: metals exposure assessment among children

In order to trace the exposure and co-exposure profiles to eight known and suspected carcinogenic elements (As, Be, Cd, Co, Cr, Ni, Pb and Sb), we performed a cross-sectional study on 143 healthy Italian children (5-11 years), attending a primary school located in the urban area of Rome. Inductively coupled plasma-mass spectrometry (ICP-MS) was employed to determine simultaneously the selected metals on urinary samples, collected at the end of the day, the last before sleeping. The u-creatinine concentrations were determined using the method of Jaffé (Henry, 1974). Because health effects due to co-exposures to toxic metals are further affected by behavioral and cultural practices (e.g. smoking), information about possible interfering factors were obtained from questionnaires. In these questionnaires we considered not relevant the influence of diet on the intake of the monitored trace elements as their urinary concentrations are representative of a recent exposure and all the participants had the same lunch in the school canteen during the sampling day. In order to avoid any possible interference due to the dietary

source, mercury, despite being a highly toxic element was not considered in our study because its large source of exposure is diet for most individuals. In any case, for metals such as arsenic and nickel, that are found naturally in the diet, the failure to consider dietary sources of metals may result in a misinterpretation of the exposure. Considering metal exposure to be effective for each subject when its urinary concentration exceeds the median values calculated among all participants, we found that the great part (83.2%) of the studied children resulted co-exposed to at least two of the monitored elements. Univariate logistic regression analyses performed to assess the contribution of some factors (gender, age, athletic activity during the sampling day, educational level of parents and ETS exposure) to co-exposure to target metals found only male gender as significant predictor of co-exposure ($p=0.047$). Given the importance of protecting children's health and the risk related to the exposure to carcinogenic metals, especially when it occurs simultaneously, other researches in this field are strongly recommended.

Ann Ig 2017; 29: 494-503 doi:10.7416/ai.2017.2180

Exposure to individual and multiple carcinogenic metals during paediatric age: an experience from an Italian urban scenario

C. Protano[1], M.L. Astolfi[2], S. Canepari[2], R. Andreoli[3], A. Mutti[3], F. Valeriani[4], V. Romano Spica[4], A. Antonucci[1], V. Mattei[5], S. Martellucci[5], M. Vitali[1]

Key words: Urinary carcinogenic metals, children, gender differences, concurrent exposure, Italy
Parole chiave: Metalli cancerogeni urinari, bambini, differenze di genere, esposizione contemporanea, Italia

Abstract

Background. *Exposure to single and multiple carcinogenic metals and/or semimetals represents a major environmental risk factor for public health. In particular, children are more susceptible to environmental pollutants than adults, but specific studies are still limited. The aims of the present study were: 1) to trace the exposure and co-exposure profiles to eight known or suspected carcinogenic metals and semimetals (As, Be, Cd, Co, Cr, Ni, Pb, and Sb); and: 2) to evaluate the influence of some possible interfering/confounding factors on the exposure to these elements during childhood.*
Study design. *Cross-sectional study.*
Methods. *We recruited 159 healthy Italian children attending a primary school of the urban area of Rome, Italy. Selected metals were determined by inductively coupled plasma mass spectrometry on urinary samples collected at the end of a "typical" day (one sample for each child), while information about possible confounding/interfering factors were collected via questionnaires.*
Results. *The great part of the studied children resulted co-exposed to the monitored metals: 83.2%, 69.2%, 51.0% and 29.3% of the participants were concurrently exposed to at least two, three, four and five trace elements, respectively. Gender was the only one among the investigated variable that significantly influenced the co-exposure, with females resulting at lower risk (OR = 0.392; 95 IC = 0.156 – 0.989; p < 0.047).*
Conclusions. *Given the importance of protecting child's health and the risks related to the exposure to carcinogenic metals, especially when they occur simultaneously, other researches in this field are strongly recommended.*

[1] *Department of Public Health and Infectious Diseases, Sapienza University of Rome, Italy*
[2] *Department of Chemistry, Sapienza University of Rome, Italy*
[3] *Department of Clinical and Experimental Medicine, Laboratory of Industrial Toxicology, University of Parma, Italy*
[4] *Public Health Unit, University of Rome "Foro Italico", Rome, Italy*
[5] *Laboratory of Experimental Medicine and Environmental Pathology, Polo Universitario di Rieti "Sabina Universitas", Rieti, Italy*

Introduction

The presence of carcinogenic metals in the living and working environment is an issue of great concern for public health. The exposure to these elements, in fact, is considered one of the major risk factors associated with the increase in cancer incidence over the last decades, both in occupational and general scenarios (1). Initially, the attention of the researchers has been focused chiefly on those population groups who have had very high exposure, such as people living in contaminated areas, or on work-related "cases" of cancers (2, 3). Afterwards, an increasing number of scientific evidences has been reported about carcinogenic effects related to the exposure to low/very low levels of metals, involving also the general population (4, 5). Indeed, certain metals and semimetals such as arsenic (As), beryllium (Be), cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), nickel (Ni), and vanadium (V), are toxic even at low levels of exposure, and they have been recognized as carcinogens by the major public health agencies and authorities (6, 7**).** Besides other metals, such as Cu, Fe, Se and Zn, that are essential for human health in trace amounts, can determine adverse effects up to carcinogenesis as a result of chronic and extensive exposure (7). An important question about metal-induced carcinogenesis is related to the co-exposure to more than one carcinogenic metal and the multiplicative effects determined by this co-exposure, yet evidenced for such elements in previous studies both for occupational and general population, including children (8-11). Thus, since the most of exposure to environmental pollutants does not occur in isolation (12-14), the increased risk of adverse effects exceeding the sum of effects determined by each metal alone cannot be negligible. This is of particular concern for paediatric age due to the different behaviour of children respect to adults, both in term of exposure and effects. Indeed, children present a higher breathing rate, and they ingest more food and water per body weight unit respect to adults; besides, the organism of a child is still in a period of development and the defensive system is not fully efficient to defend the child from potential risks related to pollutants exposure (15). Furthermore, an early exposure to environmental pollutants may determine an increased risk for developing related diseases not only during childhood, but also in adult age, especially for those diseases characterized by a prolonged latency (16-19).

Given these premises, we performed a cross-sectional study among a sample of healthy Italian schoolchildren aged 5-11 years in order to: 1) trace the exposure and co-exposure profiles to eight known or suspected carcinogenic metals and semimetals (As, Be, Cd, Co, Cr, Ni, Pb, and Sb) and 2) evaluate the influence of some possible interfering/confounding factors on the exposure to these elements during childhood.

Material and methods

Study design, studied population and monitoring campaign

The research consisted in a cross-sectional study performed on 143 healthy Italian children, attending a primary school of the urban area of Rome, Italy. Enrolment was well-described in previous reports (20-23). Briefly, the research project was presented to all the students and their parents, and all materials for participating (questionnaire, polypropylene bottle for urine sample, informed consent, etc) were delivered by the research group. The monitoring campaign was conducted in the academic years 2007–08, during a typical weekday (Wednesdays) of the winter season as the sampling day. During the sampling day, participants' parents fulfilled a self-

administered questionnaire elaborated *ad hoc* to collect information about the children (gender, age, Environmental Tobacco Smoke – ETS - exposure status, and sport and other activities taking place during the sampling day) and their parents (ethnicity, maternal and paternal educational level). In the same day, parents collected one urine sample of the participant in the evening (the last urine emiction of child before bedtime) and immediately stored the sample in the domestic refrigerator (about +4 °C); next morning each participant brought to school the filled in and signed informed consent and the questionnaire, together with the urine sample. The research team collected documents and samples at school and transported urine to the laboratory in refrigerated containers. Each urine sample was immediately partitioned into plastic tubes, coded and frozen at −20 °C until the analytical determinations of urinary metals and urinary (u) creatinine took place.

Analytical determinations

The procedure used for the analytical determination of target elements is reported in detail in a previous paper (24). Briefly, As, Be, Cd, Co, Cr, Ni, Pb and Sb were quantified by using the Bruker (Bremen, Germany) 820-MS Inductively Coupled Plasma Mass Spectrometer (ICP-MS), equipped with a MicroMistTM (0.4 mL/min) glass nebulizer, at operating specific conditions previously detailed (25).

Evaluation of recoveries was performed using a standard reference material (Seronorm trace urine elements, lot no. 0511545, purchased from SERO AS, Billingstad, Norway). During routine analyses, certified samples were run both after calibration and every 20 samples.

Procedure blanks (laboratory blanks) were performed following the same procedure of samples.

Limits of Detection (LODs) and Limits of Quantification (LOQs) were calculated, respectively, as three and ten times σ/b, where σ is the standard deviation (SD) of blank determination from ten replicates and b is the slope of the calibration curve. LODs and LOQs are reported in a previous paper (24).

The u-creatinine levels were determined using the method of Jaffé (27). When we found u-creatinine concentrations < 0.3 g/L or ≥ 3.0 g/L (very diluted or concentrated urine samples, respectively), samples were excluded from the subsequent determinations according to the recommendations of international public health agencies (28, 29).

The urinary concentrations of the monitored trace elements were expressed both as µg/L and µg/g creatinine because the use of u-creatinine as normalisation factor is under debate (30, 31).

Covariates used for the statistical elaboration

Information about gender, age, athletic activities during the sampling day, ETS exposure of children, and educational levels of each parent of the participant were collected via questionnaire, while participants' co-exposure to two or more carcinogenic metals were elaborated according to the results of the analytical determinations. In particular, regarding the information obtained by the questionnaire, gender of each participant was classified as 0 = male and 1 = female, while age was based on the primary-school grade attended by each participant: 0 = 1st, 2nd, or 3rd grade (the younger group) and 1 = 4th or 5th grade (the older group). This classification was chosen in order to avoid the construction of a variable with seven dummies (ages of 5, 6, 7, 8, 9, 10, 11 years); thus, we divided children in two groups according to the age of puberty onset (9–10 years old) (32). Athletic activities were assessed by a question regarding the participation in at least one organized sport outside school hours (no/yes, coded as 0 =

no and 1 = yes). The maternal and paternal educational levels were assessed by a closed-ended question. The respective responses were coded to produce a dichotomous variable: "< junior high school (< 8)" or "≥ junior high school (≥ 8)".

ETS exposure in domestic environment was investigated by the question: "Are there smokers living with the child?" The possible response was either "yes" or "no" (categorised as 0 = no and 1 = yes).

Referring to the co-exposure of children to multiple carcinogenic metals, a child was considered to be co-exposed when the urinary concentrations of at least two metals exceeded their median values calculated among all participants.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 21 software (IBM Corp., Armonk, NY, USA). First of all, the normality of each metal concentration was tested by Kolmogorov-Smirnov test, and the results showed that the levels of the monitored substances were not normally distributed. Thus, univariate analyses were conducted with non-parametric techniques (Spearman's rho correlations).

Logistic regression analyses were run on the entire sample of children, to assess the contribution of gender, age, athletic activity during the sampling day, educational levels of parents and ETS exposure to the co-exposure to studied elements. The significance level for all tests was $p \leq 0.05$ (two-tailed).

Results

The main characteristics of the study population are summarised in Table 1. Participants were similar according to gender, age and Environmental Tobacco Smoke (ETS) exposure. Besides, 31.4% of children practiced an organized sport during the sampling day. Regarding to the parental educational level, more than half of parents had at least lower secondary educational level, with a percentage slightly higher for mothers respect to fathers (64.1% vs. 57.4%). With respect to the co-exposure to multiple metals, the great part of the studied children resulted co-exposed to at least two of the monitored compounds (83.2%), while 69.2%, 51.0% and 29.3% of participants were co-exposed to at least three, four and five trace elements, respectively.

Summary statistics of urinary concentrations of each monitored element are shown in Table 2. The greatest variability was observed for urinary concentrations of Ni and As, that ranged from 1.53 to 98.93 µg/L (2.20 – 159.60 µg/g creatinine) and from 0.60 to 30.40 µg/L (0.60 – 49.40 µg/g creatinine), respectively. Results related to Be are not shown because its concentration was always below the LOD.

Table 3 reports the Spearman's correlation coefficients between the monitored compounds. Most of the metals were positively correlated, while Ni was not associated to any other element.

The results of univariate logistic regression analyses are presented in Table 4. The only significant predictor of co-exposure to at least two trace elements was gender (males as reference), with an OR equal to 0.392 (95 IC = 0.156 – 0.989; $p = 0.047$).

Discussion and conclusions

The present study was aimed to trace a profile of exposure to individual and multiple carcinogenic metals and semimetals in a samples of Italian healthy children living in an urban area. In fact, it has been shown that environmental exposure occurs almost always to a mixture of pollutants rather than in isolation. Consequently, it is essential to promote studies for assessing the concurrent exposure to multiple environmental

Table 1 - Characteristics of the study sample

Variable	n (%)	Missing values
N° of subjects	143	
Gender		
M	73 (52.1)	3
F	67 (47.9)	
Grade of primary school		
1st, 2nd, 3rd	77 (54.6)	2
4th, 5th	64 (45.4)	
Physical activity during the sampling day		
No	96 (68.6)	3
Maternal education (years)		
< junior high school (< 8)	51 (35.9)	1
≥ junior high school (≥ 8)	91 (64.1)	
Paternal education (years)		
< junior high school (< 8)	60 (42.6)	2
≥ junior high school (≥ 8)	81 (57.4)	
Exposure to Environmental Tobacco Smoke (ETS)[a]		
No	69 (48.3)	-
Yes	74 (51.7)	
Co-Exposure to at least two metals[b]		
No	24 (16.8)	-
Yes	119 (83.2)	
Co-Exposure to at least three metals[c]		
No	44 (30.8)	-
Yes	99 (69.2)	
Co-Exposure to at least four metals[d]		
No	70 (49.0)	-
Yes	73 (51.0)	
Co-Exposure to at least five metals[e]		
No	101 (70.6)	-
Yes	42 (29.3)	

[a]A child is considered to be exposed to ETS if he/she lives with at least one smoker
[b]A child is considered to be exposed to multiple metals when the urinary concentration of at least two metals exceeds its median value among all subjects
[c]A child is considered to be exposed to multiple metals when the urinary concentration of at least three metals exceeds its median value among all subjects
[d]A child is considered to be exposed to multiple metals when the urinary concentration of at least four metals exceeds its median value among all subjects
[e]A child is considered to be exposed to multiple metals when the urinary concentration of at least five metals exceeds its median value among all subjects

Table 2 - Urinary levels of Arsenic (As), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Nickel (Ni), Lead (Pb), Antimony (Sb) in the study population (n = 143)

	As		Cd		Co		Cr		Ni		Pb		Sb	
	µg/L	µg/ creatinine	µg/L	µg/g creatinine	µg/L	µg/g creatinine	µg/L	µg/g creatinine	µg/L	µg/g creatinine	µg/L	µg/g creatinine	µg/L	µg/g creatinine
Mean	5.28	5.98	0.68	0.77	1.25	1.31	0.34	0.38	7.87	9.23	2.08	2.36	0.11	0.12
Median	3.00	3.40	0.57	0.60	1.01	1.16	0.33	0.34	6.57	6.75	1.83	1.86	0.11	0.10
SD	5.58	7.62	0.49	0.58	0.88	0.76	0.12	0.20	8.33	13.55	1.57	1.71	0.06	0.09
Minimum	0.60	0.60	0.09	0.10	0.15	0.32	0.12	0.11	1.53	2.20	0.50	0.37	0.03	0.02
Maximum	30.40	49.40	3.94	3.10	7.05	4.83	0.89	1.32	98.93	159.60	14.13	9.68	0.24	0.51
25 Percentile	2.20	2.10	0.38	0.40	0.74	0.79	0.26	0.25	5.23	5.20	1.09	1.13	0.04	0.06
75 Percentile	6.60	6.40	0.77	0.91	1.43	1.53	0.40	0.44	8.66	9.70	2.52	2.95	0.15	0.17

contaminants, in order to characterize at its best the risk for human health, especially for the most susceptible groups of population such as children (33, 34). In the last few years, scientific evidences on exposures to multiple metals and other environmental pollutants are increasing; however, they are typically related to specific sources or risk behaviors, such as living in very contaminated areas, while exposure profiles of children living in areas without specific environmental problems are very limited (12). Besides, a very recent review in this field recommends to consider children's environmental exposure, and the associated health risks, as a research agenda of the scientific community (15).

In terms of public health, the first relevant finding of the present study is the proportion of children (more than a half) that are exposed in domestic environments to ETS, one of the main health risk factors during paediatric age. This percentage is higher than in another research carried out in the same area in 2014 (35), probably as a consequence of many educational interventions on smoking cessation implemented in Italy in the last few years. Also the smoking bans applied in all indoor and some outdoor public settings have been successful in reducing smoking addiction. Our result is interesting although it needs further investigation by a specific study.

The second major finding is related to the percentage of children co-exposed to multiple carcinogenic elements: 83.2%, 69.2%, 51.0%, 29.3% of studied children resulted exposed to at least two, three, four, and five carcinogenic metals, respectively. Co-exposure of monitored children to multiple metals is also supported by the significant positive association observed for most of the target elements. These results are of particular concern for the health of children, given the possible association between the concurrent exposure to multiple carcinogenic elements and health threats evidenced by previous studies. Wong et

Table 3 - Spearman's rho correlation coefficients between urinary levels of Arsenic (As), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Lead (Pb), Nickel (Ni), Antimony (Sb)

	Cd	Co	Cr	Ni	Pb	Sb
As	0.018	-0.041	**0.264****	0.068	0.033	**0.170***
Cd	-	**0.481****	0.045	0.156	**0.223****	0.064
Co	-	-	**0.323****	-0.146	0.041	-0.027
Cr	-	-	-	0.040	**0.293****	**0.310****
Ni	-	-	-	-	**0.208***	-0.027
Pb	-	-	-	-	-	**0.242****

*$p < 0.05$, **$p < 0.01$

al. (9), for example, found that children with both high urinary As and Cr had the highest urinary levels of 8-hydroxy-2′-deoxyguanosine, a biomarker widely used for assessing the association between exposure to environmental pollutants and oxidative stress (36, 37). It has been demonstrated that oxidative stress can determine a cellular redox imbalance and an increase in oxidative DNA lesions, implicated in several diseases including cancer (38). Besides, oxidative stress has also been linked to metal-related carcinogenic process. Indeed, even if the molecular mechanisms of this process are not completely understood, research in this field demonstrated three main modes of action associated to the ability of carcinogenic metals to: (1) induce oxidative stress and alteration of cellular components, especially with nucleic acid, (2) determine genomic instability by impairing DNA repair system, and (3) disturb the normal cell growth and proliferation, interfering with signaling pathways and regulation of oncogenes or tumor suppressor genes (39).

Another important finding is related to the influence of some investigated variables on the co-exposure to multiple metals in childhood: males resulted at higher risk of concurrent exposure respect to females. This result is in line with those already reported for metals exposure profiles (12, 24), and

Table 4 - Variables associated with co-exposure to at least two trace elements in univariate analysis

Variable	OR	95 IC	*p*	
Gender	M (Ref.) F	0.392	0.156 – 0.989	0.047
Grade of primary school	1st, 2nd, 3rd (Ref.) 4th, 5th	0.800	0.332 – 1.928	0.619
Physical activity during the sampling day	No (Ref.) Yes	1.777	0.614 – 5.143	0.289
Maternal education (years)	< junior high school (< 8) (Ref.) ≥ junior high school (≥ 8)	0.872	0.345 – 2.206	0.773
Paternal education (years)	< junior high school (< 8) (Ref.) ≥ junior high school (≥ 8)	0.677	0.267 – 1.719	0.412
Exposure to Environmental Tobacco Smoke (ETS)	No (Ref.) Yes	0.726	0299 – 1.765	0.480

it is probably related to gender differences either in uptake and intake of environmental pollutants and in typical behaviours (i.e. males spend more time playing in potential contaminated outdoor environments). With respect to the other investigated variables such as age, physical activities, etc, we did not find significant differences, according to a previous similar study (13). This lack of significant differences can be attributed to the small sample size studied here. Given the relevance of this issues and the great number of children found as co-exposed, it should be very interesting to investigate the impact of further potential predictors of co-exposure profile during childhood.

The present study had some limitations. First of all, analytical determinations were carried out only on one spot sample, collecting the urine just before sleeping. Considering the possible differences in terms of bioaccumulation and times of metabolisation and excretion, it should be interesting to perform a biomonitoring campaign for evaluating potential variations in the urinary excretion of the target elements collecting urine in different periods of the day. Moreover, we did not consider the possible impact of the diet of the participants on urinary concentrations of the selected metals. However, it is important to note that urinary concentrations of metals are related to recent exposure. Consequently, the role of the diet on the metals excretion can be considered not relevant, as all studied children consumed the same lunch in the school canteen during the sampling day. Additionally, other potential confounding factors, such as ponderal status, genetic polimorfisms, etc, can influence the urinary excretion of metals, and they should be considered in further studies in this field.

