Analysis of metal deposit distribution in ants (Crematogaster scutellaris) at the Florence external scanning microbeam

Research Article

X-RAY Spectrometry

Received: 6 September 2010 Revised: 12 January 2011 Accepted: 13 January 2011 Published online in Wiley Online Library: 2 March 2011

(wileyonlinelibrary.com) DOI 10.1002/xrs.1308

Analysis of metal deposit distribution in ants (*Crematogaster scutellaris*) at the Florence external scanning microbeam

E. Gramigni,[a] S. Calusi,[b,c]* G. Chelazzi,[a,d] F. Del Greco,[e] G. Delfino,[a] N. Gelli,[b] L. Giuntini,[b,c] M. Massi[b,c] and G. Santini[a,d]

Metals are one of the major classes of environmental contaminants and raise concerns for their adverse effects on ecosystems. Ants are good candidates as bioindicators for metal contamination assessment; previous studies indeed showed that ants are able to selectively accumulate some metals within their tissues. Available works provide only whole-body burdens of these contaminants, with scarce information on the fine-scale localisation in tissues and organs, although this information is important to better understand the behaviour of metals in living organisms and to clarify their effects in ecosystems. At the Florence external scanning microbeam, we are carrying on a Particle Induced X-ray Emission (PIXE) study on a common ant species sampled from sites with different environmental metal availabilities. Measurements were carried out on resin-embedded, self-standing sections for a direct localisation of metal deposits and an easy determination of their content. The combined use of the PIXE and the external scanning microbeam made it possible to map element distributions with good spatial resolution and sensitivity, restricting quantitative analyses to the metal accumulation regions. To determine in which tissues/organs metals concentrated, we compared PIXE maps with histological images on sections contiguous to the analysed slices. Measurements in the external set-up allowed us to avoid sample damaging. Differences in metal concentrations in ants from different sites resulted from quantitative PIXE analyses.

Introduction

Heavy metals are considered one of the major classes of contaminants, in both terrestrial and aquatic environments, and their noxious effects have been described for all ecosystem components.[1]

Biological monitoring of metal contaminants is a promising approach, which has recently made considerable progresses. Several types of organisms have been used as bioindicators of environmental metal content.[2,3] Available studies[4] showed that ants are able to selectively accumulate metals within tissues, making these elements detectable even when their environmental concentration is very low: ants therefore can be properly addressed as bioindicators of environmental contamination.[5] Furthermore, ants possess several other ecological and biological features, such as worldwide distribution, key role in ecosystem functioning and strengthening their choice as bioindicators.[6]

Despite these attractive features, examples of the use of ants in biomonitoring are still scanty, if compared with other arthropods.[2] In addition, only whole-body burdens of these contaminants have been usually reported in the literature, thus little is known about the accumulation of metals within specific organs or tissues.[7] This information is however of the utmost importance to point out the behaviour of metals within living organisms and clarify their toxicity mechanisms.[8,9]

This article aims to explore the potentialities of the external micro-PIXE analysis in determining accumulation of metals within tissues and organs of a common ant species, *Crematogaster scutellaris*, widespread throughout the Mediterranean basin, in both natural and human-managed ecosystems.

Micro-PIXE studies on the biological samples have normally been carried out adopting in-vacuum set-ups,[10,11] although the use of external-beam analyses in the biological field is reported since the mid-1970s ([12] and references therein). In general, *ex vacuo* analyses offer the advantage of lower sample damage, thanks to heat dissipation in atmosphere; this feature is especially important for biological samples, normally characterised by considerable sample degradation when analysed in vacuum.[13] External-beam studies allow avoiding sample drying (widely used in sample preparation for in-vacuum analyses), which could induce redistribution of elements in tissues. *Ex vacuo* set-ups are characterised by a spatial resolution, which can be as good as a few microns, typically adequate for many biological applications.[14]

Notwithstanding the favourable characteristics of the nuclear microprobe for eco-biological applications, only a few papers are dedicated to in-vacuum studies on metal distributions in insects[11,15,16,17–20] and no work at all exploits the advantages of

