European Journal of Soil Biology 58 (2013) 24–31

Contents lists available at SciVerse ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Ants as bioaccumulators of metals from soils: Body content and tissue-specific distribution of metals in the ant *Crematogaster scutellaris*

Elisa Gramigni [a], Silvia Calusi [b], Nicla Gelli [b], Lorenzo Giuntini [b], Mirko Massi [b], Giovanni Delfino [a], Guido Chelazzi [a,c], David Baracchi [a], Filippo Frizzi [a], Giacomo Santini [a,c,*]

[a] Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino, Italy
[b] Department of Physics and Astronomy, University of Florence, INFN Sezione di Firenze, Via Sansone 1, 50019 Sesto Fiorentino, Florence, Italy
[c] CESPRO, University of Florence, Via Galcianese 20H, 59100 Prato, Italy

ARTICLE INFO

Article history:
Received 7 March 2013
Received in revised form
24 May 2013
Accepted 27 May 2013
Available online 7 June 2013

Handling editor: Stefan Schrader

Keywords:
Ants
Metals
Crematogaster scutellaris
Micro-PIXE

ABSTRACT

Ants possess several features that make them good candidates as indicators of environmental contamination. Concentrations of six metals (Cu, Cd, Ni, Mn, Pb and Zn) were investigated in *Crematogaster scutellaris*, a myrmicine ant common throughout the Mediterranean basin. Concentrations of metals in ant bodies and soil samples from polluted and unpolluted sites were compared. Tissue-specific distribution of metals in a non-soluble form was examined using a novel technique that coupled histological imagery and micro-PIXE analysis. Zinc and cadmium accumulated in ants with respect to the soil. Copper body burdens were independent of soil concentration, while lower concentrations of nickel, manganese and lead were found in ants than in the soil, although ant body content was correlated with soil concentrations. Most of the metals were concentrated in the midgut, the Malpighian tubules and fat body, supporting the role of these organs as primary sites of metal storage and contaminant immobilization.

1. Introduction

Metals represent one of the major classes of contaminants in both terrestrial and aquatic environments [1–3]. The deleterious effects of some metals are well known, and have been described at all levels of biological organization, ranging from cells to ecosystems [4,5]. Several kinds of organisms have been used to study the impact of metal pollution in terrestrial ecosystems [6,7]. Among these, ants possess several features that make them good candidates as indicators of environmental contamination [8–11]. Their worldwide distribution and abundance, ease of collection and identification all play a crucial role [12–14]. Furthermore, ants have a key role in ecosystem functioning and occupy all trophic positions from herbivores to predators, with a predominance of omnivores [15,16], and therefore potentially act as efficient collectors of pollutants within ecosystems [17]. In addition, ant colonies can be seen as a type of perennial "superorganism", whose life expectancy (>10 years in some cases) generally exceeds the life span of the majority of other invertebrates [18], making long-term effects of pollutants more easily detectable. Despite these features, reports on the use of ants in studying the impact of metals are rare when compared to other arthropods (see Ref. [9] for a review).

Selective accumulation of specific metals within the ant body occurs in several ant species [9–11,19–22]. Evidence has shown that metal pollution may affect ant behaviour [23] and impair immune responses [24]. Finally, effects on population structure and community composition along metal pollution gradients have also been reported [25–27].

The available literature reveals both taxonomic and geographical biases. The majority of studies, in fact, predominantly considered Formicine ants (and among these *Formica*, *Lasius* and *Camponotus* spp.), mainly from north and central Europe. Comparatively less is known for Myrmicinae or other subfamilies and, to the best of our knowledge, no report exists on species from southern latitudes. Since different species show different accumulation patterns [9,20,28], obtaining more information on metal

* Corresponding author. Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino, Italy. Tel.: +39 055 457 4721; fax: +39 055 4574906.
E-mail address: giacomo.santini@unifi.it (G. Santini).

1164-5563/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejsobi.2013.05.006

uptake in other species from different sites is necessary to ascertain whether ants can be used in routine monitoring schemes. Furthermore, details of metal accumulation and metabolism in ants have been poorly investigated. The majority of available studies have provided only whole-body burdens of these contaminants, with little or no information on the microscopic localization of such elements within specific organs and tissues. Notable exceptions are the studies by Rabitsch [11] and Carneiro et al. [29], examining metal contents in different ant species. This information is, however, important to elucidate the behaviour of metals within living organisms and clarify their toxicity mechanisms. The detection of metals in specific organs is a first step to a fuller comprehension of the physiological basis of metal tolerance in ants.