In conclusion, we recovered a great evidence of concurrent exposure to multiple carcinogenic compounds in the investigated sample of children. Given the importance of protecting child's health and the risks related to the exposure to carcinogenic metals, especially when it occurs simultaneously, other researches in this field are strongly recommended.

Riassunto

Esposizione singola e multipla a metalli cancerogeni durante l'età pediatrica: un'esperienza da uno scenario urbano Italiano

Premessa. L'esposizione singola o multipla a metalli cancerogeni rappresenta un rilevante fattore di rischio ambientale per la salute pubblica. In particolare, i bambini sono più suscettibili agli inquinanti ambientali rispetto agli adulti, ma ricerche specifiche sono a tutt'oggi insufficienti. Gli obiettivi del presente studio sono stati: 1) tracciare il profilo di esposizione e co-esposizione ad otto noti o sospetti metalli cancerogeni (As, Be, Cd, Co, Cr, Ni, Pb, e Sb) e 2) valutare l'influenza di alcuni possibili fattori confondenti/interferenti sull'esposizione a questi metalli durante l'infanzia.

Disegno dello studio. Studio trasversale.

Metodi. 159 bambini Italiani sani sono stati reclutati in una scuola primaria dell'area urbana di Roma. I metalli selezionati sono stati determinati su campioni di urine raccolti alla fine di una giornata "tipo" (un campione per ogni bambino) mediante uno spettrofotometro di emissione a plasma accoppiato induttivamente a un rivelatore di massa, mentre le informazioni sui possibili fattori confondenti/interferenti sono state raccolte mediante questionari.

Risultati. In maggioranza i bambini sono risultati co-esposti ai metalli monitorati: rispettivamente 83,2%, 69,2%, 51,0% e 29,3% dei partecipanti risultavano esposti contemporaneamente ad almeno due, tre, quattro e cinque elementi in traccia. Tra le diverse variabili investigate, il genere è risultata l'unica ad influenzare significativamente la co-esposizione, e le femmine sono risultate a minor rischio (OR = 0,392; 95 IC = 0,156 – 0,989; $p < 0,047$).

Conclusioni. Data l'importanza di proteggere la salute dei bambini e i rischi connessi con l'esposizione a metalli cancerogeni, specialmente quando questa si verifica contemporaneamente, sono fortemente raccomandate ulteriori ricerche in questo campo.

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Corresponding author: Matteo Vitali, Department of Public Health and Infectious Disease, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Roma, Italy
e-mail: matteo.vitali@uniroma1.it

CHAPTER 3

SENSITIVE MULTIRESIDUE METHOD BY HS-SPME/GC-MS FOR 10 VOLATILE ORGANIC COMPOUNDS IN URINE MATRIX: A NEW TOOL FOR BIOMONITORING STUDIES ON CHILDREN

Antonucci A, Vitali M, Avino P, Manigrasso M, Protano C. *Sensitive multiresidue method by HS-SPME/GC-MS for 10 volatile organic compounds in urine matrix: a new tool for biomonitoring studies on children*. Anal Bioanal Chem. 408 (2016); 5789-800. DOI: 10.1007/s00216-016-9682-x.

Framework of the present study of my research program

Outdoor – indoor air pollution and human exposure to multiple chemicals

The heterogeneity of chemical substances recovered in air represents one of the most relevant obstacles to the human exposure assessment. The largest percentage of these contaminants are produced by anthropogenic activities, such as industrial, agricultural and urban activities, although also terrestrial vegetation (biogenic organic compounds) and physical activities (e.g. brush fires, geyser fumes and volcanic eruptions) may release hazardous chemicals in the environment. Although precise identification and assessment of atmospheric contaminants is always profitable, many studies have shown that measurements of outdoor concentrations fail to adequately assess individual exposure, mainly in industrialized countries, where people spend most of their time indoors (Quackenboss et al., 1986; Adair and Spengler, 1989; Kepleis, et al., 2001; Leech et al., 2002). Over the last thirty years, indoor air pollution has been recognized as an important threat to human health as a consequence of introduction of a variety of new materials and chemicals into the home environment and public buildings (e.g. schools, offices, gyms) (WHO, 2010-2014). For this reason, recent studies aimed to assess human exposure have been extended to include not only traditional industrial and vehicular traffic sources, but also the consumer goods (cleaning products, fragrances and air fresheners), building materials and finishes, including paints and floor coverings (Missia et al., 2010). Indoor sources may include also heaters, cooking stoves, and fuel (coal, wood, gas, kerosene or liquid petroleum gas), each of which causes indoor emission of a wide range of pollutants. Actually, many types of exposure to environmental pollutants that occur indoors arise in large part from indoor pollutant sources among which the habits, such as cigarettes (Wallace, 1996) or activities of the people using the indoor environment (cooking, maintenance and remodeling activities, ventilation-air exchange), play a key role. However, indoor air quality can be affect largely by outdoor air, which serves as a major source of particulate and gaseous pollutants for indoor air (Freijer and Bolemen, 2000; Diapouli et al,. 2013; Wan et al., 2015). The degree of contamination of an enclosed environment by external pollutants may depend on several factors, related to the specific characteristics of the place (volume, structure, presence of airtight windows, circulating airflow rate, filtration of entering air) and to the proximity with heavy vehicular traffic or other sources of pollutants (garages, petrol stations or industrial sites) (Dodson et al., 2008; Langer and Beko, 2013). Also great seasonally variability and pronounced differences between day and night were observed (Schlink et al., 2004; Jia et al., 2012). Further, some volatile organic compounds (VOCs) have

been found to be ubiquitous in residential indoor where their air concentration levels typically exceed those outdoors as a consequence of the entry and accumulation of VOCs from outdoor sources and the presence of many other VOCs indoor sources (Weisel et al., 2005; Batterman et al., 2012). In light of this, time spent in indoor environments has been identified as the most contributor of personal exposure to these contaminants, even if also the proximity of individuals to vehicular traffic while in transit has shown elevated exposures to several VOCs (Chan et al., 1991; Chan 2003; Kaur et al., 2007).

Children exposure to air pollution

Over the years valuable information about personal and indoors VOC concentration levels has been generating by many studies worldwide (Clayton et al., 1999; Hoffmann et al., 2000; Edwards et al., 2001; Schneider et al., 2001; Saarela et al., 2003; Son et al., 2003; Lai et al., 2004; Serrano-Trespalacios et al., 2004; Järnström et al., 2008; Salthammer et al., 2010). Compared to the studies on the general population and occupational exposures, which predominantly occurs through inhalation, only a few cases have been reviewed for preschool and school children environmental exposure to multiple volatile toxic compounds (Adgate et al., 2004; Conrad et al., 2010; Pearson et al., 2011). Most children exposure originates from environmental tobacco smoke (ETS), emissions from building materials and furnishings through inhalation because they spend most of the time indoors (homes, school, gyms) where they are exposed to a complex mixture of pollutants at concentration levels that are often several times higher than outdoors. Besides, breathing car exhaust gases on the way to school, or playing outside, children may increase environmental pollutants exposure.

Target air pollutants

Because of the complexity and plurality of the involved factors, synthetic biological indicators of exposure to air pollution should be very useful. However, when the cumulative risk related to multiple chemicals exposure has to be evaluated it is essential to perform an estimation of exposure as exhaustive as possible, to better define the correlation between exposure and emerging symptoms (*cumulative risk anlysis*). Based on previous scientific literature (Perbellini et al., 2002; Fustinoni et al., 2010; Campo et al., 2011; De Palma et al., 2011), we decided to consider two important groups of airborne contaminants found in the urban environment as a consequence in most cases of vehicular traffic: monocyclic aromatic chemicals (benzene, toluene, ethylbenzene, o, m, p-xylenes - BTEX) and oxygenated compounds belonging to the class of ethers (methyl ter-butyl ether, MTBE; ethyl ter-butyl ether, ETBE; diisopropyl ether, DIPE; ter-amyl methyl ether, TAME). Such chemicals are mainly used in gasoline as additives to enhance

octane rating and improve the combustion process, so that their presence in outdoor air has been largely associated with fuels, as component of both vehicular exhaust fumes and vapors. BTEX are ubiquitous air pollutants, diffused in outdoor and indoor environments, both in occupational and general ambient. As they arise from incomplete combustion of organic matter, their presence in air could have multiple sources, both anthropogenic (traffic, industry) and natural emissions. Moreover, they are components of several household products for cleaning, personal care, and painting, representing additional indoor air contaminants. Among BTEX, the biological control of exposure to benzene (in 1982 classified by IARC as known human carcinogen, group 1) is of great importance to prevent its toxic and carcinogenic effects and it is therefore very relevant to develop methods that permit an evaluation of its environmental concentration levels (Duarte-Davidson, 2001; Weisel, 2010). Benzene recovers its major sources for general population exposure in combustion of motor vehicles and smoking habits (smokers, in general, are exposed to 10 times more benzene than non-smokers), so that the degree of air pollution in residence area and the ETS were the most important factors affecting benzene exposure in people not professionally exposed. It has been widely demonstrated that this ubiquitous pollutant can be used for providing a synthetic evaluation of the qualitative state of air atmospheric pollution (Protano et al., 2010). On the other hand, ethers present in outdoor air have been directly related to gasoline, being emitted into atmosphere through tailpipe gases and evaporation of fuel. As their main source in urban air is automobile traffic, this class of chemicals may well represent specific vehicular emission tracers.

Biomarkers of exposure for children's evaluation

In order to characterize environmental and domestic children exposure to pollutants, it becomes necessary to provide an analytical procedure allowing the monitoring of proper biomarkers at trace levels. Several studies conducted on general population, suggest urinary unmodified pollutants as good biomarkers of exposure to very low airborne levels of these agents (Johnson et al. 2007; Barbieri et al., 2008; Lovreglio et al., 2010; Campagna et al., 2011). Recently, many researchers have found that unmodified benzene levels in children's urine (u-UB) are greatly influenced by ETS exposure and urbanization of residence areas. Consequently, it would be important to develop a method able to discriminate the ETS exposure from airborne pollutants closely related to urban traffic, in which urinary unmodified ethers could be used as specific biomarkers for assessing vehicular exposure. However, such application present some challenges, such as the necessity of a more sensitive sampling procedure, since the not metabolized chemical agents eliminated through urine in unchanged form are present at extremely low concentrations (about 1-5 % of the total output) (Bartolucci et al., 2003). Moreover, children usually show lower

levels of benzene then those of exposed workers and smokers, as they represent general population surely not exposed to benzene in occupational settings or from the habit to smoke.

Technical support equipment

In order to improve the sensitivity and precision of measurement of the concentration of organic compounds in heterogeneous samples (e.g. food, biological and pharmaceutical samples), solid-phase microextraction (SPME) technique has been proved an efficient method for purificating, extracting, concentrating and injecting at one time. In the last years, SPME has been applied to various biological samples such as urine, plasma and hair (Junting et al., 1998; Theodoridis et al., 2000; Peng et al., 2016). This efficient sampling procedure guarantees a solvent-free extraction and avoids sample purification and concentration before analysis as usually needed for such complex matrix, in addition to a number of advantages such as simplicity, low cost, compatibility with analytical systems and automation. On the other hand, the use of gas chromatography - mass spectrometry coupled technique provides highly sensitive responses and allows the quantification of trace chemical agents.

Our study: a new approach for biomonitoring study on children

Starting from the considerations discussed in the previous paragraphs and gathered from the literature on exposure of general population to pollutants and biomonitoring, we developed a smart procedure for simultaneous determination of ten environmental agents at very low concentration levels (BTEXs, MTBE, ETBE, DIPE, TAME), even down to 50 ng/l, in urine samples by solid-phase micro-extraction (SPME) – gas chromatography/mass spectrometry (GC/MS), to be used in an integrated risk assessment process. In the first step of our study, a headspace SPME protocol was optimized for extraction and concentration of investigated analytes from urine. Various parameters influencing the SPME extraction efficiency have been considered and optimized: fiber type/thickness, temperature and time of extraction, ionic strength and sample agitation. In the optimized procedure, the analytes were extracted for 15 min at 30 ° C in the headspace of each sample, using the 85μm-CAR-PDMS SPME fiber. In a second phase, some specific parameters were calculated to evaluate the analytical performances of the developed method. For all analytes considered, the method proved to be specific and robust, linear in the concentration range of interest (0-1500) ng l^{-1} with high coefficients of correlation ($0.9972 < R^2 < 0.9997$), characterized by high precision (intra / inter-day < 5.5 % for all analytes) and accuracy (< 15 % of the nominal concentration). The sensitivity of the developed method, with quantification limits within the range of 2.9 ÷ 7.5 ng l^{-1}, allowed yielding reliable results also in biological samples from the general population. Finally, the selected experimented conditions were validated by applying the method to the biological monitoring of 40 urine samples from children (5-11 years) exposed to different environmental levels of the

target compounds. Time-activity diary and personal information questioners were also obtained because frequented locations, performed activities and cohabitant habits were found to be explanatory for the variation in exposure to benzene and the other volatile organic compounds. Over time in fact, these types of investigations have become an integral part of risk management, especially to better understand the role of living environment to pollutants exposure in childhood (Wallace et al., 1989-1991; Thomas et al., 1991-1993). Analytical results were then compared with data obtained from questionnaires about main potential exposure factors of each participant. The developed analytical procedure proved to be valid for simultaneous quantitative determination of biomarkers of interest. Except for the ethers DIPE and TAME, detected at very ultra trace levels, the other biomarkers of vehicular traffic exposure (MTBE and BTEX) resulted significantly higher for children living in high traffic area and not exposed to ETS. The same results, but to a lesser extent for MTBE, were found for children exposed to ETS. Our surveys evidenced that ETS was the most important contributor to benzene exposure during childhood, in line with previous reports (Protano et al., 2012a; Andreoli et al., 2012), underlining the need for educational health campaigns specific for smokers in order to protect subjects passively exposed to smoke.

The choice of biological samples easy to collect for large scale surveys and non invasive for susceptible populations such as children, the high sensitivity of the developed analytical method, its ease of application and low cost of execution were the main goals of our research. In particular, the possibility of easily obtaining urinary exposure profiles for children makes this procedure an appealing tool for environmental epidemiology. The outcome of the assay confirmed the u-UB as the best biomarker of exposure to very low levels of benzene and suitability of this multiresidue method as a one-step assessment tool for biomonitoring studies to assess a large number of environmental exposures.

Anal Bioanal Chem (2016) 408:5789–5800
DOI 10.1007/s00216-016-9682-x

RESEARCH PAPER

Sensitive multiresidue method by HS-SPME/GC-MS for 10 volatile organic compounds in urine matrix: a new tool for biomonitoring studies on children

Arianna Antonucci[1,2] & Matteo Vitali [2] & Pasquale Avino[3] & Maurizio Manigrasso [3] & Carmela Protano[2]

Received: 12 April 2016 / Revised: 24 May 2016 / Accepted: 1 June 2016 / Published online: 16 June 2016

Abstract A HS-SPME method coupled with GC-MS analysis has been developed for simultaneously measuring the concentration of 10 volatile organic compounds (VOCs) (benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*-xylene, methyl *tert*-butyl ether, ethyl *tert*-butyl ether, 2-methyl-2-butyl methyl ether, and diisopropyl ether) in urine matrix as a biomonitoring tool for populations at low levels of exposure to such VOCs. These compounds, potentially toxic for human health, are common contaminants of both outdoor and indoor air, as they are released by autovehicular traffic; some of them are also present in environmental tobacco smoke (ETS). Thus, the exposure to these pollutants cannot be neglected and should be assessed. The low limits of detection and quantification (LODs and LOQs <6.5 and 7.5 ng L^{-1}, respectively) and the high reproducibility (CVs <4 %) make the developed method suited for biomonitoring populations exposed at low levels such as children. Further, the method is cost-effective and low in time-consumption; therefore, it is useful for investigating large populations. It has been applied to children exposed to traffic pollution and/or ETS; the relevant results are reported, and the relevant implications are discussed.

Keywords Volatile organic compounds · Biomonitoring · Urine · Children · HS-SPME · GC-MS

Matteo Vitali
matteo.vitali@uniroma1.it

[1] Department of Ecological and Biological Sciences, Tuscia University, Via S. Maria in Gradi, 4, 01100 Viterbo, Italy

[2] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Piazzale Aldo Moro, 5, 00185 Rome, Italy

[3] Department of Technological Innovations, INAIL, Via IV Novembre 144, 00187 Rome, Italy

Introduction

Volatile organic compounds (VOCs) are a heterogeneous group of compounds; many of them are airborne pollutants of both outdoor and indoor environments. As regards to outdoor pollution, VOCs are emitted into urban atmosphere by motor vehicles [1], domestic heating systems, and many productive processes [2, 3]. Besides, a large amount of VOCs, known as biogenic VOCs, are emitted into the atmosphere by terrestrial vegetation; terpenes are the primary products of these natural sources and, due to their high chemical reactivity, may lead to the formation of secondary biogenic VOCs [4].

Regarding indoor pollution, previous studies show that indoor VOC levels are the sum of their outdoor concentrations and their emission from indoor sources, such as environmental tobacco smoke (ETS), combustion, and consumer product use [5–8].

It is well-known that VOCs exposure is a clear threat to human health, directly [8] or indirectly, contributing to the formation of ozone and other photochemical oxidants associated with urban smog [9]. Thus, the characterization of risks associated to VOCs is a key process to protect human health. In this context, programs of exposure assessment are strongly recommended because they increase the knowledge to what extent, how, and when individuals and communities are exposed [10]. Several methodological approaches are commonly used for exposure estimation/measurement, such as collection of information through questionnaires, environmental, and/or biological monitoring. The first two approaches just allow an estimate of the exposure by theoretical assumptions and modeling techniques; biological monitoring, instead, permits the identification of the actual and systemic exposure to environmental

contaminants through the measurement of the chemicals themselves and/or their metabolites in body fluids. In this way, it is possible to take into account all exposure routes (inhalation, ingestion, and dermal contact) and contaminant concentration differences in all the resident environments [10, 11].

The importance of the biological monitoring for protecting public health is emphasized by the use of consolidated biological exposure indices (BEI) for workers' surveillance, along with programs carried out on the general population: an example is the survey organized by the National Health and Nutrition Examination Survey (NHANES) and performed routinely by the Centers for Disease Control (USA). However, both international and Italian biomonitoring studies performed on the general population mainly concern adults, whereas specific studies on pediatric population are still scarce if compared with the need for scientific evidence [12]. In this regard, several years ago the World Health Organization (WHO) Task Force for Children's Environmental Health recommended considering children as "unique" individuals [13]; indeed, children differ from adults because of differences in intake of contaminants, their metabolization, and susceptibility to adverse effects from pollutant exposure [14–19]. Consequently, pediatric populations need focused studies. This has also been highlighted by the recent WHO Global Plan of Action for Children's Health and the Environment 2010–2015, which promotes periodic biomonitoring programs for the pediatric population [20]. In order to achieve this recommendation, it is essential to develop useful assessing tools for biomonitoring studies of children. The VOC exposure evaluation by biological monitoring in pediatric age involves certain critical considerations, some of them linked to all types of biological monitoring, some others related to children as the study population, and still others associated to VOCs. First, it is necessary to develop useful assessment tools, such as analytical methods with high sensitivity for evaluating exposures occurring at low levels like those observed for children living in an urban environment. These methods should also be simple and cost-effective, so that they can be applied routinely on large samples of a population [12]. Besides, it is essential to identify biological samples easy to collect for large-scale surveys, and noninvasive for avoiding ethical and practical constraints typically related to studies performed on children and other susceptible populations [21]. Further, VOCs are always present in the environment as a complex mixture of different compounds; in particular, it should be noted that in recent years, several studies focused the attention on some relevant aromatic VOCs in occupational and environmental settings, such as benzene, toluene, ethylbenzene, *o*-, *m*-, *p*-xylenes (BTEX) [22–28]. The importance of these compounds is justified by their environmental ubiquity and toxicity for humans. Benzene is a hematotoxic compound and it was classified as a known carcinogen to humans by the International Agency for Research on Cancer (group 1) [29]; toluene, ethylbenzene, and xylenes are neurotoxic whether exposure occurs to single compounds or to their mixtures [30]. Other relevant VOCs contaminating indoor and outdoor air are methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), 2-methyl-2-butyl methyl ether (TAME), and diisopropyl ether (DIPE), the use of which as fuel additives has increased over time, drawing the attention of many research in the field [27, 28, 31–33]. However, to our knowledge, there is no analytical method that allows determining all these VOCs concurrently into a biological matrix and, therefore, to assess the human exposure to them.