* *Correspondence to: S. Calusi, Department of Physics and Astronomy and National Institute of Nuclear Physics (INFN), via Sansone 1, 50019 Sesto Fiorentino, Florence, Italy. E-mail: calusi@fi.infn.it*

a *Department of Evolutionary Biology 'Leo Pardi', University of Florence, via Romana 17, 50125 Florence, Italy*

b *National Institute of Nuclear Physics (INFN), via Sansone 1, 50019 Sesto Fiorentino, Florence, Italy*

c *Department of Physics and Astronomy, University of Florence, via Sansone 1, 50019 Sesto Fiorentino, Florence, Italy*

d *CESPRO, University of Florence, via Galcianese 20H, 59100 Prato, Italy*

e *Experimental Physics Department, University of Torino, Via Giuria 1, 10125 Torino, Italy*

an external microbeam. In addition, to the best of our knowledge, no study with ion beam analysis techniques has been carried on to obtain information about elemental accumulation in ant tissues/organs. For this task, at our external scanning micro-PIXE set-up,[21] we analysed samples of *C. scutellaris* from sites with different metal concentrations.

Materials and Methods

Samples and preparation procedure

C. scutellaris is a monomorphic ant species, with limited or null differences in size of foraging workers. Ant samples were collected from three different sites around Prato (a town in northern Tuscany, Italy), chosen to represent different level/types of exposure to metal contamination:

1. Urban site: an urban boulevard with heavy vehicle traffic;
2. Ophiolitic site (Galceti), naturally characterised by excess concentrations of several metals (e.g. Fe, Cu, Zn, etc.)[22];
3. Control site (Travalle), far from roads and sources of pollution.

Prior studies[8,9], exploiting combined transmission electron microscope observations and X-ray microanalyses, revealed that within arthropod cells, excess metals may accumulate as insoluble deposits in discrete granules (known as electron-dense granules). In this study, the analysis was focussed on such insoluble deposits within organs and tissues in the abdomen, where excess metals are stored and detoxification mechanisms are carried out.[7] Other storage sites are however possible; for instance, several metals are known to accumulate in the exoskeleton, particularly on mandibles, tarsi or ovipositors, where they contribute to enhance the toughness of these body parts.[23] Although such structures may contribute to total body burdens, it is less clear whether and how they contribute to metal detoxification in adult insects and have not been taken into account in our work.

Samples were prepared according to standard histological methods using chemical fixation, known not to affect metals stored in a non-soluble form (e.g. [8,9,24,25] and references therein).

The first step consisted of cryo-cutting: to facilitate the penetration of fixative and embedding medium (see below) into the tissues, a Leica-CM1510 was used to remove a small slice of the cuticle from the abdomen. Fixation was carried out by immersing the frozen samples in cold (~3 °C) 10% aqueous formalin for 3 h, then rinsing in de-ionised water and successively dehydrating in ethanol series at low temperature (~3 °C), according to standard procedure. Chemical (aldehyde) fixation has proved to be suitable for preserving metal deposits in their specific storage organelles.[8,9] Samples were soaked in propylene oxide and embedded in Spurr resin (Spurr low viscosity embedding kit, Polysciences Inc.).

Using an ultra-microtome (LKB, type: 4801A) and glass knives, we obtained:

1. 2-µm-thick (nominally) sections, stained with toluidine blue and used for organ identification under light microscope (LM); LM images provided valuable help for identification of the regions of interest in the thicker, contiguous sections (see next point) and improved the micro-PIXE analysis;
2. 15-µm-thick (nominally) sections, contiguous to the previous ones, for micro-PIXE analyses.

The sections to be analysed by PIXE were glued onto plastic holed tiles to avoid contributions to the spectra from the support. Due to the low thickness of the sections, contributions from various tissues at different depths were excluded. Target cooling was carried out by blowing He both on the front and the rear sides of the specimen. The combined use of the holed tiles, thin, self-supporting samples and He blows proved to be very efficient in limiting target warming.

Micro-PIXE set-up

Measurements were carried out exploiting 3-MeV proton beams, 0.5–2 nA typical intensities, in He atmosphere, for 30–50 min (50 for quantitative measurements). Actual beam dimensions were ~10 µm on sample surface as well as throughout ant sections, as the beam spread inside the sections produces a negligible beam enlargement (less than 1 µm, SRIM code simulation[26]). For the PIXE analyses, we used our standard two detectors set-up, optimised for low (D1) and medium-high (D2) energy X-rays detection. Subtended solid angles were 6 msr for D1 and 250 msr for D2; D2 was equipped with a 450-µm thick Mylar absorber. Beam charge on sample was measured by exploiting the yield of Si X-rays produced by the beam in the exit window.[21] Spectra and map acquisition was carried out exploiting the new OMDAQ 2007 hardware and software, adapted by Oxford Microbeam Ltd.[27] for our external set-up and recently installed at the microbeam.