Therefore, the aim of the present research is to develop and validate a multiresidue method as a "one-step" assessment tool for biomonitoring studies performed on children, useful to determine low levels of BTEX, MTBE, ETBE, TAME, and DIPE in the matrix urine. For this purpose, an analytical procedure was developed using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS). HS-SPME and GC-MS analyses were optimized. The proposed method was validated by testing its linearity, precision, recovery, accuracy, and detection and quantitation limit values. Finally, an application "in field" of the developed assay was performed using urine samples of children exposed to different environmental levels of the target VOCs. Thus, according to the two major contributors of BTEX and ethers for children exposure, namely urban traffic and ETS, the analytical procedure was applied to analyze urine samples of (a) children not exposed to ETS and living in an area of very low traffic density, (b) children exposed to ETS and living in an area of very low traffic density, (c) children not exposed to ETS and living in an area of very high traffic density, and (d) children exposed to ETS and living in an area of very high traffic density.

Materials and methods

Chemicals, reagents, and other materials

A standard mixture containing BTEX, MTBE, ETBE, and TAME in methanol (0.201 ± 0.001 mg mL^{-1}) was purchased from Ultra Scientific (Bologna, Italy). A standard solution of benzene-d_6 in methanol (0.201 ± 0.001 mg mL^{-1}) was supplied by Supelco (Bellefonte, PA, USA). DIPE (≥99 %) and MTBE-d_3 (≥99 %) were obtained by Sigma-Aldrich (Milan, Italy). Analytical grade sodium chloride (NaCl) was delivered by Carlo Erba (Milan, Italy). Methanol of high purity grade was supplied by Merck (Darmstadt, Germany). Polypropylene specimen containers for urine collection were supplied by VWR (Milan, Italy); 10 and 500 μL syringes, 20 mL glass vials, septa (silicone/PTFE, 20 mm diameter), and perforated aluminum seals were purchased from Agilent (Santa Clara, CA, USA); 2 mL amber glass vials with screw

top, 85 µm carboxen/polydimethylsiloxane (CAR/PDMS) fibers, and solid-phase microextraction (SPME) fibers holder for manual use were obtained from Supelco.

Standard and samples preparation

DIPE and the standard mixture containing BTEX, MTBE, ETBE, and TAME were diluted with methanol and mixed to obtain a mixed stock standard solution of 10 analytes, each one at 0.2 mg L^{-1} concentration. This solution was freshly made and used for preparing working and calibration standards. MTBE-d_3 and the standard solution of benzene-d_6 were used to prepare the internal standard (IS) stock solution containing the two deuterated compounds at a concentration of 2.0 mg L^{-1} in methanol. This solution, prepared and stored in the dark at −20 °C until use, was added to all samples just before carrying out the analytical procedure. All methanolic stock solutions were transferred into 2 mL amber glass vials without headspace in order to minimize evaporation of the solvent and the analytes, especially during handling of the solutions.

A pool of urine collected from children low-exposed to the monitored compounds, as verified by preliminary analysis, was used for the preparation of blanks, calibration, and working standards. This urine matrix was used in place of purified water for compensating matrix effect. Beforehand, the pool of urine was flushed with nitrogen gas (purity grade equal to 99.9999 % v/v; from Alphagaz, Milan, Italy) to further decrease analytes concentration.

In order to apply the developed method в in field,^ additional 40 urine samples, used as unknown samples, were collected from children exposed to different levels of the monitored compounds according to their living environments and ETS exposure.

Blanks, working and calibration standards, and unknown urine samples were prepared in the same way, as follows: 20 mL glass vials containing 3 g of dried NaCl were filled with 14 mL of urine matrix and sealed with Teflon-lined septa and holes aluminum caps. Then, the following additional treatments were performed for preparing working and calibration standards as follows:

- 20 µL of the mixed stock standard solution (0.2 mg L^{-1}) was added to 14 mL aliquots of urine to obtain working standards containing all investigated compounds, each at a concentration of about 300 ng L^{-1}; these standards were used to develop and optimize the assay;
- proper amounts of mixed stock standard solution (0.2 mg L^{-1} of each analyte), ranging from 2.8 to 110 µL, were added to 14 mL aliquots of urine to obtain six calibration standards containing all the investigated compounds, each at a concentration of 40, 100, 400, 800, 1000, and 1500 ng L^{-1}, respectively. Each calibration level was prepared in triplicate and was used to draw calibration curves. Unspiked samples of the same pool of urine were kept as blanks. Two more calibration standards at concentrations of 200 ng L^{-1} and 600 ng L^{-1} were prepared in the same way (additional volume of mixed stock standard solution equal to 14 µL and 42 µL, respectively) for assessing the repeatability and the accuracy of the method.

All blanks, working, and calibration standards were prepared on the same day and stored at −20 °C. Similarly, unknown urine samples were collected and prepared on the same day and stored at −20 °C.

Before analysis, each standard or unknown urine sample was thawed at room temperature and then spiked with 2 µL of IS stock solution in order to obtain a constant urinary concentration of MTBE-d_3 and benzene-d_6 of about 300 ng L^{-1}.

Equipment

Investigations were carried out by means of a Trace GC ULTRA gas cromatograph coupled to a Focus DSQ mass spectrometer (Thermo Fisher Scientific, Rodano, Milan, Italy) running in electron impact ionization mode (EI, 70 eV, source at 250 °C). GC was equipped with a split-splitless injector, supporting an inlet liner for SPME, 0.75 mm i.d. (Supelco). Chromatographic separation was performed on a SPB-624 capillary column (30 m × 0.25 mm × 1.4 µm film thickness; Supelco) employing helium (purity grade ≥99.9999 % v/v) from Alphagaz as carrier gas. Xcalibur software (Thermo Fisher Scientific) was used for data acquisition/processing and instrument management. An aluminum block device (VWR) was used for providing agitation and temperature control during incubation process and HS-SPME sampling of monitored VOCs from urine.

GC-MS analysis

The GC split/splitless injection port operated in splitless mode at 240 °C. Thermal desorption of analytes from SPME was carried out for 0.2 min. During the desorption time, the liner split was closed; after this period, the liner was purged by GC carrier flow. Helium was used as carrier gas at 1 mL min^{-1} constant flow. The oven temperature program was as follows: 35 °C held for 5 min, ramped to 50 °C at 3 °C min^{-1}, and finally ramped to 230 °C at 30 °C min^{-1} and held for 4 min (total run time 20 min). The MS detection was performed in the following conditions: transfer line temperature 250 °C; solvent delay 5.4 min. The optimization of retention times and chromatographic resolution were carried out in scan mode using a standard solution at 1 µg mL^{-1} for each analyte; the ion range *m/z* (50−350) was scanned (1.6 scan s^{-1}) and the analytes were univocally characterized on the basis of their retention times and their mass-to-charge ratios. To quantify

the analytes in the urine samples, selected ion monitoring (SIM) mode was then chosen, and characteristic ions for each compound were selected in order to increase the sensitivity of the mass detection. The instrument operated in SIM mode in four acquisition windows with total scan times of 0.14 or 0.21 s, depending on the number of selected ions, and each one with a dwell time of 100 ms. The ions chosen for detection/quantification were those that exhibited the strongest intensities in the full-scan EI/MS spectra. Quantification was based on the ratio between the chromatographic peak area of the analyte and benzene-d_6 for BTEX and MTBE-d_3 for ethers, respectively. Benzene-d_6 and MTBE-d_3 used as ISs were monitored with *m/z* 84 and 76, respectively. The retention times, the chromatographic windows of acquisition, and the mass to charge ratio values selected to detect/quantify each investigated compound are listed in Table 1.

SPME optimization

Several factors can influence the analyte extraction efficiency as well as the expected concentrations of target compounds in children's urine are very small. Therefore, the development of SPME procedure for determination of urinary analytes generally requires the optimization of variables related to both extraction and desorption steps to provide acceptable peak areas [34]. In order to develop and optimize our multiresidue method, some parameters, which may affect the extraction, were preliminarily selected based on literature studies [35–39], on our previous researches [40–43], and laboratory experiences. Other parameters, such as desorption times, extraction temperatures, and extraction times have been investigated and optimized. For this purpose, we performed several preliminary experiments using working standard solutions at 300 ng L^{-1} of each analyte.

Thus, initially, according to the chemical characteristics of different commercially fiber coatings, scientific literature [44–48], and our preliminary experiments, 85 μm carboxen/polydimethylsiloxane fiber was used in order to assess the best extraction efficiency and the lowest equilibrium time. Before the use, the fiber was conditioned for 1 h in a GC injection port at 300 °C as recommended by the manufacturer. Then, every day before working, it was cleaned by heating in the injection port of the chromatographic system (15 min at 250 °C).

Secondly, headspace sampling was preferred to the fiber immersion because it has been proven to be more sensitive in extracting volatile compounds [49]. Besides, headspace technique is frequently chosen for analyzing complex matrices or unclean samples in order to avoid loss of performance due to the fast degradation of fiber coating and matrix effect [50, 51]. Fourteen mL of urine for each sample was considered to be a good compromise between restriction in collecting biological fluids and the positive influence of large volume on the response. The extraction was performed in the presence of salt (NaCl) to increase the ionic solution strength and decrease the aqueous solubility of organic chemicals, so as to improve the analyte partitioning from the aqueous solution to the headspace. On the other hand, urine is a very complex matrix, different salt concentrations of which may affect the correct analyte quantification. Therefore, to promote and standardize the transfer of analytes from the urine matrix to the gas phase in the vial headspace, the same quantity of NaCl (3 g) was added to all urine standards and unknown samples. Such amount, about 20 % w/v, was judged suitable for our purpose because it yielded the best results for all compounds (ionic strength increase and measurement repeatability). Preliminary tests showed that all the salt added to each vial dissolved completely during thawing at room temperature followed by manual agitation of the solution for 30 s. Sample agitation was also selected in this study and was maintained throughout the sampling time in order to accelerate the mass transport into the aqueous phase.

We tested three different desorption times (2, 1, and 0.2 min) with the GC injection port set at 240 °C. Since the investigated analytes are highly volatile compounds, the influence of the extraction temperature was evaluated sampling the

Table 1 GC/MS parameters for the investigated urinary compounds and internal standards (ISs)

	Retention time (min)	Chromatographic window (min)	Detecting ions (*m/z*)	Scan time (s)	Quantifying ion (*m/z*)
MTBE-d_3 (IS)	5.99	5.40–7.00	76–73	0.14	76
MTBE	6.05				73
DIPE	7.30	7.00–10.50	59–87	0.14	87
ETBE	8.25				59
Benzene-d_6 (IS)	10.68	10.50–12.60	73–78–84	0.21	84
Benzene	10.75				78
TAME	11.01				73
Toluene	12.98	12.60–16.00	91–106	0.14	91
Ethylbenzene	14.10				91
m,p-xylene	14.18				91
o-xylene	14.44				91

analytes at 30, 40, 50, 55, and 60 °C, always for 15 min. Then, in a second step of the HS-SPME optimization process, the extraction time was assessed, testing, respectively, 5, 15, 25, 35, and 45 min, always at 30 °C. Different time intervals were selected in order to keep the total extraction time compatible with the chromatographic run time.

The immersion depths of the fiber both into the headspace above the sample during extraction and the injector during desorption were always kept the same (1.5 cm into the headspace and 2.5 cm into the injector, respectively). After desorption, the fiber was still kept in the liner for an additional time to remove possible memory effects. If high concentrations of organic compounds were detected in urine samples, a blank injection of the fiber was performed to confirm removal of impurities from the GC system. When further regeneration for the fiber coating was necessary, it was performed by heat-cleaning in the injection port of a chromatograph (15 min at 250 °C) under high flow of inert nitrogen gas.

All injections with the SPME unit were performed manually and in triplicate.

Analytical performance

Linear range, limit of detection (LOD), limit of quantification (LOQ), inter- and intra-day precision, and accuracy at different concentration levels were investigated for the validation of the HS-SPME/GC-MS method under the optimized conditions. The linearity of the proposed method was studied for all the analytes of interest over a concentration range corresponding to the expected variation of the analytes content in the unknown samples (0−1500 ng L^{-1}). The IS calibration curves were constructed with six calibration standards covering the aforementioned range. Thus, calibration standards were sequentially analyzed in order of crescent concentrations. Each analysis was performed in triplicate in order to generate an average value for each point. Least-squares linear regression analysis was used to fit experimental data and estimate the slopes (b) and intercepts (a) of the calibration curves $y = bx + a$, where x is the nominal concentration (ng L^{-1}) in spiked calibration standards and y is the ratio of chromatographic peak areas between each analyte and the IS. The calculated six-point linear plots were used for the quantification of analytes.

LOD and LOQ were calculated as follows:

LOD ¼ xm þ 3SD
LOQ ¼ xm þ 10SD

where xm corresponds to the arithmetic mean of sample blank values obtained by the analysis of 15 consecutive blanks, and SD was the standard deviation of xm [52].

The precision of the method was evaluated based on its repeatability. Intra-day precision expressed as percent of variation (CV%) was determined by analyzing five replicates for each concentration of two calibration standards (200 and 600 ng L^{-1}) on the same day. The same processing procedure was repeated in three different days in a week in order to test the inter-day precision of the method and to evaluate procedure robustness. Accuracy, expressed as percent of recovery (% Rec), was calculated as percent ratio between the value calculated from the calibration curve and the theoretical value, respectively.

Analytes stability in urine matrix

The analyte loss due to evaporation/degradation, possibly occurring between the sample collection and the analytical determination, was evaluated. A set of 12 working standards at a concentration of 300 ng L^{-1} was prepared on the same day starting from a freshly made mixed stock standard solution. Three working standards were immediately analyzed, whereas the remaining nine were stored in the same conditions as for real samples (−20 °C in the dark) and analyzed in triplicate after 3, 6, and 9 mo, respectively. Stability was expressed as mean % ± SD of loss compared with the values obtained at zero time.

Application in field of the optimized HS-SPME/GC-MS

The assay was applied to the analysis of BTEX and ethers as biomarkers of exposure to traffic-related pollution and to ETS, on urine samples from a group of 40 children (5- to 11 y old). We selected four groups of children (10 subjects for each group) as follows:

- children living in an area of very low traffic density and not exposed to ETS;
- children living in an area of very low traffic density and exposed to ETS;
- children living in an area of very high traffic density and not exposed to ETS;
- children living in an area at very high traffic density and exposed to ETS.

Traffic density was evaluated according to the municipal data, whereas ETS exposure was assessed by parental questionnaire: children were considered to be exposed to ETS if they lived in households where at least one person was a smoker. The questionnaire also required information about sociodemographic characteristics, lifestyle factors, living environment, ETS exposure, and activities engaged in on the sampling day for each participant.

In order to study the feasibility of the developed method, one urine sample for each child was collected in the evening (the last urine before going to sleep) in a benzene-free polypropylene bottle and immediately stored in the refrigerator at 0−4 °C. The next morning, the sample was placed into a polystyrene cooler containing an ice pack and transported to the laboratory.

After coding, samples were prepared as reported above (standard and samples preparation section) stored at −20 °C until analysis, which was performed within 30 d from collection.

The protocol of this part of the study was approved by the local Ethical Committee. Informed consent was signed by both parents of each participant and by older children (9- to 11 y old).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22 software (IBM Corp., Armonk, NY, USA). Values below LOQ were designated as half the LOQ value. In order to assure normal distribution, all data were log transformed. In order to assess differences in HS-SPME optimization (sampling times and temperatures) and stability of standard solutions and to compare the results of the ʁin field^ study, we used Student's *t*-test or ANOVA one-way with post hoc Bonferroni test. The results were considered statistically significant when p-values were <0.05.

Results and discussion

Method development

GC-MS detection

Figure 1 shows a GC-MS (SIM mode) chromatogram of a calibration standard solution (400 ng L^{-1}) obtained under optimized working conditions. The resolution was considered

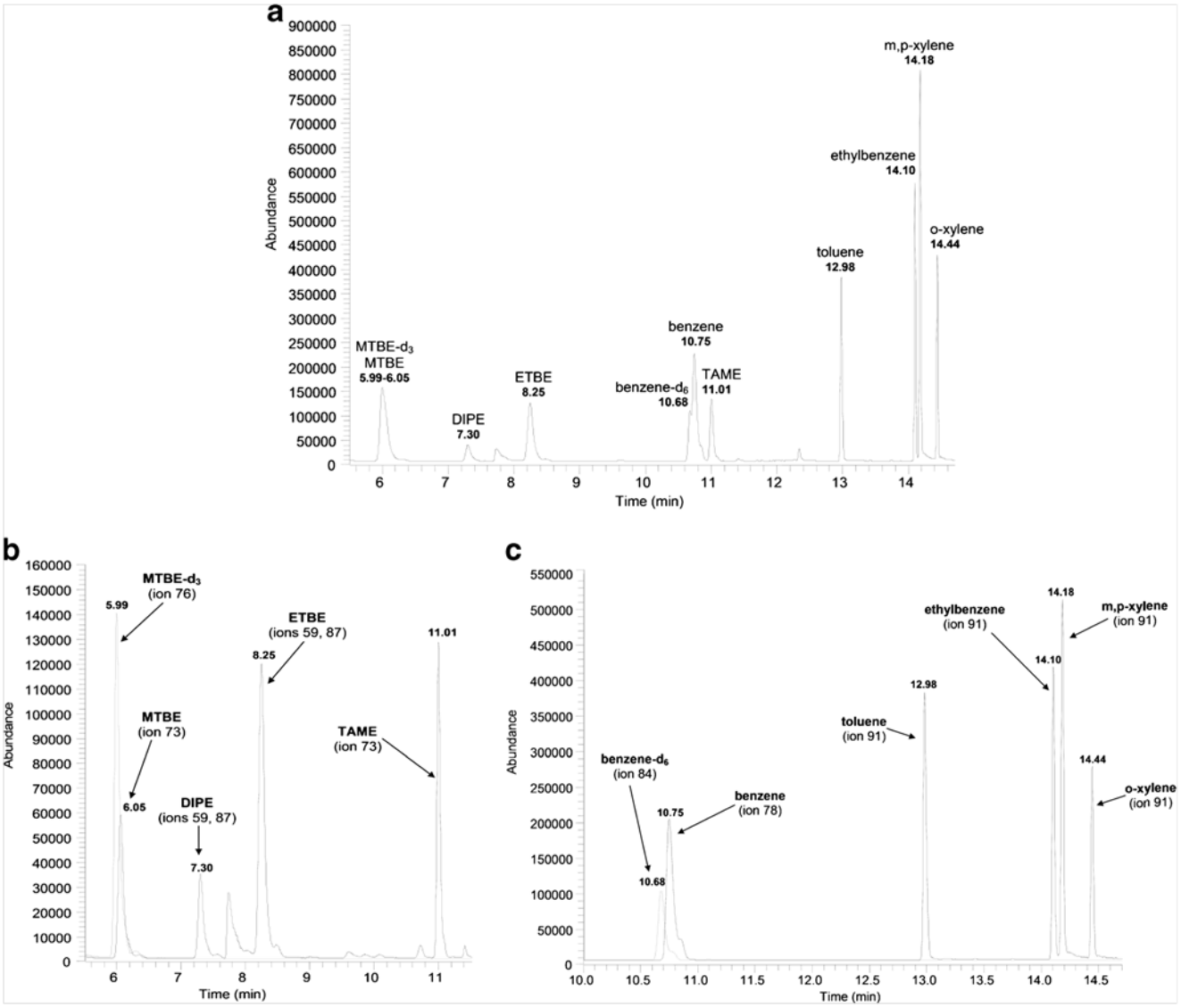

Fig. 1 Chromatogram of a calibration standard at concentration of 400 ng L^{-1}: (A) total ion chromatogram (TIC); (B) ethers − extract ion chromatogram (EIC): m/z = 76 (MTBE-d_3, RT = 5.99 min); m/z = 73 (MTBE, RT = 6.05 min; TAME, RT = 11.01 min); m/z = 59 (DIPE, RT = 7.30 min); m/z = 87 (ETBE, RT = 8.25 min); (C) Aromatics − EIC: m/z = 84 (benzene-d_6, RT = 10.68 min); m/z = 78 (benzene, RT = 10.75 min); m/z = 91 (toluene, RT = 12.98 min; ethylbenzene, RT = 14.10 min; m,p-xylenes, RT = 14.18 min; o-xylene, RT = 14.44 min)

satisfactory: the peaks of MTBE, DIPE, ETBE, benzene, TAME, toluene, ethylbenzene, and *o*-xylene were clearly detected. *m*- and *p*-xylenes were not resolved, and consequently they were calculated together as reported by other researchers [53]. When the developed method was used to quantify the monitored compounds in urine samples collected from 40 children, *o*-, *m*-, and *p*-xylenes were presented as the sum of the three isomers because from a toxicologic point of view, it is relevant to evaluate the average daily intake of total xylenes. Indeed, xylenes are present as a mixture in fuels and in other products such as paint, coatings, etc; thus, the general population is expected to be exposed mainly to xylenes as a mixture rather than to the individual isomers [54].

HS-SPME procedure

HS-SPME technique is particularly suited for the isolation of organic pollutants from complex matrices and has become the best method for carrying out simultaneous extraction and concentration of many compounds in urine samples. Indeed, this extraction technique is solvent-free and very simple because it enables the injection of the entire extracted sample with no further sample preparation step; it allows an important reduction of sample manipulation. However, as several factors can impact the extraction efficiency of the monitored analytes, it is necessary to optimize the HS-SPME procedure [34].

The optimization steps for the extraction and desorption conditions were performed by using the real matrix, in order to ensure the best extraction efficiency (highest chromatographic peak area) for all selected analytes in terms of extraction temperature, and extraction and desorption times.

We preliminary observed (data not shown) that the decrease of the desorption time from 1 to 0.2 min determined only a 5 % drop in the measured peaks areas but, simultaneously, it caused a great chromatographic resolution enhancement. Because the most part of analytes desorption was achieved within 0.2 min, longer times were not taken into consideration to avoid peak tailing phenomenon and resolution decrease. The fiber was then left at 240 °C in the GC injection port for an additional 5 min during the injection purging to complete the analyte desorption and to eliminate memory effects. Fiber blank injections confirmed the efficacy of this post-injection cleaning procedure.

Once the desorption time was selected, two more parameters were optimized: the extraction temperature and the extraction time. Results of the HS-SPME optimization for the extraction temperatures and times are shown in Figure 2. The temperature increase from 30 to 60 °C, at the same extraction time conditions, determines a significant decreasing trend in the extraction

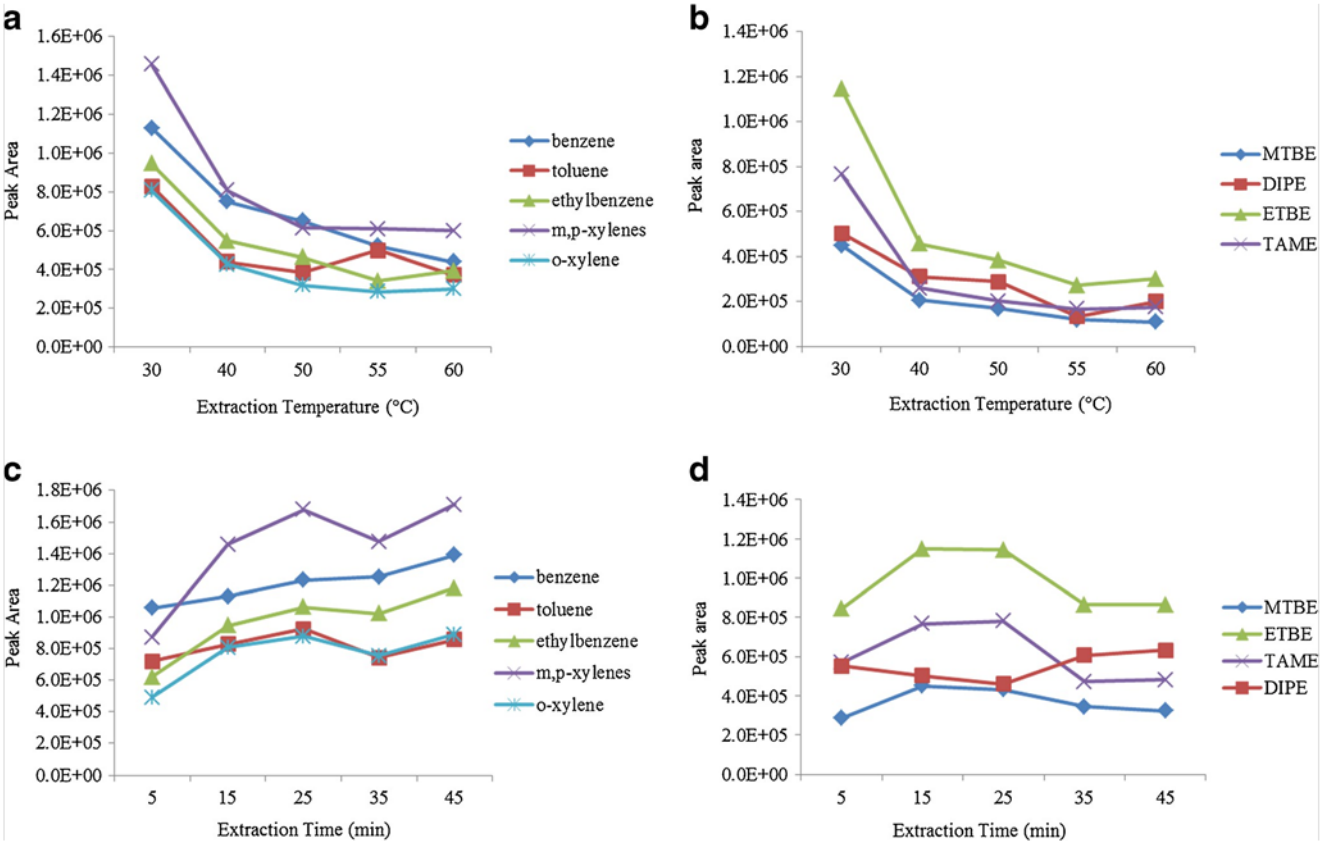

Fig. 2 HS-SPME parameter optimization using a CAR/PDMS-85 μm fiber, 3 g of NaCl and agitating the sample. Influence of extraction temperature (graph A for BTEX and graph B for ethers) and extraction time (graph C for BTEX and graph D for ethers) on the extraction efficiency of the fiber

Table 2 Correlation coefficients, limits of detection (LOD) and quantification (LOQ) for the determination of urinary analyte using the proposed method

	R^2	LOD (ng L^{-1})	LOQ (ng L^{-1})
MTBE	0.9997	4.9	5.4
DIPE	0.9988	4.9	6.1
ETBE	0.9972	4.7	5.3
benzene	0.9995	6.1	7.4
TAME	0.9979	2.3	2.9
toluene	0.9994	6.3	7.3
ethylbenzene	0.9993	5.7	6.8
m,p-xylene	0.9995	5.9	7.4
o-xylene	0.9997	5.6	6.8

efficiency ($p < 0.05$ for each analyte); the lowest tested temperature was found to yield the best analytical signal for all compounds. These results are in agreement with the findings of Prado et al. [55]. The most probable cause of the drop in analytes recovery at higher temperatures could be related to the competitive adsorption (by the fiber) of a large amount of lowest volatile compounds usually present in the urine matrix.

As regard to the tested extraction times, it was observed that the analytical signal for BTEX compounds was significantly enhanced for increasing times ($p < 0.05$), whereas MTBE, ETBE, and TAME showed an inverse trend, and DIPE had a fluctuating trend. Based on these results, an extraction time of 15 min was chosen as the best compromise to obtain a good peak response for all monitored analytes. After optimization experiments, the entire procedure was performed under the following working conditions: after being thawed at room temperature, each sample was kept under agitation at 30 °C in a block heater device for 60 min before sampling. Then, the SPME fiber was exposed to the vial headspace, and analytes were extracted for 15 min.

Analytical performance

Once the optimum working conditions were established, the validation of the proposed method was carried out in terms of linearity range, LODs and LOQs, precision, and accuracy. Correlation coefficients (R^2), LODs and LOQs for each analyte are reported in Table 2. The application of regression line with least-squares method to experimental points showed a linear range behavior over the entire concentration range tested for all analytes leading to R^2 ranging from 0.9972 for ETBE to 0.9997 for *o*-xylene and MTBE. The highest LOD and LOQ values (equal to 6.3 and 7.4 ng L^{-1}, respectively) underline that the developed method is able to quantify very low concentrations of monitored compounds in urine samples, as it is advisable for its practical application.

Table 3 shows recoveries, intra-day and inter-day precision obtained in urine at two spiking levels (200 and 600 ng L^{-1}). Values highlight good accuracy and precision of the developed method. Indeed, the %Rec values are satisfactory

Table 3 Average recovery (%Rec) and Coefficient of Variation (CV%) at two concentration levels (200 and 600 ng L^{-1}), of the developed method for the determination of monitored compound

	Investigated range (ng L^{-1})	Day 1		Day 2		Day 3		Overall	
		CV%	%Rec	CV%	%Rec	CV%	%Rec	CV%	%Rec
MTBE	200	1.89	105.0	2.52	105.2	3.00	103.2	0.96	104.5
	600	1.86	98.4	1.26	99.1	1.33	97.8	0.61	98.4
DIPE	200	3.22	86.4	5.24	89.6	7.30	88.3	1.89	88.1
	600	3.94	90.2	3.47	91.7	3.55	90.9	0.84	90.9
ETBE	200	5.68	92.1	2.89	95.5	7.55	93.7	2.23	93.7
	600	2.83	86.6	3.19	94.2	1.04	94.9	1.35	91.9
benzene	200	3.88	102.6	2.84	96.9	6.03	95.4	3.56	98.3
	600	2.93	98.9	1.58	97.9	3.14	97.8	0.59	98.2
TAME	200	4.69	98.2	2.95	99.5	3.99	99.1	0.79	98.9
	600	2.91	100.4	3.33	100.6	1.89	100.9	0.28	100.6
toluene	200	2.43	102.7	2.32	99.9	4.69	98.1	2.03	100.2
	600	1.59	102.2	1.76	102.5	2.56	103.4	2.56	102.5
ethylbenzene	200	3.54	91.9	5.09	90.2	6.15	89.6	1.25	90.6
	600	2.69	101.0	1.24	103.2	2.07	104.2	1.52	102.8
m,p-xylene	200	2.18	93.4	5.75	92.1	5.39	90.7	1.37	92.1
	600	2.61	103.3	2.05	103.9	1.92	104.5	0.57	103.9
o-xylene	200	2.01	92.8	4.34	93.8	4.78	93.9	0.67	93.5
	600	2.98	101.1	2.64	101.4	2.71	102.6	0.78	101.7

Table 4 Stability of monitored compounds in working standards stored at −20 °C in the dark for 3, 6, and 9 mo, expressed as mean % ± SD of loss compared with the values obtained at zero time

Months	3 mean % ± SD	6	9
MTBE	9.6 ± 1.4	19.9 ± 3.0	27.2 ± 5.2
DIPE	5.7 ± 0.7	12.4 ± 2.3	20.6 ± 4.4
ETBE	6.3 ± 0.8	6.4 ± 1.1	18.1 ± 4.0
benzene	2.5 ± 0.2	2.4 ± 0.4	5.9 ± 1.5
TAME	11.1 ± 2.4	10.5 ± 1.8	25.2 ± 6.0
toluene	3.1 ± 0.1	1.7 ± 0.3	5.4 ± 1.8
ethylbenzene	3.7 ± 0.3	0.7 ± 0.3	1.6 ± 0.9
m,p-xylene	7.3 ± 1.0	3.5 ± 0.5	3.9 ± 0.9
o-xylene	6.5 ± 0.9	3.5 ± 0.8	2.6 ± 0.7

because they always result close to 100 % with very low relative SDs. Also, CV% ranges between 0.28 and 3.56 % for all studied concentrations and for all compounds.

Analytes stability in urine matrix

The influence of the storage time can affect the analyte concentrations. For investigating this effect, experiments at different storage times (0, 3, 6, 9 mo, respectively) were carried out. The results, shown in Table 4, demonstrate that the difference between each analyte's values found at time 0 and those recovered after 9 mo range between 0 and 30 %, depending on the specific compound. In particular, aromatic compounds are relatively stable, and no significant concentration changes

Table 5 Urinary levels of monitored compounds, expressed as median (min − max) in ng L^{-1}, among children grouped according to traffic intensity of residence area and environmental tobacco smoke (ETS) exposure

		Children (N)					
		Living in low traffic area		Living in high traffic area		*p*-value[a]	*p*-value[b]
Monitored compounds		Not exposed to ETS (10)	Exposed to ETS (10)	Not exposed to ETS (10)	Exposed to ETS (10)		
MTBE	Median	8.6	13.5	18.0	18.7	0.007	0.489
(N < LOQ= 6)	Min	<LOQ	<LOQ	6.7	8.1		
	Max	26.7	135.2	75.7	34.1		
	p-value[c]	0.253		0.765			
DIPE	Median	<LOQ	<LOQ	<LOQ	<LOQ	-	-
(N < LOQ= 40)	Min	<LOQ	<LOQ	<LOQ	<LOQ		
	Max	<LOQ	<LOQ	<LOQ	<LOQ		
	p-value[d]	-		-			
ETBE	Median	<LOQ	6.1	6.2	7.7	0.462	0.358
(N < LOQ= 14)	Min	<LOQ	<LOQ	<LOQ	5.8		
	Max	25.4	25.2	29.8	43.9		
	p-value[d]	0.650		0.497			
Benzene	Median	33.6	59.1	66.8	137.5	<0.001	0.006
(N < LOQ= 0)	Min	13.8	38.0	28.1	41.9		
	Max	40.8	101.8	148.3	314.0		
	p-value[d]	<0.001		0.019			
TAME	Median	<LOQ	<LOQ	<LOQ	<LOQ	0.474	0.506
(N < LOQ= 23)	Min	<LOQ	<LOQ	<LOQ	<LOQ		
	Max	11.4	13.5	14.0	5.4		
	p-value[d]	0.424		0.599			
Toluene	Median	108.4	206.4	81.5	106.8	0.416	0.253
(N < LOQ= 0)	Min	40.9	61.7	41.3	52.4		
	Max	488.2	315.3	225.4	238.4		
	p-value[d]	0.184		0.090			
Ethylbenzene	Median	27.5	30.7	51.5	69.1	0.045	0.014
(N < LOQ= 0)	Min	18.3	18.6	19.3	17.2		
	Max	57.3	69.3	213.7	247.0		
	p-value[d]	0.587		0.460			
o,m,p-xylenes	Median	77.6	82.8	136.3	153.8	0.036	0.036
(N < LOQ= 0)	Min	39.8	53.8	48.9	56.6		
	Max	129.9	177.9	261.7	312.1		
	p-value[d]	0.122		0.289			

[a] Unpaired *t*-test was used to compare low traffic and high traffic areas (ln-transformed data) in children not exposed to ETS.

[b] Unpaired *t*-test was used to compare low traffic and high traffic areas (ln-transformed data) in children exposed to ETS.

[c] Unpaired *t*-test was used to compare not exposed and exposed to ETS (ln-transformed data) living in low traffic and high traffic areas, respectively.

within the mentioned period are noticed (<10 % loss). These results agree with those reported previously [23]. Conversely, all the concentrations of the investigated ethers decrease over the entire 9-mo period; after 3 mo the decrease is not significant and ranges between 5.7 and 11.1 %. Higher decreases are found after 9 mo, with a maximum of 27.2 % for MTBE ($p < 0.05$). For this reason, it would be advisable to analyze unknown samples within at most 3 mo of storage at −20 °C.

Application in field of the optimized HS-SPME/GC-MS procedure

The method developed and optimized in the present study was used for quantifying selected VOCs in urinary samples collected from 40 children exposed to different levels of the monitored compounds according to living environments and ETS exposure. The results are reported in Table 5. Notice that DIPE and TAME are detected at very ultra-trace levels; in many samples their concentration is below the LOQ. It is presumable that these findings are related to the low use of these ether compounds in Italian fuels. Indeed, other indicators of exposure to vehicular traffic pollutants, such as MTBE, benzene, ethylbenzene, and xylenes, resulted significantly higher for children not exposed to ETS and living in high traffic area compared with those living in low traffic area, with *p*-values equal to 0.007, <0.001, 0.045, respectively, and 0.036 for children not exposed to ETS. These results were confirmed also for children exposed to ETS, except for MTBE. However, mean urinary MTBE concentration of children exposed to ETS and living in high traffic environment were 1.5 times higher than those recovered for children exposed to ETS and living in low traffic environment; it is presumable that an increase in the number of monitored individuals could reveal more significant differences. Thus, it should be relevant to apply the developed assay to a large number of children.

Furthermore, significant differences were observed in the excretion of urinary benzene in children exposed to ETS and to those not exposed, considering both low and high traffic areas of residence. This result still confirms the utility of urinary benzene as tobacco-related carcinogen biomarkers, as previously reported [24].

Conclusions

The HS-SPME GC-MS method developed for the determination of 10 VOCs with a unique chromatographic analysis shows good analytical performance and is reliable and efficient for detecting and quantifying urinary compounds at trace/ultra-trace concentrations. At the same time, it is a simple and economic procedure. Thus, this assay represents an assessing tool useful for studies performed for evaluating the exposure to environmental pollutants of pediatric populations (a scenario of very low exposure). Finally, the cost-effective requisite of the method makes it suitable also in the case of high sampling size, which is advisable for biomonitoring studies.

Acknowledgements The authors are thankful to the Sapienza University of Rome for financial support of the study. Grant 2014 prot. N. C26A14AZYY.

Compliance with Ethical Standards

Conflict of Interest The authors certify that they have no affiliations or involvement with any organization or entity with any financial or nonfinancial interest in the subject matter or materials discussed in this manuscript.

Research involving Human Participants The protocol of the study involving human participants was approved by local Ethical Committee (Policlinico Umberto I/Sapienza University of Rome; protocol code 2894).

Informed consent Since participants were children aged 5- to 11 y old, informed consent was signed by both parents of each participant and by older children (9- to 11-y old).

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CHAPTER 4

ASSESSING INDOOR AIR QUALITY OF SCHOOL ENVIRONMENTS: TRANSPLANTED LICHEN PSEUDOVERNIA FURFURACEA AS A NEW TOOL FOR BIOMONITORING AND BIOACCUMULATION

Protano C, Owczarek M, ***Antonucci A***, Guidotti M, Vitali M. *Assessing indoor air quality of school environments: transplanted lichen Pseudovernia furfuracea as a new tool for biomonitoring and bioaccumulation*. Environ Monit Assess. 189 (2017); 358. DOI: 10.1007/s10661-017-6076-2.

Framework of the present study of my research program

Alternative air quality monitoring tools

In order to control chemicals emissions and prevent the risks for public health derived from exposure to air pollutants, the assessment of air quality by continuous monitoring of these compounds in environmental matrices is one of the main strategies. The concentrations measured at air monitoring quality stations, which work continuously for short periods, provide useful temporal data, though referred to limited time intervals (sampling time). Anyway, once emitted in atmosphere, organic pollutants tend to be rapidly diluted by air, reaching such low levels to make difficult and expensive their detection in sampled air with the use of conventional instruments. Besides, the use of conventional instruments in selected point monitoring stations presents several limitations when wide areas have to be inspected. Frequently, instrumental monitoring stations are only placed where emissions of atmospheric pollutants are expected to be significant (industrial or urban station) or where the background values should be used as a reference. The use of biomonitors, such as mosses and lichens, as passive collectors of atmospheric pollution has been increasing in recent years since they can provide a measure of integrated exposure over a given time interval with high spatial resolution (Villeneuve et al., 1988; Calamari et al., 1991; Garty, 1993-2001; Conti et al., 2001; Owczarek et al., 2001; Wolterbeek, 2002; Szczepaniak et al., 2003; Guidotti et al., 2003; Zechmeister et al., 2004; Basile et al. 2008; Augusto et al., 2010; Wu et al., 2014). Among bioindicators, lichens, resulting of a symbiotic association of a fungus and an alga and/or a cyanobacterium, have been proved to be useful tools for monitoring airborne pollutants that they absorb directly from the air, as well as water and nutrients, because of lacking roots. The high ratio of surface to volume of their tissues, other than their slow growth, helps the accumulation of pollutants. Trace elements can accumulate in the cell wall and outer surface of the plasma membrane, at intracellular sites or as particles on the surface (Bargagli, 1998). These living organisms are abundant and widely distributed because of their tolerance and sensitivity to a wide range of pollutants and weather conditions, thus providing suitable spatial resolution data. These factors suggest that lichens can operate both as efficient bioindicators, by mapping frequency and cover of all species present in a specific area, and as bioaccumulating organisms employable as biomonitors to trace pollutants originated in different environmental contexts. The most toxitolerant species have been widely used for more than 30 years for assessing the atmospheric deposition of trace elements and radionuclides in polluted areas (Garty and Ammann, 1987; Biazrov, 1994; Nimis and Bargagli, 1999; Carreras and Pignata, 2002). More recent campaigns have shown the lichens ability in accumulation of

detectable concentrations of a wide range of air pollutants (e.g. metals, PAHs, PCBs) inside their tissues. The significant correlation between most of the chemicals accumulated by lichens and relative air concentrations evidences their ability to represent atmospheric contamination (Scerbo et al., 2002; Brunialti et al., 2007; Shulka et al., 2009-2012; Van der Watt et al., 2015; El Rhzaoui et al., 2015; Douibi et al., 2015; Parzych et al., 2016). The gathering capacity is influenced by the characteristics of compounds (such as solubility, vapor pressure, coefficient of distribution between the gaseous phase and the solid phase), lichen species features, climate conditions (mainly temperature and precipitations) and time of exposure of lichen to pollutants (Baptista et al., 2008; Augusto et al., 2013). Pollutants accumulation can be assessed in native species over a long time, through the individual sampling of lichen species and measurement of pollutants that accumulate in the thallus. Alternatively, lichens transplanted from an uncontaminated area to a contaminated one (lichen bags), used when lichens are rare or absent in the investigated area (Bergamaschi et al. 2007; Ayrault et al., 2007; Sorbo et al., 2008), could be advantageously placed to monitor air pollution in arduous sites. Transplant technique allows the precise selection of the sampling points for a biomonitoring campaign, but lichens employed in such cases work better as indicators for short-time monitoring, until three-six months exposure to pollutants, depending on the features of the monitored environment (geographic area, weather conditions, temperature, degree of humidity).