Both the target viewing and the sample positioning systems described by Giuntini *et al*.[21] proved to be crucial features for these analyses: typical areas of interest are tiny and very difficult to find, as they are similar to the surrounding environment, and require frequent position adjustments.

Results and Discussion

The study of the many tens (>60) of samples allowed us to document some characteristic correlations between elemental deposit sites and specific organs/tissues, identified in the maps, thanks to the availability of the histological images contiguous with the analysed slices. In view of the use of ants as bioindicators, the knowledge of the accumulation sites for the different elements is of course a mandatory requirement.

The first evidence concerns the gut, where we pointed out a consistent and noticeable association of P and Zn as well as Ca and S in the wall throughout all samples. Cu, when detected, was also correlated with the previous elements in the gut wall. A typical accumulation pattern of Ca, P, S, Sr, Zn and Fe is presented in Fig. 1, together with the LM image of the contiguous histological section.

In many sections, we also found the Malpighian tubules (excretory and osmo-regulatory organs), where a significant correlation of Ca, P, Zn and Sr (when present) was observed (see e.g. Figure 1).

The second evidence concerned Fe, whose accumulation sites always coincided with the fat body. This tissue was recognised on account of the typical round/polyhedral shapes of component cells (adipocytes) containing transparent lipid droplets. The fat body is a metabolically active tissue, storing lipids, proteins and carbohydrates and is in charge of detoxification and excretion of foreign elements.[28] An example of the apparent correlation of Fe with the fat body is shown in Fig. 2, where the metal deposits appear as discrete spots fitting adipocytes in both size and locations.

Evidence of tissue-specific localisation of metal accumulation in ants represents a notable advance with respect to previous mean-body contents reported in the literature. The occurrence of

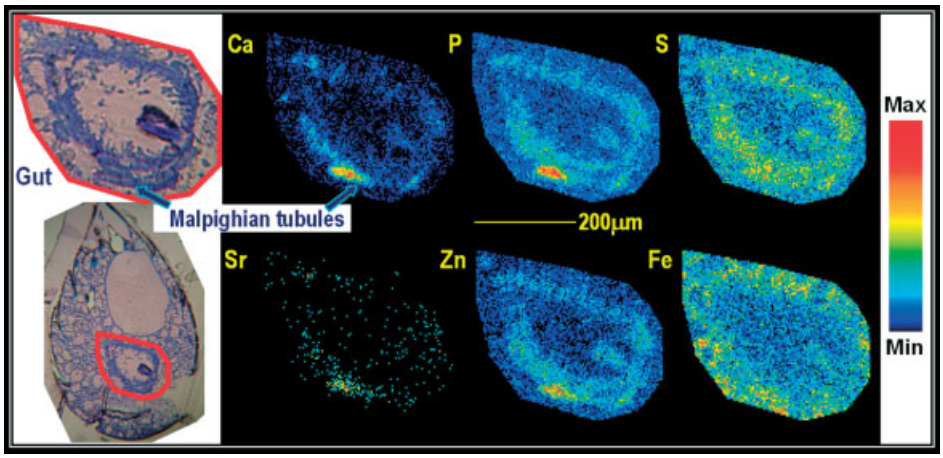

Figure 1. Left: histological image of ant's abdomen section (bottom) and blow up of the gut region, with the fat body surrounding the gut wall (top); right: Ca, P, S, Sr, Zn and Fe deposit maps corresponding to the analysed region, delimited by the red line on the histological image (red = maximum counts, black = minimum counts); the region corresponding to Malpighian tubules is also highlighted.