Pseudovernia furfuracea lichen species : monitoring at school buildings

Several environmental biomonitoring works are known for using the epiphytic lichen species *Pseudovernia furfuracea* in the assessment of atmospheric agents in different countries, proving its ability to provide a direct correlation with pollution levels **(**Nascimbene et al., 2014; Loppi et al., 2015; Malaspina et al., 2014). This foliose species is easy to collect and presents a large contact surface for atmospheric pollutants. Most remarkable, significant correlations between trace element and air pollutants contents were found in *P. furfuracea* bags and their atmospheric concentrations (Bari et al., 2001; Giordano et al., 2005; Guidotti et al., 2009; Protano et al., 2014-2015). These data are indicative of the effectiveness of this lichen as a bioaccumulator and its ability to assess air contamination. It would be appreciable to have the prospect of employing routinely this lichen species in closed environments, especially in the case of places that are considered sensitive, such as schools. It is now official finding that indoor air quality and ventilation in school buildings may affect the health of the children (Simons et al., 2011; Choo and Jalaludin, 2015). This is mainly because children spend most of their time in school buildings whose indoor concentrations are typically higher than outdoor ones. Consequently, the constant

monitoring of air quality in these environments represents an essential strategy in order to control and minimize child exposure and then to protect the health of children.

Our study: the use of transplanted lichen for monitoring indoor air quality

In the present study, we tested the suitability of transplanted lichen *P. furfuracea* to successfully biomonitor and bioaccumulate typical urban airborne pollutants in urban indoor environments and to represent an alternative tool for the constant monitoring of indoor air quality in schools environments. In particular, the accumulative nature of lichens is advantageous over direct air sampling because they provide concentrations of trace levels of air pollutants that would be otherwise detectable with the exclusive use of sensitive instrumentation. This is important when studying these pollutants because their associated health risks are often related to chronic exposure. In addition, such alternative monitoring tool would be efficient, applicable in locations that are less accessible to bulky equipment, cheap, educational for attending children and less difficult to use in schools than standard air sampling methods. We used the transplanted lichens biomonitoring, a technique usually applied for outdoor studies, both indoor and outdoor of school environments in order to get parallel information that are important to identify pollution sources and assess outdoor contribution to the indoors. Anyway, lichen bags exposed in indoor environments may be subject to physiological stress determined by specific conditions, such as temperature and humidity, so to negatively modify their bioaccumulation process. One of the main goals was therefore to evaluate the indoor functionality of these transplanted lichens by assessing their ability to reveal chemical level differences arising from the investigated environments, each of which is characterized by different pollution sources. For our study, we transplanted 14 bags of *P. furfurac*ea, both indoor and outdoor, at five primary school districts located in urban (Rome) and rural (Rieti province) areas, for two months (February – April 2014). We examined the concentrations of 19 contaminants related to vehicular traffic, which is supposed to be the main source of pollution in urban areas, in order to assess pollution sources that contribute to a poor indoor air quality in schools. Specifically, after the proper extraction procedure, graphite furnace - atomic absorption spectrometry was used to quantify 7 metals (As, Cd, Cr, Cu, Hg, Ni, and Pb), while gas chromathography - mass spectrometry coupled technique was used to investigate on air concentrations of 12 selected polycyclic aromatic hydrocarbons (PAHs) (fluorene [Flu], phenanthrene [Phen], anthracene [Anth], fluoranthene [Fla], pyrene [Pyr], benzo(a)anthracene [B(a)A], chrysene [Chry], benzo(b,k)fluoranthene [B(b,k)F], benzo(a)pyrene [B(a)Py], indeno(1,2,3cd)pyrene [IP], benzo(g,h,i)perylene [B(g,h,i)P] and dibenzo(a,h)anthracene [D(a,h)A]). Except for Cd, Fla, Pyr and Chry, all monitored analytes were mostly higher than those recovered in control sample, both in the highly urban area and in all rural areas. The higher

levels found in urban samples with respect to those in rural samples assured the ability of this lichen species to accumulate air pollutants at rates that are generally proportional to traffic density. Several differences were found between urban and rural school environments: in urban settings, except for Cd and B(a)Py the outdoor levels of pollutants were higher than the indoor settings; instead, in rural settings Hg and 10 PAHs showed higher indoor levels. These differences can be justified considering the different levels of outdoor air pollutants that are strongly influenced by vehicular traffic in urban areas, but do not seem to influence the air quality of the indoor school environments. On the other hand, in rural areas, probably due to the scarce air exchanges conditioned by the adverse weather conditions, outdoor atmospheric pollutants strongly influenced the indoor contaminants concentrations. However, to better understand the discrete contribution of the microclimatic conditions to the accumulation process, it would be interesting to repeat the same monitoring campaign in a warmer season, when the internal heating is off and the indoor and outdoor climatic conditions are similar. Moreover, to understand what is the best exposure range window, it would be interesting to repeat the monitoring campaign by placing different samples of lichens at the same time, and then removing each of them individually at different times. Our final results proved the ability of transplanted *P. furfuracea* to bioaccumulate metals and PAHs, both outdoors and indoors and confirmed the feasibility of using this lichen species to discriminate between different levels of air pollution related both to urbanization extent and peculiarities of accommodation environments. These results are greatly significant because they open the doors to the employment of transplanted lichen strategy in air quality monitoring programs in school scenarios. The availability of this new easy tool should be useful to improve air quality monitoring programs in school environments and thus support the advancement of child health prevention interventions.

Environ Monit Assess (2017) 189:358
DOI 10.1007/s10661-017-6076-2

Assessing indoor air quality of school environments: transplanted lichen *Pseudovernia furfuracea* as a new tool for biomonitoring and bioaccumulation

Carmela Protano & Malgorzata Owczarek & Arianna Antonucci & Maurizio Guidotti & Matteo Vitali

Received: 25 October 2016 / Accepted: 15 June 2017

Abstract The aim of this research is to evaluate the ability of transplanted lichen *Pseudovernia* (*P*). *furfuracea* to biomonitor and bioaccumulate in urban indoor environments. The elements As, Cd, Cr, Cu, Hg, Ni and Pb and 12 selected polycyclic aromatic hydrocarbons (PAHs) were used to assess *P. furfuracea* as a biomonitoring tool for the indoor air quality of school environments. To achieve this purpose, lichen samples were exposed for 2 months in the outdoor and indoor environments of five school settings located in urban and rural areas. The results demonstrated that transplanted lichen *P. furfuracea* is a suitable biomonitoring tool for metals and PAHs in indoor settings and can discriminate between different levels of air pollution related to urbanisation and indoor conditions, such as those characterised by school environments. A transplanted lichen biomonitoring strategy is cost-effective, ʙgreen^, educational for attending children and less ʙinvasive^ than traditional air sampling methods. The feasibility of indoor monitoring by *P. furfuracea* is a relevant finding and could be a key tool to improve air quality monitoring programmes in school scenarios and thus focus on health prevention interventions for children, who are one of the most susceptible groups in the population.

Keywords Indoor air quality · School · Heavy metals · Persistent organic pollutants · Biomonitoring · Lichen

C. Protano : A. Antonucci : M. Vitali (*)
Department of Public Health and Infectious Diseases, Sapienza University of Rome, Piazzale Aldo Moro, 5, 00185 Rome, Italy
e-mail: matteo.vitali@uniroma1.it

M. Owczarek : M. Guidotti
Arpa Lazio, Regional Agency for Environmental Protection, Sede di Rieti, Via Salaria per l'Aquila, 8, 02100 Rieti, Italy

A. Antonucci
Department of Ecological and Biological Sciences, Tuscia University, Via S. Maria in Gradi, 4, 01100 Viterbo, Italy

Introduction

Indoor air quality is an issue of great concern for public health, as evidenced by the increase in attention and the number of studies performed by researchers in this field over time. This interest is related to two essential reasons: first, the indoor concentration of air pollutants is typically higher than those of outdoor air (WHO 2010), and second, people spend most of their time (>90%) in confined environments (Hellweg et al. 2009). In this context, the school environment and its air quality are of particular importance because young people (especially the youngest groups) represent a portion of the population who are more susceptible to exposure to environmental pollutants, including those that contaminate outdoor and indoor air.

Several studies have shown that indoor air pollution in school environments can negatively affect the health of ʙattending actors^ and that having a healthy air quality in schools is essential to offer a safe, productive and comfortable environment for students, teachers and other employers (Annesi-Maesano et al. 2013). Consequently, the assessment of school air quality represents an essential strategy to protect the health of these

Published online: 27 June 2017

subjects. However, standard sampling methods generally used for monitoring indoor air quality (air pumping, air filters, etc.) can be difficult to use in schools because of the sampling apparatus characteristics (for example, noise) that can distract students and teachers during lessons (Canha et al. 2012, 2014). Thus, studies have been carried out to assess the suitability of alternative tools for the monitoring of air quality in schools (Annesi-Maesano et al. 2013). Different plant species have demonstrated this ability and can be used as bioindicators (Hijano et al. 2005; Suzuki et al. 2008) or bioaccumulators of airborne contaminants (Augusto et al. 2007, 2015; Bermudez et al. 2008; Blasco et al. 2008; Loppi et al. 2015). Results have shown that lichens are efficient bioindicating and bioaccumulating organisms because they are perennial, grow slowly and do not change their morphology over time. Moreover, lichens have no roots and adsorb water and nutrients directly from the air while co-adsorbing other airborne substances, including pollutants.

In our previous studies, we have demonstrated a valid alternative method for biomonitoring airborne contamination in several outdoor scenarios by using lichen *Pseudovernia* (*P.*) *furfuracea* through a transplanted technique (Guidotti et al. 2009; Protano et al. 2014, 2015). In indoor environments, some specific conditions (i.e., temperature and humidity) may determine a physiological stress that affects the bioaccumulation process in lichens (Canha et al. 2012). In this regard, some recent studies (Canha et al. 2012, 2014) have demonstrated that transplanted lichen *Flavoparmelia caperata* can be used to biomonitor inorganic air contaminants in indoor environments. Based on our knowledge, scientific evidence in this field remains scarce, and new findings are needed to evaluate other species of lichen and their ability to biomonitor and bioaccumulate both inorganic air contaminants and other types of typical indoor airborne pollutants. It is well known that the main source of air pollution in urban areas is vehicular traffic and that heavy metals and polycyclic aromatic hydrocarbons (PAHs) are the main contaminants related to this source. The aims of the present research study are (1) to evaluate the ability of transplanted lichen *P. furfuracea* to biomonitor and bioaccumulate typical airborne pollutants (As, Cd, Cr, Cu, Hg, Ni, Pb and 12 selected PAHs) in indoor urban environments and (2) to assess *P. furfuracea* as a biomonitoring tool for the indoor air quality of school environments.

Materials and methods

The monitoring campaign was carried out in five primary school districts (attending children of 5–11 years old) of the Latium region (Central Italy). One school is located in a highly urban area (HUA), whereas the others are situated in four rural areas (RA1, RA2, RA3 and RA4) (Fig. 1). We selected different rural settings because the air at these sites generally has low levels of pollution. Consequently, the lichen accumulation of airborne contaminants in some of the monitored areas would have been scarce, and thus indicates no evidence of pollution. The global positioning system coordinates for each area are 41.912685° N/12.519236° E (HUA), 42.215757° N/12.795055° E (RA1), 42.198362° N/12.799020° E (RA2), 42.212453° N/12.754942° E (RA3) and 42.231482° N/12.807086° E (RA4).

The monitoring sampling stations were chosen based on relevant urbanisation and air pollution indicators. In particular, the urbanisation of selected areas was preliminary evaluated on the basis of the resident population, population density, density of green space and motorisation rate, which were obtained from the National Institute of Statistics and the Italian Automobile Club. Air pollution was assessed on the basis of the mean concentrations of carbon monoxide and nitrogen dioxide, which were available from the Regional Environmental Protection Agency (ARPALAZIO). Details on the indicators and characteristics of the selected areas were previously reported, since the same study areas were also used in another research study carried out by our team (Protano et al. 2012). Each of the monitored buildings did not use air conditioning or has forced ventilation; air changes were observed when windows were opened. Methane gas boilers and radiators were used for winter heating in all cases. No other indoor sources of monitored pollutants were identified.

For the present study, thalli of the epiphytic lichen *P. furfuracea* were collected from the bark of beech trees at approximately 1 m above ground level from Mount Terminillo (1670 m), an area located in the same region of the areas selected for the monitoring campaigns; thus, Mount Terminillo presents similar meteorological conditions of the study areas in the absence of any local natural or anthropogenic sources of air pollutants. Lichen thalli were collected and prepared for exposure following the same procedure reported previously (Guidotti et al. 2009; Protano et al. 2014, 2015). Briefly, the thalli were transferred into the laboratory, divided

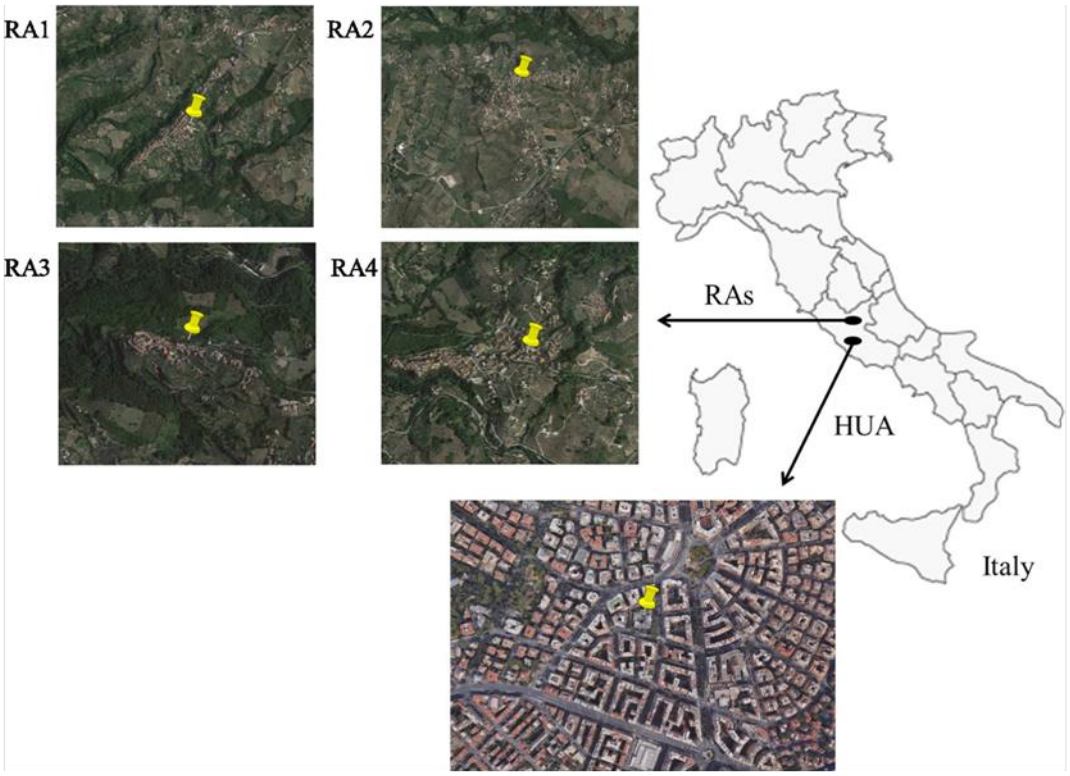

Fig. 1 Study area and sampling sites. *HUA* high urban area, *RA* rural area

into 21 homogeneous subsamples of approximately 10 g each and analysed based on the following criteria: 1 subsample (blank or unexposed control sample) was immediately analysed for all of the monitored compounds, 6 subsamples were used for quality control, and the remaining 14 subsamples were each placed in a 20 × 20 cm bag of 1-cm mesh nylon netting.

Each of the 14 subsamples of lichen thalli was transplanted 2 m above ground level on adequate supports in the monitored sites for 2 months from 12 February 2014 to 12 April 2014. In particular, subsamples were located as follows:

- HUA: two subsamples (HUAout-east and HUAout-west) were placed in a garden outside of the school in an urban area, whereas four subsamples (HUAin-east, HUAin-west, HUAin-south and HUAin-north) were placed in four different classrooms (one for each corner of the ground floor of the building).
- RA1, RA2, RA3 and RA4: two subsamples were placed in the garden and in a classroom of each selected school (RA1out, RA1in, RA2out, RA2in, RA3out, RA3in, RA4out and RA4in).

After the exposure period (2 months), each lichen sample was retrieved from its monitoring site and transported to the laboratory for the analyses. Study analytes included As, six heavy metals (Cd, Cr, Cu, Hg, Ni and Pb) and 12 selected PAHs (fluorene [Flu], phenanthrene [Phen], anthracene [Anth], fluoranthene [Fla], pyrene [Pyr], benzo(a)anthracene [B(a)A], chrysene [Chry], benzo(b,k)fluoranthene [B(b,k)F], benzo(a)pyrene [B(a)Py], indeno(1,2,3cd)pyrene [IP], benzo(g,h,i)perylene [B(g,h,i)P] and dibenzo(a,h)anthracene [D(a,h)A]).

The pretreatment of lichen samples and the analytical determinations of As, heavy metals and selected PAHs were carried out following the procedures described in detail in our previous studies (Guidotti et al. 2009; Protano et al. 2014, 2015). Briefly, As and heavy metals were determined using a graphite furnace atomic absorption spectrometer (AAS Varian Spectra AA 220Z, Varian, CA, USA). A standard reference material certified by the European Commission Community Bureau of Reference (BCR) (BCR No. 482; heavy metals in lichens), which was analysed in six replicates, was used to evaluate the replicability of the method. The certified values, recorded values, average recovery, relative

standard deviation (RSD) and limits of quantification (LOQs) for each compound are reported in Table 1.

Twelve selected PAHs were determined by a gas chromatograph mass spectrometer (GC-MS HP 6890 GC fitted with HP 5973MS, Hewlett Packard, CA, USA) operating in the selected ion-monitoring mode and equipped with an HP5-MS column (25 m × 0.25 mm × 0.25 μm) (Hewlett Packard, CA, USA). The linearity of the method was assessed in the range of 5–500 μg/kg for each PAH, and the relative calibration curves had r^2 values that were always >0.99. The repeatability of the method was assessed by analysing six samples of lichen, which had previously been extracted according to the method. After extraction, the six subsamples were supplemented with a standard mixture of the monitored PAHs at a concentration of 100 μg/kg (USEPA PAH standard mixture at 2000 μg/ml in methanol; Supelco, PA, USA) and analysed. The average recovery rate (%), RSDs and LOQs are reported in Table 1.

The ability of *P. furfuracea* to be used as a biomonitoring tool for indoor air quality was evaluated using the sample exposed-to-control ratios (E/C) according to the method proposed by Frati et al. (2005). In particular, E/C ratios are calculated by dividing the concentration of each compound recovered in each exposed sample by its respective concentration obtained from the blank sample. When the exposed concentration value resulted in values <LOQ, E/C ratios were not calculated; when the blank concentration was found lower than LOQ, it was replaced with the LOQ. Then, the resulting E/C ratios were classified according to the following accumulation categories for each pollutant: ʙsevere loss-based^ (0–0.25), ʙsomewhat loss-based^ (0.25–0.75), ʙnormal^ (0.75–1.25), ʙaccumulation^ (1.25–1.75) and ʙsevere accumulation^ (>1.5) (Frati et al. 2005).

Results and discussion

Indoor air quality is a relevant determinant of human health; the occurrence of airborne pollutants in indoor environments has been associated to both cancer and non-cancer risks for exposed subjects (Sarigiannis et al. 2011). In

Table 1 Certificated and recorded values for all monitored elements according to BCR No. 482: Heavy metals in lichens, and average recovery rates (%), relative standard deviations (RSDs) and limits of quantification (LOQs) for each analyte

Heavy metal	Certified value ± uncertainty (μg/g dw)	Average recorded value (6 replicates) (μg/g dw)	Average recovery (%)	RSD (%)	LOQ (μg/g dw)
As	0.85 ± 0.07	0.88	103.5	12.4	0.02
Cd	0.56 ± 0.02	0.53	94.6	13.1	0.02
Cr	4.12 ± 0.15	3.79	92.0	7.7	0.03
Cu	7.03 ± 0.19	6.37	90.6	8.6	0.03
Hg	0.48 ± 0.02	0.451	94.0	13.7	0.005
Ni	2.47 ± 0.07	2.64	106.9	9.3	0.03
Pb	40.9 ± 1.4	38.29	93.6	5.8	0.01
PAH			Recovery rate (%)	RSD (%)	LOQ (ng/g dw)
Flu			78	6.5	1
Phen			83	5.2	1
Anth			88	4.8	1
Fla			81	4.5	1
Pyr			79	5.5	1
B(a)A			84	4.7	1
Chry			85	5.3	1
B(b,k)F			81	4.8	1
B(a)Py			79	3.8	1
IP			86	4.4	1
B(g,h,i)P			83	5.1	1
D(a,h)A			81	3.5	1

this research field, one of the most studied populations is school-aged children, and school environments are considered a critical scenario for exposure to airborne pollutants during paediatric age. Scientific evidence highlights that indoor air quality in school buildings is often not in compliance with the established standards, and the risk of adverse effects derived from exposure to indoor air pollutants cannot be negligible (Simons et al. 2011; Choo and Jalaludin 2015). Consequently, the constant monitoring of air quality in these environments is needed in order to protect the health of children. The present study was performed to assess the ability of transplanted lichen *P. furfuracea* to biomonitor and bioaccumulate a number of typical airborne pollutants in urban indoor environments and evaluate the possibility of using *P. furfuracea* as a biomonitoring tool for the indoor air quality of school environments.

The first relevant result of the present study is related to the concentrations of each monitored compound found in the blank and exposed lichens (indoor and outdoor), which are reported in Table 2.

The comparison between the mean levels of analytes recovered in the blank and in lichens exposed to the outdoors demonstrated the ability of *P. furfuracea* to biomonitor and to bioaccumulate As, heavy metals and PAHs. Indeed, after the period of exposure to outdoor air, the concentrations of As, heavy metals and total PAHs were almost always higher than those recovered in the negative control samples, both in the highly urban area and in all rural areas. After exposure, the highest concentrations with respect to blank levels were recovered for As, Cu, Hg, Pb, Flu, Phen and Anth. An exception to this trend was found only for Cd recovered in two rural area samples (R2out and R4out) and some individual PAHs (Chry in all cases and Fla and Pyr in rural areas). Overall, these results endorse the possibility of successfully using lichen *P. furfuracea* as a biomonitoring tool for typical urban airborne pollutants, thus confirming previous results (Guidotti et al. 2009; Protano et al. 2014, 2015).

The comparison of concentrations recovered in urban and rural samples exposed to outdoor air showed that the levels of each analyte were always higher in urban samples with respect to those in rural samples. This finding demonstrates the ability of *P. furfuracea* to discriminate between different levels of urban air pollution and is in line with its capacity to accumulate air pollutants at rates that are generally proportional to traffic density, which has been reported by Guidotti et al. (2009).

With regards to the ability of *P. furfuracea* as a monitoring tool of indoor air quality, it is important to initially note that the levels of all heavy metals and As (except for Cd in the rural schools) in lichens sited in the school buildings were higher after exposure with respect to the blank samples. These results are in line with those reported by Canha et al. (2012, 2014). With regards to PAHs, the majority of the monitored compounds resulted in higher levels with respect to the blank, whereas levels of Fla, Pyr and Chry showed opposite figures. Based on our knowledge, our data cannot be compared with any other studies, as no other research to date has been published on the indoor air monitoring of PAHs by lichens.

The results obtained in the studied settings show that the use of *P. furfuracea* is feasible for the long-term monitoring of indoor air quality, although lichens may undergo relevant physiological stress in closed environments (Marques and Freitas 2005; Canha et al. 2013).

In this regard, note that there are several differences between the results obtained in the urban and rural school buildings. In the urban settings, the outdoor levels are generally higher than the indoor settings for As, heavy metals and PAHs, with the exception of Cd and B(a)Py. However, in the rural scenarios, Hg and most of the PAHs (10 out of 12) show higher indoor levels. It is evident that the urban environment is strongly influenced by vehicular traffic, and the indoor air at the school is only partially affected by outdoor air pollutants. In contrast, the rural schools are less affected by traffic-derived pollution, which is shown by the outdoor levels of both metals and PAHs with respect to those recovered in urban areas. Higher indoor levels of PAHs in rural schools may be due to the scarce number of air exchanges, as windows are usually closed during the winter season and the weather is colder in rural areas than in urban areas (mean winter air temperatures are 3.8 and 7.7 °C for rural and urban areas, respectively).

Figure 2 shows the mean E/C ratios for each monitored setting (urban outdoor, urban indoor, rural outdoor and rural indoor) and for each analyte. As shown in Fig. 2, the E/C ratios summarise the previously described results. In particular, mean E/C ratios for As and heavy metals were almost always greater than 1.25 (ʙaccumulation^ categories), with the exception of ʙrural^ Cd, which fell into the ʙnormal^ category.

The E/C ratios of total PAHs fell into the accumulation categories in both urban and rural settings. Regarding the single compounds, only Flu, Pyr, Chyr and

Table 2 Mean concentrations of As and heavy metals (μg/g dw) and individual and total PAHs (ng/g dw) found in both blank and exposed *Pseudovernia furfuracea* lichens at each sampling station

	Blank	HUAout-east	HUAout-west	HUAin-east	HUAin-west	HUAin-south	HUAin-north	RA1out	RA1in	RA2out	RA2in	RA3out	RA3in	RA4out	RA4in
As	0.12	0.46	0.45	0.17	0.24	0.31	0.20	0.38	0.26	0.32	0.28	0.47	0.34	0.73	0.19
Cd	0.200	0.227	0.400	0.433	0.494	0.431	0.472	0.265	0.194	0.194	0.188	0.209	0.182	0.171	0.185
Cr	1.58	3.44	2.56	2.88	2.15	1.88	2.16	1.94	1.71	2.36	1.68	2.13	1.98	4.14	4.29
Cu	2.95	6.33	5.93	5.18	4.45	4.58	4.96	3.89	3.00	4.17	3.05	4.72	4.33	6.29	5.24
Hg	0.095	0.375	0.430	0.210	0.190	0.412	0.322	0.157	0.207	0.165	0.293	0.182	0.334	0.431	0.442
Ni	1.02	2.47	2.12	2.64	2.14	1.87	1.94	2.28	2.04	1.81	1.97	1.95	1.89	2.80	3.16
Pb	4.17	11.14	7.48	8.05	7.24	5.46	6.55	7.40	7.21	7.68	7.17	8.92	8.20	11.13	10.55
Flu	16	60	43	57	52	65	41	58	60	46	60	36	56	45	43
Phen	73	162	158	140	137	148	121	115	134	121	133	128	117	124	113
Anth	3	17	23	15	16	15	12	16	17	20	15	19	13	15	16
Fla	61	86	123	54	56	52	54	31	39	34	63	32	31	33	33
Pyr	30	58	68	33	35	32	36	18	24	22	46	23	19	20	19
B(a)A	<LOQ	7	7	9	5	3	7	3	3	3	5	5	6	4	5
Chry	18	14	17	6	8	6	10	4	7	5	9	10	7	8	4
B(b,k)F	4	18	19	15	13	10	22	10	13	9	12	10	10	10	8
B(a)Py	<LOQ	7	7	8	6	13	9	3	5	5	5	3	5	4	4
IP	<LOQ	6	6	4	5	4	7	3	4	3	4	3	3	3	3
B(g,h,i)P	<LOQ	6	6	3	4	4	6	2	4	4	4	3	3	3	3
D(a,h)A	2	2	2	2	<LOQ	<LOQ	3	<LOQ	<LOQ	<LOQ	<LOQ	2	<LOQ	<LOQ	<LOQ
∑ PAHs	207	443	479	346	337	381	328	263	310	272	356	274	270	269	251

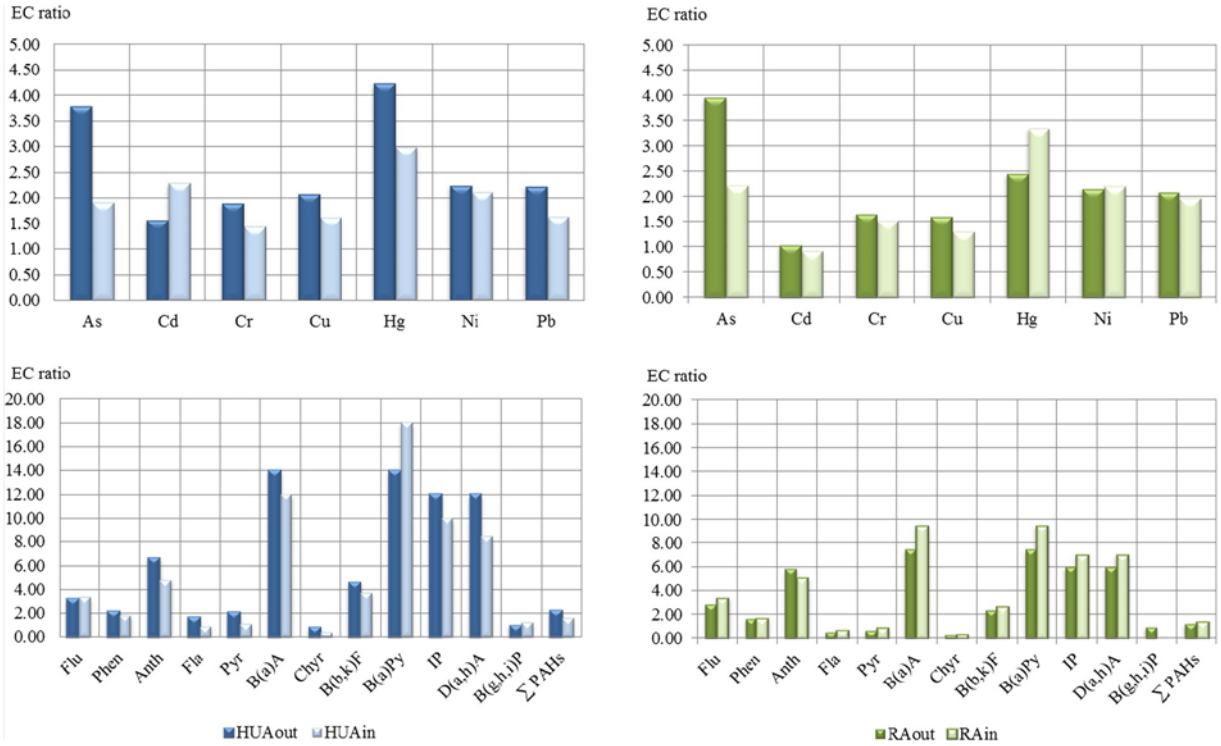

Fig. 2 Exposed to control ratio (E/C ratio) found in *Pseudovernia furfuracea* lichen at each sampling station for each monitored analyte

B(g,h,i)P fell into the normal or ʙloss^ categories both in urban and rural lichens.

The increasing and decreasing levels of metals and PAHs during the period of lichen exposure were reported by previous studies, both for *P. furfuracea* and other different lichen species (Conti and Cecchetti 2001; Bergamaschi et al. 2007; Sorbo et al. 2008; Guidotti et al. 2009; Protano et al. 2014, 2015). Several factors affected the accumulation patterns in lichen samples, such as differences in uptake ability between different lichen species and in climate conditions of the exposure period; moreover, it has been hypothesised that the accumulation process may achieve a saturation stage or an equilibrium between loss and accumulation depending on the lichen species and/or the levels of airborne contamination.

The findings from the present study should be interpreted based on several limitations. First, although the ʙindoor^ lichens demonstrated that they can bioaccumulate both metals and PAHs, we have no information regarding the independent contribution of the indoor microclimatic conditions on the accumulation process. It would be interesting to repeat the same monitoring campaigns in a warmer season when heating systems are off and indoor and outdoor climatic conditions are similar. In addition, we evaluated only a single period of exposure (2 months); thus, we are not able to identify the best window of exposure. It would be interesting to perform the biomonitoring campaign by placing different lichen samples at the same time in each sampling point and retrieving each sample after different periods.

Conclusion

The results confirm that the transplanted lichen *P. furfuracea* is a suitable biomonitoring tool for metals and PAHs in outdoor environments. To the best of our knowledge, this is also the first demonstration of the ability of this lichen species to bioaccumulate metals and PAHs in indoor settings. Finally, the monitoring results show that transplanted lichen *P. furfuracea* is able to discriminate between different levels of air pollution related to urbanisation and to indoor conditions, such as those characterised by school environments. A transplanted lichen biomonitoring strategy is cost-effective, ʙgreen^, educational for attending

children and less ʙinvasive^ than traditional air sampling methods. The feasibility of indoor monitoring by *P. furfuracea* is a relevant finding and could be a key tool to improve air quality monitoring programmes in school scenarios and thus focus on health prevention interventions for children, who are one of the most susceptible groups in the population.

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CHAPTER 5

NATIVE LICHENS AS A TOOL FOR ASSESSING AIR QUALITY: A STUDY IN FIELD ON XANTHORIA PARIETINA

Native lichens as a tool for assessing air quality: a study in field on Xanthoria parietina. Draft manuscript.

1. Introduction

Among substances detected in air samples, persistent organic pollutants (POPs), such as polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and polycyclic aromatic hydrocarbons (PAHs) have been shown to exhibit potentially harmful effects in man and the environment (Zhou, 2004; Zhou et al., 2008; Ciesielczuk et al., 2012). These compounds are widely spread in the environment, mainly due to anthropogenic activities, often in small quantities of the order of μg/kg or ng/m^3, associated to other pollutants, such as heavy metals. Their combined effects on ecosystems and on human health are related to long-term exposure to low levels (chronic exposure) (IARC, 1987; Kogevinas, 2001). In particular, heavy metals are largely used in industrial, domestic, agricultural, medical and technological applications (Tchounwou et al. 2012). Due to their ubiquitous presence in the environment, persistence and liposolubility, POPs tend to accumulate over time in tissues and organs of humans and animals. Additionally, thanks to their ability to be stably bound to lipids, the concentration of these substances and their effects can increase up through the food chain (biomagnification), adversely affecting health (Norstrom et al., 1998; Muir et al. 2006; Lohman et al., 2007; Klanova et al., 2008; Nash, 2011). Among polychlorinated compounds, PCDD/Fs are not intentionally produced but are accidental byproducts of various activities such as industrial or combustion processes; in particular they have been detected in emissions from waste and materials containing chlorine (e.g. plastics) combustion (USEPA, 1997; Davy, 2004; Chang et al., 2004; Quaβ et al., 2004). Due to their chemical-physical properties PCBs and PBDEs are chemicals widely synthesized for industrial uses. They have been used in many different products, including electrical equipment, surface coatings, inks, adhesives, and paints, flame-retardants added to commercial and household products (e.g. foams, textiles, electronic components) (Biterna and Voutsa, 2005; Hedman et al., 2006; Hermanson and Johnson, 2007; Weber et al., 2008; de Wit et al., 2006-2010; Hung et al., 2010; Letcher et al., 2010; IARC, 2016). PAHs derive predominantly from incomplete combustion of organic matter such as coal, petroleum, oil or biomass derivatives, which can occur in incinerators, fires and internal combustion engines (Lin et al., 2001; Garban et al., 2002; Dyke et al., 2003; Mastral et al. 2003). They are transported through atmosphere both in the gas state and adsorbed onto the solid suspended fraction depending on their number of rings and their molecular weight (Augusto et al., 2009; Dat and Chang, 2017). Due to their ubiquitous presence in the environment and their potential toxicity for human health, the continuous monitoring of these compounds in environmental matrices play a key role in the environmental and human health risk assessment process. As regards the monitoring of airborne pollutants, data

produced by routine environmental monitoring campaigns to evaluate air quality is complicated by several factors, such as the great number of substances potentially toxic for human health to monitor and the resulting necessity for multiple monitoring methods and tools, and the need for dedicated monitoring programs related to different emission sources. Indeed, the main challenge of environmental monitoring is to be able to distinguish between different pollution sources in order to identify major contributing sources of contaminants in an area of interest. In this context, biomonitoring of atmospheric contaminants by biomonitors and bioaccumulators is an approach of great interest for the simplicity and rapidity with which it can be performed. For this reason, the use of biomonitors and bioaccumulators such as mosses, lichens, ferns, tree bark and higher plants, as passive collectors of atmospheric pollutants has been increasing in recent years (Protano et al. 2015). In particular, biomonitoring with lichens has proven to provide valuable information about the concentration and source of PAHs in the ambient air (Guidotti et al. 2003; Shukla and Upreti 2009; Augusto et al., 2010; Shukla et al. 2010). Our previous researches demonstrated that lichen *Pseudevernia furfuracea*, by the use of the transplanted technique, was a useful tool for monitoring both heavy metals and POPs in air of several outdoor and indoor scenarios (Guidotti et al. 2009; Protano et al. 2014-2017a). At today, different kind of lichen species was assessed as air biomonitors such as *Usnea sp.*, *R. farinacea*, *Evernia prunastri*, *Pseudevernia furfuracea*, *Parmelia caperata*, *Parmotrema reticulatum*, *Ramalina canariensis*, *Ramalina fastigiata*, *Xanthoria (X.) parietina (L) Th.Fr.* The latter type of lichen belongs to a very abundant and easily recognizable species and it is very resistant to the adverse effects of air pollution (Olsen et al. 2010).

The aims of the present study were (1) to evaluate the ability of native *X. parietina* to biomonitor and bioaccumulate heavy metals, PAHs, PCDDs, PCDFs, PCBs and PBDEs; (2) to assess the use of the native *X. parietina* as a multi-tracer tool for different scenarios; (3) to investigate the presence of punctual source of the monitored pollutants. For this purpose, we performed a monitoring study to examine the air quality of Latium region (central Italy) by investigating the airborne concentrations of 78 substances, including some metals and POPs in native lichen *X. parietina* and correlating the compound levels with the presence of pollutants' potential sources in the monitored areas. For our study we selected the epiphytic *X. Parietina* lichen species because it can penetrate heavily anthropized environments and it is absent only in heavily polluted areas, resulting one of the most widespread biomonitor in the study area. Specifically, it is widely distributed in the central Italy and the preliminary evaluation of the studied areas confirmed the high distribution of this kind of lichen.

2 Materials and methods

2.1 Study area

Samples of *X. parietina* lichen species were collected from different areas located in Latium region (central Italy). The studied region covers an area of approximatively 17,200 Km^2 and is located on the Tyrrhenian coast of Mediterranean sea behind the Apennine Mountains. A preliminary evaluation of the potential anthropic pollution sources, such as industrial, civil and agricultural activities were taken into account for selecting the locations for our monitoring campaign. According to this, three different typologies of location were classified according to the presence and the number of air pollution sources, and grouped as green (G), residential (R) and industrial (I) area. Later, further and more selective information, such as the urbanization rate and some other air pollution indicators were evaluated to improve criteria adopted for sites classification. The urbanization of each site was evaluated on the basis of inhabitants' density (DEMOISTAT reference), green areas density and the motorization rates, collected from national databases (National Institute of Statistics and Italian Automobile Club). Air quality of the monitored areas was assessed according to the mean air concentrations of carbon monoxide and nitrogen dioxide, which were available from the chemical measuring stations of Regional Environmental Protection Agency (ARPALAZIO). According to these information we selected 16 monitoring stations. First, six sampling sites with high vegetation density and low levels of pollution, identified as "green areas", included one regional park and more generally five rural sites without occurrence of airborne contamination directly related to human activity (G1, G2, G3, G4, G5, G6). Then, based on municipal data, 3 sampling sites, identified as "residential areas", were selected in urban districts away from the heavy traffic load (R7, R8, R9); this category, characterized by low levels of selected pollution parameters, includes areas mainly concerned with local traffic, low resident population density, limited presence of commercial activities and absence of industrial activities. Finally, for the locations identified as "industrial areas" we selected seven (I10, I11, I12, I13, I14, I15, I16) sampling sites with a high rate of industrial development and scarcity of residential buildings; these areas are surrounded by significant pollution sources, mainly due to combustion processes related to energy production and chemical plants (steelworks, power plants, cement plants, paper mill, industrial centers, landfill). Once the location typology was selected, the choice of suitable points for the study was based on several factors including the presence of the sampling matrix, the accessibility of the site in safe conditions and the occasional occurrence of local natural sources of air pollutants (e.g. brush fires). Sampling was carried out in accordance with the guidelines for the use of epiphytic lichens as trace elements bio-accumulators (ANPA 2001). Figure 1 shows the map of the study area with

marked points identifying our monitoring stations and the 20 units monitoring network belonging to the Regional Air Quality Assessment Program distributed in the regional territory. Detailed information about the sampling locations are given in Table 1.