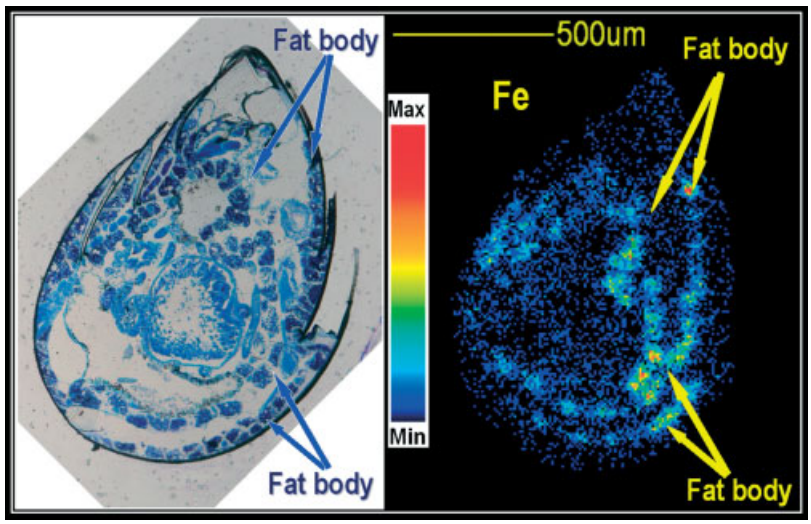

Figure 2. Ant's abdomen section and related Fe deposits (red = maximum counts, black = minimum counts).

deposits of several elements in the gut, fat body and Malpighian tubules supports the role of these organs as primary targets for metal accumulation and suggests their possible role as active defence sites, where toxic metals are stored in a non-biologically active form ([8,9] and references therein). Little is known on tissue/organ bioaccumulation of metals in ants, with the only notable exception of the study by Rabitsch[7] where, however, metal contents were obtained through standard chemical techniques on excised organs, an approach possible only for large bodied species. Interestingly, although metal accumulation was reported in the gut and Malpighian tubules, this study did not put into evidence metals in the fat body, suggesting a difference in metal metabolism between *C. scutellaris* and the three Formicinae species analysed in the quoted investigation.

These qualitative results on metal accumulations gave a fundamental guidance for further analyses. A subsequent, mandatory step to test the reliability of this species as a possible bioindicator of environmental contamination is the demonstration that metal accumulation differs in specimens sampled from differently polluted sites. To this purpose, we made possible the inter-comparison of the Mn, Fe, Cu, Zn and Sr contents within the gut and the fat body for selected samples from three sites with different metal concentrations. OMDAQ software allowed us to select off-line the portion of the scanned area most suitable for the elemental concentration determination.

To obtain quantitative results, it is necessary to know matrix composition and areal density, which determine proton stopping power and X-rays absorption. In our case, the aim was to compare the metal concentrations in the same tissues from different samples, so that the absolute values were not necessary; as a consequence, we adopted conventional matrix and density for all the samples:

1. Matrix composition: C 75%, H 3%, O 8%, N 12% and Cl 2%, based on standard Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analyses and on the elements introduced by our sample preparation procedure.
2. Areal density: we assumed a 1 g/cm^3 for the mass density (plausible for tissues and resin); by means of optical measurements, we found an unexpected wide distribution of the sample thickness (14–20 μm) for the same nominal value. For quantitative analyses, we thus assumed 17 ± 3 μm; the wide thickness indetermination leads to consistent errors on the

Table 1. Metal concentration in the gut and in the fat body in the ants from the three sites

Element	Control (ppm)	Ophiolitic (ppm)	Urban (ppm)
Gut tissue			
Mn	0–60	26 ± 8	90 ± 30
Fe	100 ± 30	110 ± 30	160 ± 50
Cu	28 ± 8	15 ± 5	61 ± 18
Zn	60 ± 20	80 ± 20	330 ± 100
Sr	10*	<15	20*
Fat body			
Mn	10*	34 ± 10	0–20
Fe	110 ± 30	360 ± 110	380 ± 110
Cu	0–10	10*	56 ± 17
Zn	47 ± 14	52 ± 16	90 ± 30
Sr	<20	<15	<10

When the peak of an element does not occur in the spectrum, the concentration is indicated by < MDL (minimum detection limit value); when detected quantities are of the order of the MDL, values are reported with * ; when an element is present only in some samples, the interval of the measured concentrations is reported. The errors originate from the wide indetermination on the sample thickness, as explained in the text.

metal concentrations (~30%), which is the only contribution to the error relevant to the inter-comparison of the metal content.

PIXE spectra were analysed by using GUPIXwin[29]; measured average metal concentrations in the gut tissues (five samples for each provenance) and fat body (six samples for each provenance) are reported in Table 1; the errors are by far dominated by the above-mentioned thickness indetermination.