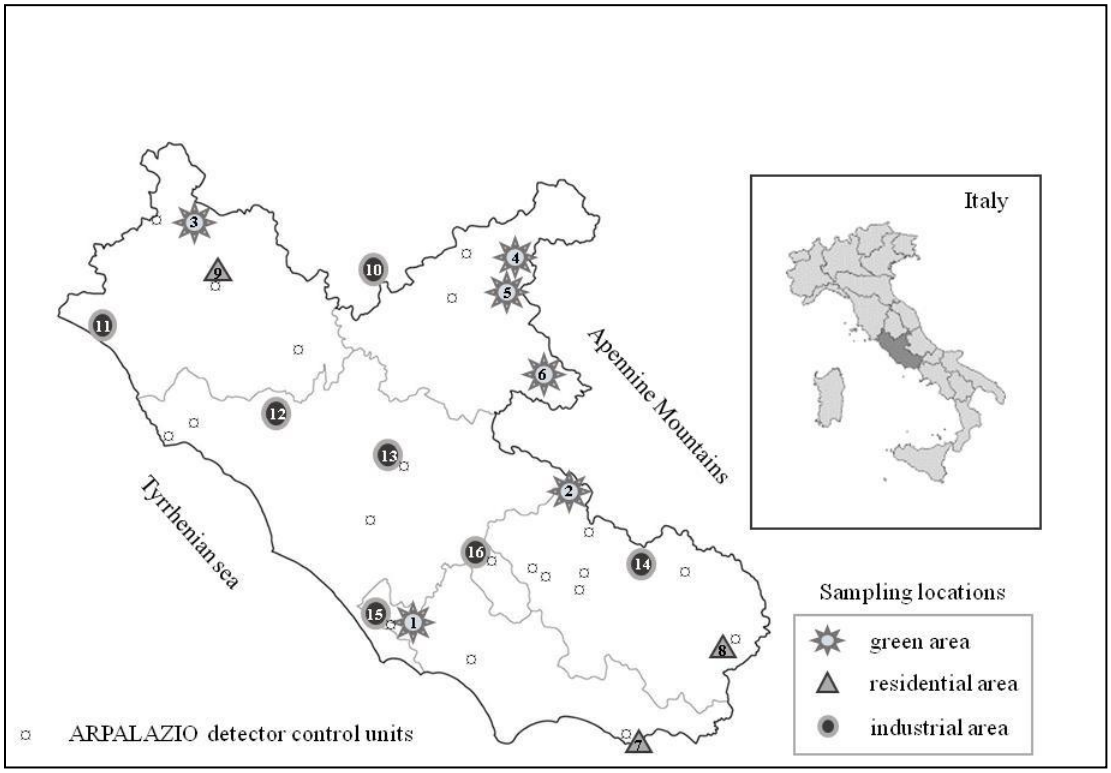


Figure 1. Map of the study area and monitoring stations.

area type	monitoring station	area description	latitude / longitude
green	G1	wildwood interspersed with Mediterranean scrub	41.510209° N/12.775141° E
	G2	ski area	41.923277° N/13.336612° E
	G3	territory near the lake of volcanic origin	42.644204° N/11.986438° E
	G4	territory near the river Velino to the north-east of the Terminillo massif	42.526140° N/13.098774° E
	G5	wide valley surrounded by three mountain system	42.421094° N/13.080453° E
	G6	territory located in the Apennine hinterland in the upper valley of the Salto river	42.192769° N/13.235852° E
residential	R7	part of the municipality of a city located on the Tyrrhenian Sea in the Gulf of Gaeta	41.210730° N/13.571429° E
	R8	part of the municipality of a city located on a tufaceous spur over the river Sacco valley	41.492271° N/13.857858° E
	R9	part of the municipality of a city located on the slopes of the Cimini Mountains	42.420677° N/12.107669° E
industrial	I10	steelworks	42.563617° N/12.642660° E
	I11	photovoltaic and thermoelectric power plants	42.351566° N/11.607010° E
	I12	cement plants	42.220572° N/12.188064° E
	I13	cement plants	41.993969° N/12.724504° E
	I14	paper mill	41.718854° N/13.613049° E
	I15	industrial establishments, especially pharmaceuticals	41.594402° N/12.656031° E
	I16	intense industrial activity, especially chemical	41.727253° N/13.003784° E

Table 1. Geographical coordinates and brief description of each monitoring site.

2.2 Sampling and treatment

The monitoring campaign was performed in 2016, during the winter season. We chose the typical winter conditions to avoid any reduction in the ability of lichen to accumulate atmospheric pollutants, mainly due to the influence of summer temperature and precipitations on lichens metabolic activity and chemicals deposition on their thalli. In this period thalli of native *X. parietina* were collected at the 16 sampling sites reported in Figure 1 from the bark of local trees at a height > 1m above the ground level. At each site, lichen samples 5 X 5 cm^2 - sized from at least three trees chosen randomly in an area 50 m by 50 m, were collected in polyethylene bags. In order to reduce the within thallus variability in chemicals content we selected lichens at a similar stage of development. Then, in order to assure a significant accumulation of compounds which are typically to be found in atmosphere at very low concentrations, the last 3 - 4 mm length of lichen material growth, corresponding approximately to one year of exposure to air pollutants, was used for the analyses. Samples were boxed, protected from sunlight and immediately stored at 4 °C. After collection, samples were transported to the laboratory for the analyses. In the laboratory lichen samples were dehydrated at 40 °C for 48 hours to constant weight, cleaned under a microscope to remove any kind of extraneous material traces (dust, leaves, soil, wood chips) and then grinded with an agate mortar. The treated samples were stored in a vacuum drier until analyses. All samples were extracted, purified and analyzed within two months, in accordance with the standard procedures for each analytical category.

2.3 Analytical procedures

Among air pollutants, 78 chemicals were selected according to environmental prevalence and toxicological property. They included As and 5 heavy metals (As, Cd, Co, Cr, Ni, and Pb), 12 PAHs (fluorene [flu], phenanthrene [phen], anthracene [anth], fluoranthene [fla], pyrene [pyr], benzo(a)anthracene [B(a)A], chrysene [chry], benzo(b,k)fluoranthene [B(b,k)F], benzo(a)pyrene [B(a)Py], indeno(1,2,3-cd)pyrene [IP], benzo(g,h,i)perylene [B(g,h,i)P], and dibenzo(a,h)anthracene [D(a,h)A]), 28 PCB congeners (dioxin-like PCBs with IUPAC Ns. 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189; marker PCBs with IUPAC Ns. 52, 101, 138, 153, 180; nondioxin-like PCBs with IUPA Ns. 95, 99, 110, 146, 149, 151, 170, 177, 183, 187), 17 toxic congeners of PCDDs and PCDFs (2,3,7,8-TCDD; 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD; 1,2,3,4,6,7,8-HpCDD; OCDD, 2,3,7,8-TCDF; 1,2,3,7,8-PeCDF; 2,3,4,7,8-PeCDF; 1,2,3,4,7,8-HxCDF; 1,2,3,6,7,8-HxCDF; 1,2,3,7,8,9-HxCDF; 2,3,4,6,7,8-HxCDF; 1,2,3,4,6,7,8-HpCDF; 1,2,3,4,7,8,9-HpCDF and OCDF) and 15 PBDEs

(PBDEs with IUPAC Ns 17, 28, 47, 49, 66, 71, 77, 85, 99, 100, 119, 138, 153, 154, 156). All analyses took place at the ISO/IEC 17025 accredited laboratory of the Latium Environmental Protection Agency (section of Rieti), where USEPA recommended procedures were performed and validated for analytical determinations of each investigated micro-pollutants class in lichen samples. The used methods generally required specific sample extraction/purification proceedings prior to instrumental analyses. In order to compensate the incomplete recovery of the analytes during the extraction and purification phases, the isotopic enrichment technique was also required for chemical determination. In order to assure the quality of our results, in particular to reveal any possible processing contamination and assess the accuracy of applied method, full intra-laboratory quality assurance and quality control (QA/QC) procedures were constantly in place with the use of laboratory blanks and matrix spike samples.

2.3.1 Metals

Selected metals (As and heavy metals) were determined following the methodology developed by Guidotti et al. (2009). Related concentrations, expressed in a dry weight basis, were obtained by using a graphite-furnace atomic absorption spectrometer (AAS Varian Spectra AA 220Z, Varian, CA, USA), according to USEPA 3051A. In preparation for analysis, about 150 mg of finely chopped lichen was previously digested in a sealed and pressurized vessel (teflon bomb) with an acid mixture of 7 ml of 63% HNO_3, 3 ml of 30 % H_2O_2, and 0.2 ml of 40 % hydrofluoric acid using the microwave heating unit MARS5 (by CEM Corportion) at 120 °C, for 3h. After cooling, mixture was transferred into a test tube and contents were first centrifuged, then filtered into graduate 100-ml flask and finally diluted to volume with bidistilled water. Quantitative analyses were performed using the standard addition method.

2.3.2 PAHs

According to the procedures described by Guidotti et al. (2003-2009) and Protano et al. (2014), USEPA method 8270D was implemented to obtain PAHs contents by using a gas-chromatograph equipped with an HP5-MS column (30 m X 0.25 mm X 0.25 μm) (Hewlett Packard, CA, USA) and coupled with a mass spectrometer (GC–MS MS HP 6890 GC fitted with HP 5973MS, Hewlett Packard, Sunnyvale, CA, USA), operating in the selected ion monitoring. Prior to PAHs analyses, 0.2 g of finely chopped lichen, added with 20 μl of marked PAHs solution (chrysene-d_{12} and phenanthrene-d_{10} at 0.1 μg/ml in methanol), was extracted with 30 ml of cyclohexane in an ultrasonic bath (Sonica Sweet System from Soltec, Mi, Italy) at room temperature for 30 min, and the final extract was filtered through filter paper No. 40 from Whatman. The extraction procedure was repeated and the combined extracts were concentrated

to 2 ml using a rotavapor system, before purification. The sample extract was purified in accordance with EPA method 3630B. 1g-silice SPE cartridges were used for clean-up of the 12 PAHs in the extraction solutions: cartridge sorbents were first conditioned by 6ml of hexane and then the extraction solutions were aspirated through the cartridges at 0.1 ml/min flow rate and eluted by 9 ml of 9:1 cyclohexane-to-dichloromethane mixture. The eluate was concentrated to 1 ml under a slight N_2 fluxing and then recovered with 50 µl of internal standard solution (perylene-d_{12} at 40 µg/ml in isooctane) before being injected in the gas-chromatograph. For each target compound different ions (isotopic labeled included) were monitored in SIM (selective ion monitoring) mode. The ions chosen for detection/quantification were those that exhibited the strongest intensities in the full-scan EI/MS spectra.

2.3.3 PCDD/Fs - PCBs - PBDEs

Dioxin and dioxin-like PCB and PBDE analyses involve detection of multiple congeners at ppt or ppq level. The quantifications of PCBs (USEPA method 8270D), PCDD/Fs (USEPA method1613B) and PBDEs (USEPA method 1614A) were performed using a gas chromatograph coupled to triple-quadrupole mass spectrometer (7890A GC coupled to a MS/MS Triple Quadrupole 7000 Series, equipped with an 7693A autosampler, all from Agilent Instruments, Santa Clara, CA, USA). The extraction of investigated four persistent organic pollutants (PCBs, PBDEs, dioxins and furans) was performed from one sample simultaneously by using a Soxhlet automated apparatus (VELP SCIENTIFICA SER 148 Solvent extractor). First, 2g of grinded thalli were added with 20 µl of $^{13}C_{12}$-labelled PCB/PBDE surrogates standards solution at 0,1 µg/ml (PCB-77, PCB-81, PCB- 105, PCB-114, PCB-118, PCB-123, PCB-126, PCB 156, PCB-157, PCB-167, PCB-169, PCB-189, BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, acetonitrile) and 10 µl of $^{13}C_{12}$-labelled dioxin/furan surrogates standards solution at 0,02 µg/ml (2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, OCDF, acetonitrile). After isotopic enrichment the sample was extracted with 50 ml of 4:1 hexane-to-acetone mixture at 130°C and 10 h reflux. Extract was centrifuged for 5min at 5000 rpm. The liquid phase was filtered into a 50-ml flask using a quantitative-grade filter paper in presence of 3g of dehydrated sodium sulfate and then concentrated at 1ml volume by rotary evaporation in an N_2 gas stream at 40°C, before performing sample cleanup approach which combines multiple columns. First, the sample passed through a multilayer column (Na_2SO_4, silice, $NaHCO_3$ - Na_2SO_4 mixture, 12 ml H_2SO_4 96% - 20 g celite mixture). The column was eluted with 60 ml hexane and eluate was retained and concentrated by rotary evaporation to about 1 ml, which was quantitatively transferred to the top

of a column packaged with basic alumina. This second column was eluted with hexane (10 ml), not collected, and 40 ml of hexane: dichloromethane mixture (1:1). This final eluate, which contains PCB, PBDDE, dioxins and furans, was brought to dryness by a slight N_2 fluxing. Finally, it was recovered with 200 µl of methanolic mixtures of $^{13}C_{12}$-labeled compounds at 2 µg/ml used as ISs solutions of PCB, PCDD/Fs and PBDEs (PCB-70, PCB-111, PCB-170; 1,2,3,4-TCDD, 1,2,3,7,8,9-HxCDD; BDE-77, BDE-138). The triple quadrupole GC/MS was operated in MS/MS-EI (electron ionization) Multiple Reaction Monitoring (MRM) mode. Quantification was performed by isotopic dilution technique applied on product ions. Each analyte and its $^{13}C_{12}$ - surrogate/internal standards were measured using the two most abundant product ions for native and ^{13}C - labelled standard, respectively.

2.4 Quantification, analytical performance and quality control

For each chemical class the relative USEPA primary standard mixture (2000 µg/ml in methanol) was used both for the quantification of investigated analytes and for the evaluation of analytical performances. Starting from these standards, concentrated stock solutions at 100 µg/ml were prepared in methanol and stored at -18 °C to be later diluted at proper concentrations, ranging within the expected variation of the analytes content in the unknown samples, and employed in the construction of calibration curves. Mixed concentrated spiking solutions of target analytes were further obtained by methanolic dilution, each at 2 mg/l concentration. These solutions were freshly made and used for preparing reference standard materials needed for the optimization of the method performances and the assessment of the results quality. Validation of the procedures described before were carried out as regards accuracy, linearity range, precision and limit of detection (LOD) and limit of quantification (LOQ). Accuracy, expressed as percent of recovery (Rec %), was calculated as percent ratio between the value calculated from the calibration curve and the theoretical value, respectively. The recoveries were considered to be satisfactory if the results were within the +/-15 % confidence interval of the nominal values. In order to evaluate the repeatability of the method, expressed as percent of variation (VC %), six replicates of lichen matrix at different levels of fortification were analyzed on the same day (intra-day precision); then the same procedure was repeated in three different days in a week (inter-day precision). Acceptable results were within the 30 VC %.

Standard materials certified from the European Commission Community Bureau of Reference (BCR) (BCR No. 482; heavy metals in lichens) were processed and analyzed to assess the method

robustness for metals. Mean total elements concentrations were within 10 % of certified values (Table 2).

In order to assess the method performances for the organic pollutants, the same reference standard material was supplemented at different concentrations with the appropriate EPA mixture. These spiked reference standard materials were then subjected to the same analytical procedure and checked for the same acceptance criteria. LOQ, defined as the concentration of the analyte that produces a signal-to-noise ratio of 9 : 1 was determined for each compound using thalli of *X. parietina* collected from an area without any local natural or anthropogenic sources of air pollutants (Mount Terminillo 1670 m) spiked at the lowest concentration level.

Correlation coefficients (R^2), recoveries percentages (Rec %), variation coefficients (VC %) and LOQs for target analytes are reported in Table 3. Values obtained for metals show high percentages of recovery (91 - 104 %) with margins of error within the certified ones. Good linearity was achieved and regression lines were characterized by correlation coefficients $R^2 >$ 0.999 for all the elements. LOQs ranged from 0.01 to 0.03 mg/Kg. The obtained seven-points ISTD calibration curves for organic pollutants showed excellent linearity over the different concentration range and were characterized by correlation coefficients (R^2) higher than 0.99 for all compounds. The recoveries were found between 85 - 115 % for all the congeners.

One reagents blank for each group of treated samples was processed and analyzed to check potential laboratory contamination that could be occurring from the contact with equipment used in the analytical procedure. In order to monitor the matrix effect on performance of the total analytical process, the analysis of at least one matrix spike and one duplicate unspiked sample were included in each analytical batch of 15 samples. Any laboratory blank, matrix spike and replicate samples were handled in exactly the same manner as actual sample.

All analyses were carried out in triplicate in order to generate an average value for each point and the recoveries of individual pollutants, used for their quantification, were determined through isotopic enrichment method. For this purpose, before extraction at the beginning of the sample processing, a known amount of surrogate standard mixtures (SS) was added to a weighted aliquot of each sample, blank, and matrix spike sample both to evaluate the recovery efficiency and to monitor unusual matrix effects. Except for metals, internal standards (ISs) employed for the quantification of target compounds, were added in a constant amount to treated samples, just before instrumental analyses, to correct for variability in injection volumes. Depending on the analyses to be performed different syringe standards were added for quantification.

mg/kg	As	Cd	Co	Cr	Ni	Pb
calculated average concentration	0.87	0.54	0.30	4.30	2.50	37.02
CRM certified concentration	0.85 ± 0.07	0.56 ± 0.02	0.32± 0.03	4.12 ± 0.15	2.47 ± 0.07	40.9 ± 1.4

Table 2. Average recovery concentration values obtained from BCR 482 certified reference materials.

	LOQ	VC %	Rec %	R^2
As	(1)0.02	6.1	102	0.999
Cd	(1)0.02	4.8	96	0.999
Co	(1)0.01	5.6	94	0.999
Cr	(1)0.03	4.5	104	0.999
Ni	(1)0.03	4.3	101	0.999
Pb	(1)0.01	3.6	91	0.999
PAHs	(2)0.5	(4)15 - 28	(4)87 - 100	0.997 – 0.999
PCBs	(3)5	(4)16 - 26	(4)85 - 110	0.997 – 0.999
PCDDs	(3)0.3	(5)8 - 26	(5)85 - 105	0.999
PCDFs	(3)0.3	(5)7 - 22	(5)88 - 115	0.996 – 0.999
PBDEs	(3)0.3	(5)4 -15	(5)85 - 105	0.9996 - 0.999

(1) Values expressed in mg/kg

(2) Values expressed in μg/kg

(3) Values expressed in ng/kg

(4) Spiked amount at 100 μg/kg

(5) Spiked amount at 100 ng/kg

Table 3. Analytical features (limit of quantification, variation coefficient, recovery percentage, linearity) of the methods developed for metals and each class of POPs.

3 Preliminary results

Figures 2, 3, 4, 5 and 6 shows the concentrations of all elements and compounds determined in *X. parietina* samples collected in each monitored area.

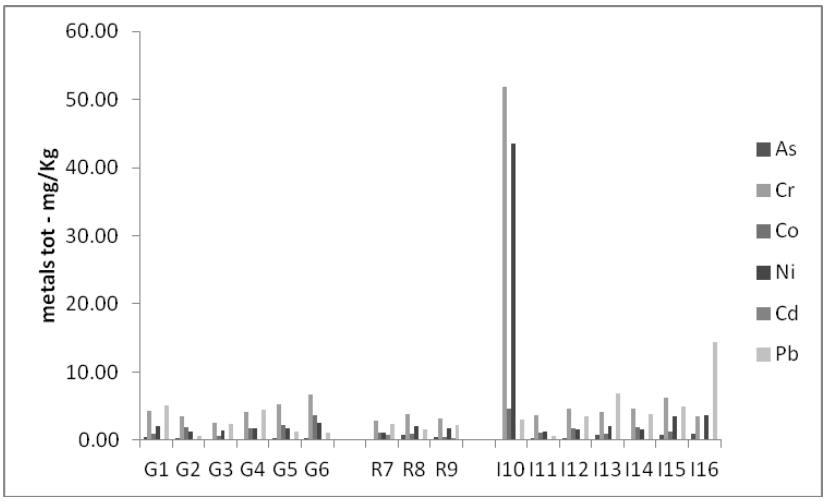
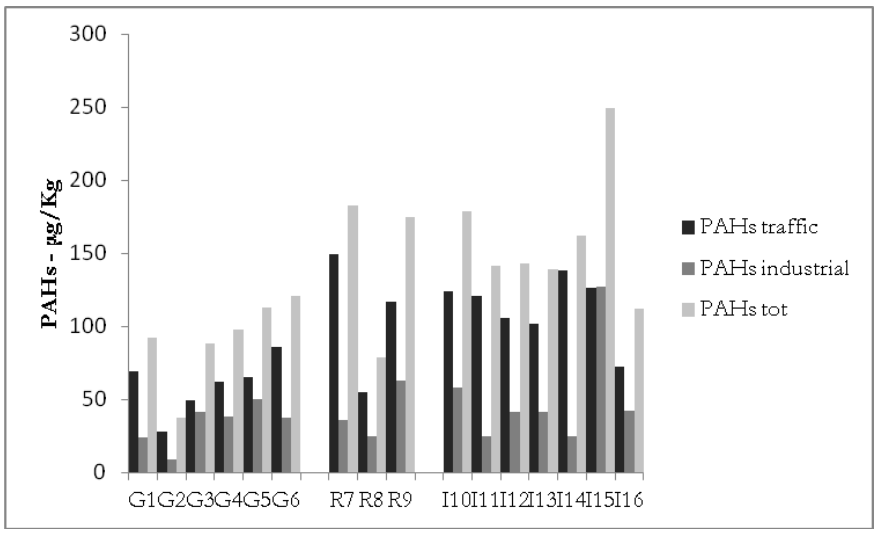


Figure 2 – Metals - The total concentration of metals ranged between 6,9 and 103,4 mg/Kg. High concentrations of chromium, nickel and cobalt were found at industrial area near steelworks (site I10). High lead concentrations were found near cement plant and manufacturing center (sites I13 and I16).



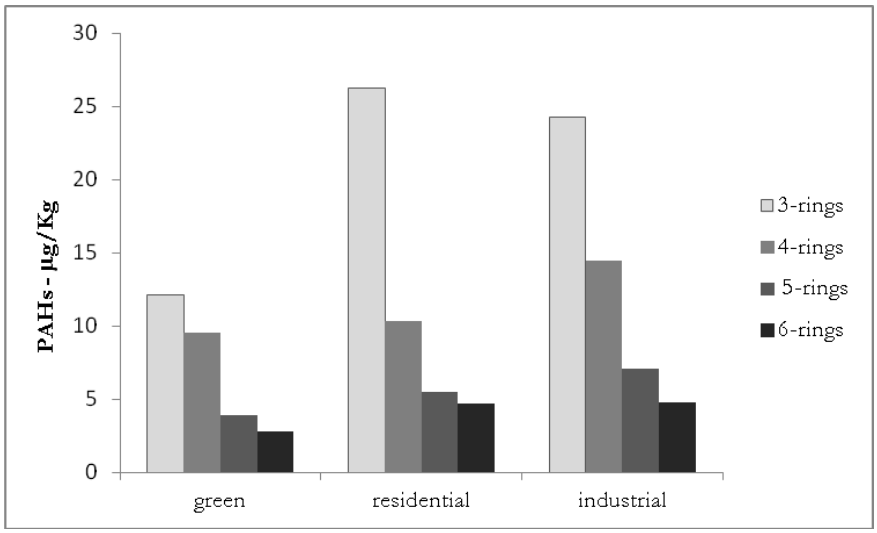
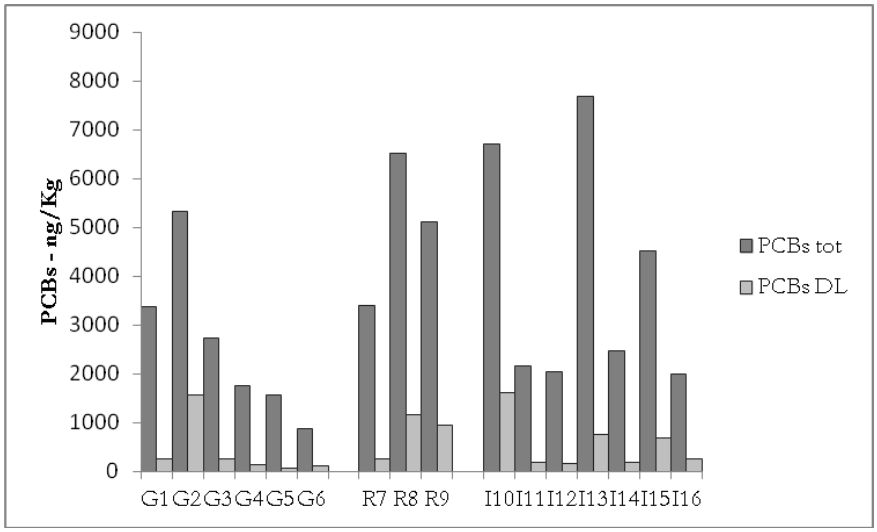


Figure 3 – PAHs - The total PAHs concentration ranged from 37 to 249 µg/Kg dw. As can be seen, care was taken for separating the PAHs that typically come from vehicular traffic from those typically produced by industrial activities. The highest values were found in industrial areas (combustion processes), followed closely by residential areas (vehicular traffic). Among PaHs, phenanthrene showed the highest concentrations at all sites explored in this study, confirming the dominant compounds found in lichens by other authors. Among 3 – 4 rings PAHs, anthracene showed the lowest concentration due to its low thermodynamics stability.



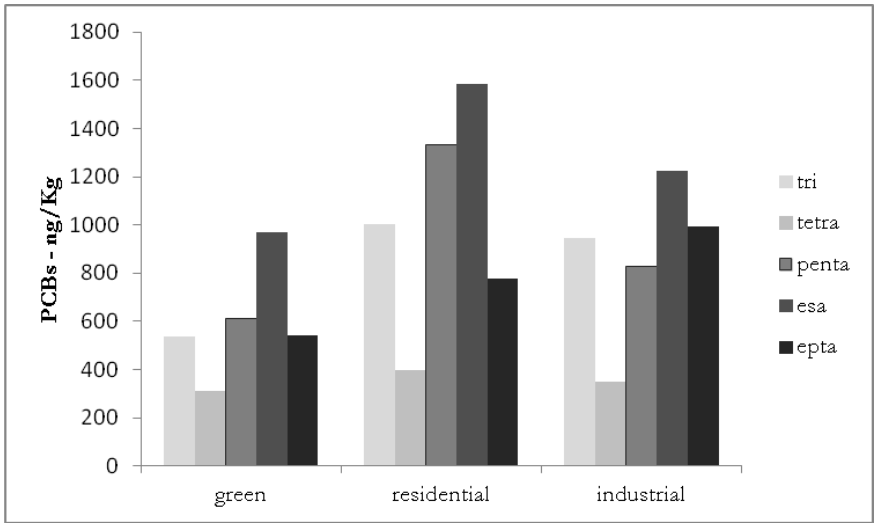


Figure 4 – PCBs - Total PCBs concentration ranged between 868 and 7685 ng/Kg. PCBs contents in lichens from residential and industrial areas were much higher than those in lichen samples from green areas. Among the sites located in industrial zones, I13 (cement plant) and I10 (steelworks) are those with the highest concentrations of PCBs. Among the residential areas the highest value is that of the site R8 where a dismantled synthetic yarn is sited. The trichlorinated PCB-28, which exists as gas in atmosphere, was the more concentrated compound in all sites. Anyway, in all sites there was a prevalence of hexa-CBs, followed by hepta- and penta-CBs.

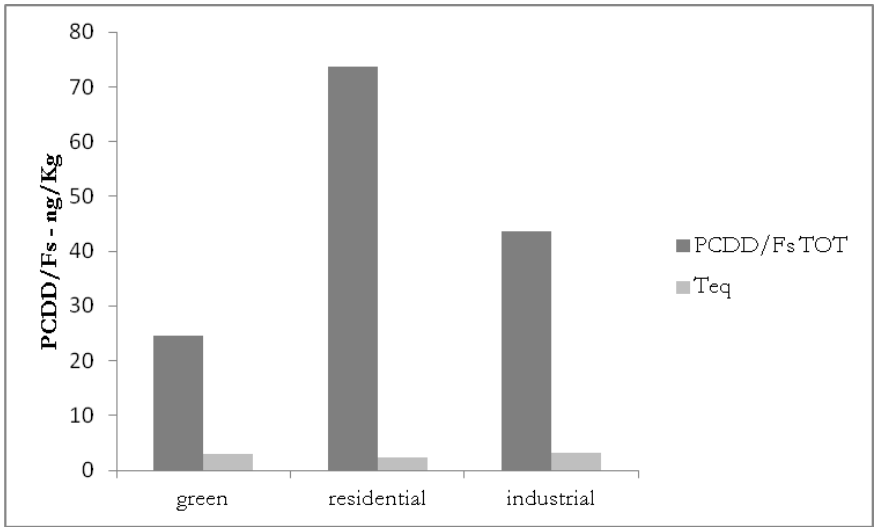


Figure 5 – dioxins/furans - In our investigation, a general low diffusion of dioxins and furans was found even in remote site, not having local inputs. In general, concentrations of the sum of the tetra- to octa-CDD/Fs homologues are between 14 and 114 ng/Kg. Specifically, the total concentrations were as follows: rural ~ 14-44 ng/Kg, residential ~ 38-114 ng/Kg and urban/industrial ~ 21-96 ng/Kg. Among dioxins the OCDD showed the highest concentration in all monitoring stations.

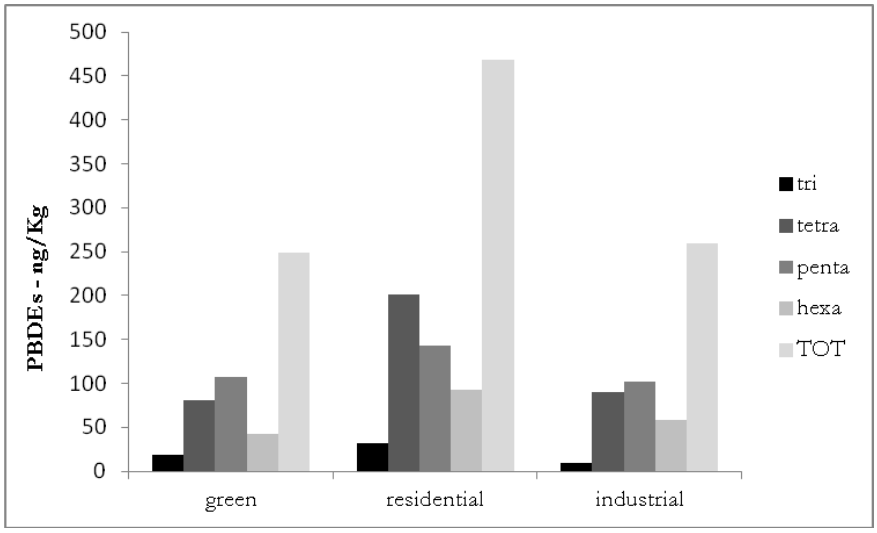


Figure 6 – PBDEs - The concentration of total PBDEs in lichens samples ranged from 130 to 779 ng/Kg dry weight, with a mean concentration of 249 ng/Kg for green areas, 469 ng/Kg for residential areas and 259 ng/Kg for industrial areas. The major congener detected were BDE#99 and BDE#47.

CHAPTER 6

CONCLUSIONS

Harmful effects to human health and to ecological systems resulting from exposure to chemical compounds (single and mixtures) are of global concern. Each environmental problem can perturb the human health, the ecology, the economic system or the quality of human life. Therefore, it is increasingly clear that the improvements in environmental matrices quality would bring substantial health benefits worldwide.

An isolated environmental agent exhibits a specific toxicological profile and represents a risk of adverse effects on humans depending on its intrinsic properties or toxicity, concentration and exposure time, the corresponding dose levels and the number and susceptibility of exposed subjects. On the other hand, the presence of multiple-combined chemicals makes the composition of environmental pollution a complex variable, accountable of multiple situations in terms of toxicological risk assessment. In particular, the information on the composition and associated additive, synergistic or antagonistic effects of real-word-mixtures are fundamental to perform an accurate assessment of related risk. In the last years, there has been an increased need of a holistic approach to risks assessment that concern real life situations of multi-chemical, multi-media, multi-route, and multi-species exposures. Moreover, the wide use of chemicals and the resulting enhanced diversified pollution make the assessment of related health risks increasingly important.

Among the different types of pollution, air contaminants represent an issue of great concern over the world due to their widespread nature, mainly in highly urbanized areas. A great number of air pollutants are common to both indoor and outdoor environments. In particular, the indoor air pollution, able to affect seriously the health of the inhabitants, has become a very important question among many scientists, as people spend most of their time (> 90%) in enclosed environments. Most of the studies on combined indoor and outdoor air quality have found indoor air pollutants' concentration higher than outdoor and have confirmed that indoor air quality is highly negatively affected both by outdoor air quality and by indoor activities. Therefore, it is important to consider both indoor and outdoor environment and conduct further studies in order to enhance our understanding on the potential health implication of indoor exposure, the cause of problems and associated control measures and prevention strategies.

Other than environmental monitoring and health surveillance, biological monitoring is an important tool in the prevention of diseases due to toxic agents in the general or occupational environment. Several measurements have been carrying out for many years within the context of biological monitoring to assess the occurrence and the extent of exposure, the early and clinical effects and the sensitivity to an external insult. Suitable biomarkers may provide the critical link

between chemical exposure, internal dose and health damage, and are valuable tools in assessment of risk. Specifically human biomonitoring, by measuring the concentration of unmodified compounds or their metabolites in body fluids (blood, urine, and breast milk) or tissues (hair, nails, fat, and bone), is an essential method to evaluate personal exposure to air pollution. It would be also useful for identifying potential health risks and supporting environmental and public health policies.

These needs are particularly urgent for populations which are at greatest risk from such threats due to characteristics that make them particularly vulnerable. Among all people exposed to pollutants, children are considered a high-risk population for both acute and chronic effects of environmental hazards. Given the well-known differences of exposure to air pollution between children and adults in terms of magnitude of exposure and susceptibility to adverse effects, it is necessary to perform focused studies for the assessment of exposure to air pollutants for children. Despite the great number of researches performed on urban pollutants exposure for adult population, relatively few data are available for children. The lack of information on the composition and associated effects of real-world-mixture exposures are fundamental obstacles for the development of children risk assessment approaches. Even though there has been recent progress within this research field, there is still a lack of knowledge on how to monitor exposures and effects of complex exposures. In order to considerably expand the scientific database and increase the knowledge on harmful exposure combinations, techniques and methods need to be further developed by both epidemiologic and laboratory research approaches.

The present PhD project concerns the development and field application of strategies both for performing human biomonitoring and environmental monitoring control programs in order to characterize the combined and cumulative exposures to concurrent contaminants at environmental concentration levels. Specifically, two human biomonitoring studies on quantitative analytical procedures, based on multiresidue analytical methods to determine chemical mixtures at levels that are likely to be encountered by general population, have been developed and then applied in monitoring studies aimed to characterize the exposure of children to different environmental pollutants occurring simultaneously. Moreover, two environmental monitoring studies were performed respectively by the use of transplanted and native lichen as innovative tools for monitoring the concentrations of a great number of air pollutants (until to 78 compounds).

Innovative procedures and analytical results were explained and discussed in three relevant articles published on pertinent scientific journals and one is in progress for future submission.

Such improved understanding would be the basis in developing procedures for risk assessment of chemical mixtures.

The main results of the present doctoral thesis are summarized below:

- the re-elaboration of data resulted from a previous monitoring study (performed in the academic years 2007-2009) about eight highly toxic metals (As, Be, Cd, Co, Cr, Ni, Pb, and Sb), which have been classified as known or suspected carcinogenic metals and semimetals, for long exposures at low concentrations (Protano et al., 2017b). The results of the statistical elaboration related to the urinary concentration of the studied elements, recovered in samples from 143 healthy Italian children (5-11 years old) attending a primary school of the urban area of Rome (Italy), evidenced that the great part of the studied children resulted co-exposed to at least two of the monitored elements (83.2 %). This finding is of great importance as metal-induced carcinogenesis is related to the co-exposure to more than one carcinogenic metal and the multiplicative effects determined by this co-exposure was yet evidenced for such elements in previous studies, both for occupational and general population, including children.

- The development and application in field of a smart multiresidue method, easy to apply and with low cost of execution, for simultaneously biomonitoring ten environmental pollutants (benzene, toluene, ethylbenzene, o,m,p-xylenes, methyl *tert*-butyl ether, ethyl *tert*-butyl ether, diisopropyl ether, *tert*-amyl methy ether) among volatile organic compounds, to be used in the assessment process of the risk arising from common urban contaminants and ETS exposures. Among the specific parameters calculated to evaluate the analytical performances of the developed procedure, the favorable values of sensitivity (2.9 ÷ 7.5 ng l^{-1}) were compatible with the analyses of biological samples from the general population. The assay has been then successfully validated through its application in field in a sample of forty children (5-11 years) environmentally exposed to different levels of airborne pollutants in an urban area of Rome and published on Analytical and Bioanalytical Chemistry (ABC) journal in June 2016 (Antonucci et al., 2016). The excellent analytical performances and the successful field validation of such smart method made it a valuable tool for assessing exposures of children living in different environmental and social contexts. The developed procedure has been further employed in a monitoring campaign conducted during the winter of the academic years 2014-2015, which involved 435 children attending at primary school located in different sites of central Italy, which could be classified as urban (Rome) and rural (Rieti) areas.

Anyway, urine samples associated to some monitored sites showed unexpected high amounts of a specific analyte among the investigated ones. In order to understand these exceptional results some aliquots of the urine samples were send to the laboratory of the SIPOMICS-Metabolomics Platform, Universitat Rovita i Virgili, Tarragona, Spain for some analytical determination by the use of the metabolomics approach. Once the additional database is completed, the results will be inserted into a robust statistical data processing for a more comprehensive interpretation of our survey. Nevertheless, preliminary analytical results, referred to almost 900 urine samples (evening and morning urine samples for each participant) and at today only partially processed, evidenced a close correlation between unmodified benzene levels in urine samples and ETS. After this study another biological monitoring campaign, funded by an ONLUS (ALCLI Giorgio e Silvia Association), was conducted in the summer of 2017 and targeted at children attending at primary school in province of Rieti. The 378 urine samples (one for each participant), collected following the tried-and-tested procedure, are currently being analyzed according to the developed method.

- The development of analytical procedures for biomonitoring typical airborne pollutants (As, Cd, Cr, Cu, Hg, Ni, Pb and 12 selected PAHs) in school environments, both indoor and outdoor, by using transplanted lichen bags of *Pseudovrnia furfuracea* epiphytic species as bioindicator and bioaccumulator devices. The present procedure was applied in a monitoring campaign, conducted in February-April 2014, at five primary school districts (attending children of 5–11 years old) of Latium region (central Italy) and was published on Environmental Monitoring and Assessment journal on August of 2017 (Protano et al., 2017). The main challenge of this environmental monitoring program was the capability of this lichen species of being able to track and distinguish between different pollution sources in order to identify major contributing sources of contaminants in an area of interest. Using transplanted lichens as air quality biomonitors, has proven to be an approach of great interest for the simplicity and rapidity with which it can be performed, as lichens are efficient, easy and cheap compared to traditional methods. A research with a similar approach was performed in winter of 2016 in Latium Region (central Italy), with the use of the native lichen species *Xanthoria parietina* to monitor the athmospheric concentration levels of 78 analytes selected among the class of POPs. The monitoring stations were green, residential and industrial areas respectively. On one side, the applied laboratory methods showed excellent analytical performance parameters, on the other side analytical results showed critical concentrations of some pollutants in the industrial

sites, confirming the great opportunities in employing native lichen species to monitor the air quality of a territory and discriminate the different features of each investigated station.

The results of my PhD experiences demonstrated the needs of further studies on environmental pollution in order to prevent the great number of negative effects on environmental and human health. This study needs for some essential conditions: 1) an integrated and holistic approach that consider both the environmental contamination and the intake of the contaminants by humans; 2) the needs for new tools and innovative but fast and cost-effective analytical methods for the simultaneous determination of a great number of compounds; 3) the necessity of a multidisciplinary approach that involves the collaboration of different professional skills, such as chemists, ecologists and public health operators.

In particular, with regard to children's health, our results suggest considerable implications for policy makers in health policies, as educational interventions on parents are essential to increase their awareness about the negative impact of environmental contaminants (i.e. tobacco smoke exposure) during childhood and to teach correct behaviors to protect health of kids, especially in household environment.

In conclusion, we can affirm that environmental contaminants must still be considered of great concern for children health, despite the use of many of them has been banned over the years or progressively reduced by rigorous worldwide regulation with occupational exposure limits and air quality standards.

For these reasons, it is essential that Public Health authorities encourage and promote monitoring programs for the assessment of children exposure to pollutants. The results should be use as objectifiable data for orienting the strategies aimed to control air pollution.

On the other hand, the same data should be included in community-level health promotion interventions. They could determine a higher awareness of the actual state of the living environments and, consequently, encourage the adoption of "health and ecological friendly" lifestyles.

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