To verify that no damage to the samples was occurring during the measurements, we compared the elemental concentrations obtained replaying just the initial and the final part of some runs; measured concentrations were identical within the fit errors (not greater than 10% even for the less abundant elements).

Despite the considerable uncertainty on the concentration values, the urban site is almost always characterised by higher content of metals, both with respect to the control and to the ophiolitic sites. The most significant comparisons involve the Zn content in the gut wall and the Fe content in the fat body. Regarding the gut tissue, we noticed that ants from the urban area present an exceedingly higher concentration of Zn (~5 times higher) with respect to those from the control site (Travalle). In the fat body, Fe shows a much higher concentration (~3 times higher) for the urban and ophiolitic provenances with respect to the control site; also, the Zn content seems to differentiate the urban from the other sites.

In the Malpighian tubules of the urban insects, we found a Zn concentration ~3 times higher than in those of the control site; however, due to the limited number of analysed tubules, our conclusions need to be confirmed by further analyses.

The observed differences in metal contents are broadly in line with their availabilities in the environment. Both Zn and Fe concentrations (higher in urban with respect to rural ants) are known to increase owing to environmental pollution. Zn, for example, is released during tyre wear and mixed combustion and is considered a good indicator for traffic pollution.[30–33] Similarly, the remarkable amounts of Fe in the fat body of the ants collected in the ophiolitic site, much higher than in the control site, reflect the presence of Fe in the soil in overwhelming percentage.[22]

Samples from the ophiolitic site appeared however poorer in Zn and Cu with respect to urban samples. Further investigation is thus needed to fully interpret the metal accumulation patterns and will involve direct measurement of metal soil contents, determination of its actual bioavailability and a full description of uptake paths in ants. Such a detailed analysis is however beyond the scope of this study.

Conclusions

The external scanning micro-PIXE analyses proved to be effective in describing the compartmentalisation of selected elements within ant tissues. We prepared the samples following plain and low-cost histology protocols, which may allow the diffusion of this kind of analysis. Although it is known that chemical fixation may not be sufficiently fast to fully prevent the loss of electrolytes and mobile elements,[24,25] the adopted sample preparation protocol is routinely followed for the detection and proper quantification of metals stored in non-soluble grains, which are retained within insect tissues.[8,9]

Coupling histological imagery and PIXE maps allowed us a fast and reliable identification of organs and tissues, so that the biological interpretation of element deposit maps was strongly simplified. We identified as accumulation sites the gut wall (Zn), the Malpighian tubules (Zn and Sr) and the fat body (Fe), confirming the role of these organs as detoxification sites (e.g. [7,8] and references therein). Beyond the morphological and topological identification of the accumulation sites, we made possible the inter-comparison of the Mn, Fe, Cu, Zn and Sr contents within the tissues. Notwithstanding the heavy indetermination (arising from the crude method for the determination of the sample thickness) on the measured elemental concentrations, we were able to put into evidence differences in metal concentrations in ants from different sites: as expected, ants from habitats with different metal availabilities have greater metal concentrations in gut tissues and fat body than ants from unpolluted sites.

Acknowledgements

This work has been carried out in the framework of the INFN experiment 'FARE'. Financial support was also given by the 'Ente Cassa di Risparmio di Firenze' (grant to G. S.). The authors wish to warmly thank P. Pollovio, M. Falorsi, A. Catelani, R. Ciaranfi, M. Montecchi, M. Manetti, G. Tobia, D. Baracchi and G. Tanteri for their assistance.

References

[1] J. M. Pacyna, Monitoring and assessment of metal contaminants in the air, in *Toxicology of metals* (Ed: L. W. Chang), **1996**, pp. 9–28.
[2] A. Heikens, W. J. G. M. Peijnenburg, A. J. Hendriks, *Environ. Pollut.* **2001**, *113*, 385.
[3] M. Nummelin, M. Lodenius, E. Tulisalo, H. Hirvonen, T. Alanko, *Environ. Pollut.* **2007**, *145*, 339.
[4] W. B. Rabitsch, *J. Appl. Ecol.* **1997**, *34*, 1455.
[5] T. Eeva, J. Sorvari, V. Koivunen, *Environ. Pollut.* **2004**, *132*, 533.
[6] W. B. Rabitsch, *Environ. Pollut.* **1995**, *90*, 239.
[7] W. B. Rabitsch, *Arch. Environ. Contam. Toxicol.* **1997**, *32*, 172.
[8] G. Pigino, M. Migliorini, E. Paccagnini, F. Bernini, *Ecotox. Env. Saf.* **2006**, *64*, 257.
[9] C. Ballan-Dufrançais, *Microsc. Res. Tech.* **2002**, *56*, 403.

[10] W. J. Przybylowicz, J. Mesjasz-Przybylowicz, V. M. Prozesky, C. A. Pineda, *Nucl. Inst. Meth. B* **1997**, *130*, 335.
[11] G. Tylko, J. Mesjasz-Przybylowicz, W. J. Przybylowicz, *Nucl. Instr. Meth. B* **2007**, *260*, 141.
[12] E. T. Williams, *Nucl. Instr. Meth. B* **1984**, *3*, 211.
[13] R. Ortega, G. Devès, Ph. Moretto, *Nucl. Instr. Meth. B* **2001**, *181*, 475.
[14] T. Sakai, M. Oikawa, T. Sato, T. Nagamine, H. D. Moon, K. Nakazato, K. Suzuki, *Nucl. Instrum. Meth. B* **2005**, *231*, 112.
[15] W. J. Przybylowicz, J. Mesjasz-Przybylowicz, P. Migula, K. Turnau, M. Nakonieczny, M. Augustyniak, E. Glowacka, *Nucl. Instr. Meth. B* **2004**, *57–66*, 219.
[16] M. Augustyniak, W. Przybylowicz, J. Mesjasz-Przybylowicz, M. Tarnawska, P. Migula, E. Glowacka, A. Babczynska, *X-Ray Spectrom.* **2008**, *37*, 142.
[17] P. Migula, W. J. Przybylowicz, J. Mesjasz-Przybylowicz, M. Augustyniak, M. Nakonieczny, E. Glowacka, M. Tarnawska, *Plant Soil* **2007**, *293*, 197.
[18] R. M. S. Schofield, J. H. Postlethwait, H. W. Lefevre, *J. Exp. Biol.* **1997**, *200*, 3235.
[19] G. Tylko, J. Borowska, Z. Banach, P. Elżbieta Pyza, W. J. Przybylowicz, J. Mesjasz-Przybylowicz, *Microchim. Acta* **2006**, *155*, 301.
[20] M. Tarnawsaka, P. Migula, W. Przybylowicz, J. Mesjasz-Przybylowicz, M. Augustyniak, *Nucl. Instr. Meth. B* **2007**, *260*, 213.
[21] L. Giuntini, M. Massi, S. Calusi, *Nucl. Instr. Meth. A* **2007**, *576*, 266.
[22] M. Angelone, O. Vaselli, C. Bini, N. Coradossi, *Sci. Total Environ.* **1993**, *129*, 291.
[23] R. M. S. Schofield, M. H. Nesson, K. A. Richardson, *Naturwissenschaften* **2002**, *89*, 579.
[24] T. Nagata, *Prog. Histochem. Cytochem.* **2004**, *39*, 185.
[25] B. Forslind, M. Lindberg, G. M. Romans, J. Pallon, Y. Werner-Linde, *Microsc. Res. Tech.* **1997**, *38*, 373.
[26] J. F. Ziegler, *Nucl. Instr. Meth. B* **2004**, *219*, 1027.
[27] OM_DAQ, Oxford Microbeams Limited © 2007. Available from: http://www.microbeams.co.uk/.
[28] V.T.P. de Oliveira, C. da. Cruz-Landim, *Biociências, Porto Alegre* **2003**, *11*, 195.
[29] J. A. Maxwell, W. J. Teesdale, J. L. Campbell, *Nucl. Instr. Meth. B* **1995**, *95*, 407.
[30] X. Huang, I. Olmez, N. K. Aras, G. E. Gordon, *Atmos. Environ.* **1994**, *28*, 1385.
[31] G. Weckwerth, *Atmos. Environ.* **2001**, *35*, 5525.
[32] R. Vecchi, M. Chiari, A. D'Alessandro, P. Fermo, F. Lucarelli, F. Mazzei, S. Nava, A. Piazzalunga, P. Prati, F. Silvani, G. Valli, *Atmos. Environ.* **2008**, *42*, 2240.
[33] P. Salvador, B. Artiñano, X. Querol, A. Alastuey, M. Costoya, *Atmos. Environ.* **2007**, *41*, 1.