The present study was conducted to investigate the patterns of accumulation of six metals (Cu, Cd, Ni, Mn, Pb and Zn), known as markers of industrial and traffic pollution [30], in the ant *Crematogaster scutellaris*. This myrmicine species is widespread throughout the Mediterranean basin [31], where it is common in both natural and human-managed ecosystems, including urban and industrial areas. This species forms large colonies (up to several thousand specimens) and can be easily recognized even by non-specialists due to its characteristic form and colour [31,32]. Finally, this highly competitive species depends both on predation and homopteran exudates as sources of energy [33–35] making pollutant uptake easier. In our study, body metal concentrations were determined in samples collected from polluted (heavy traffic roads within an industrial town) and unpolluted sites (countryside woods, located far from roads and sources of industrial pollution). These concentrations were compared with the metal content of soil at the same sampling sites. Finally, the within-body distribution of metals in a non-soluble form (such as those deposited in intracytoplasmic membrane-bound granules) was examined, to detect the specific localization of elements [29,36]. This was achieved using a novel technique based on coupling histological imagery and micro-PIXE analysis [37,38]. PIXE (Particle-Induced X-ray Emission) is a spectrographic technique that can be used for the non-destructive, simultaneous quantitative determination of all the elements in a sample bombarded with a proton beam. Proton collisions with target atoms cause the expulsion of inner shell electrons and the emission of X-rays, whose energies can be used as a signature of the emitting elements. Since this technique allows the quantification of all elements (from Na onward), other metals, such as Fe and Sr, not included in whole-body analyses, were also detected.

2. Materials and methods

2.1. Sampling

To obtain samples from sites with different metal availability, *C. scutellaris* ants and soil materials were collected from four different sites within and near Prato (northern Tuscany, Italy, 43°52′46″ N, 11°05′50″ E). Prato, with about 180,000 inhabitants, is an important industrial district of central Italy with active textiles, manufacturing (mostly metals and furniture), chemical and mechanical sectors. The growing traffic flow to the town and industrial activities contribute to atmospheric pollution and recently concerns over urban air quality have been expressed [39].

Two collection sites (hereafter referred to as “urban”) were chosen within the urban area: two large boulevards characterized by heavy vehicle traffic and bordered by a row of *Pinus pinea* trees, where *C. scutellaris* nests have been observed. The first site (“Galilei”, named after the road) is located at the north-east side of the town. The second one (“Repubblica”) lies within the town and is surrounded on each side by houses and industrial estates. The other two sites (hereafter referred to as “controls”) are located in the nearby countryside and are “Le Croci” and “Travalle” (43°56′39″ N, 11°11′1″ E and 43°53′21″ N, 11°9′52″ E, respectively). The first is a monospecific stand of *Pinus nigra* trees, located at about 500 m a.s.l. on the east facing slopes of a mountain. The second one is a mixed wood mainly composed of *Pinus pinaster* and oak trees (*Quercus pubescens*) on the south-east facing slopes of a hill (max elevation 140 m a.s.l.). The two sites are far from industrial estates and the distance from the nearest road is >2.5 km. From each site, four different colonies, spaced at least 30 m apart from each other, were sampled. Each ant sample consisted of 15 workers from the same colony. Only ants foraging outside the nest were collected since it is known that surface workers tend to accumulate higher concentrations of pollutants than those inside the nest [40]. *C. scutellaris* is a monomorphic species with few or no differences in the size of foraging workers. Care was taken, however, to collect specimens of comparable dimensions to avoid size-related variation in metal concentrations. Soil samples, collected within 1 m from each ant colony, were obtained by removing a 15 × 15 cm portion of soil to a depth of approximately 10 cm. Ants were placed in plastic vials and stored at −20 °C until analysis. Soil samples were stored at room temperature in sealed plastic bags for a maximum of two weeks.

2.2. Chemical analysis

Chemical analysis was performed to quantify the metal content of whole ant bodies and soil samples. Ants were first cleaned by washing with doubly distilled water, and then oven-dried at 60 °C for 24 h. Metal content was determined by digesting oven-dried material in 5 ml HNO_3 at 80 °C for 2 weeks, after which 2 ml of $HClO_4$ was added and the volume adjusted to 10 ml with deionized water. Soil samples were dried (60 °C for 24 h) and then milled and sieved through a 2 mm stainless steel mesh. Dissolution was carried out in 50-ml Erlenmeyer flasks using 0.5 g of sample and a mixture of 5 ml of 65% HNO_3 and 15 ml of 37% HCl. The solution was heated at 50 °C in a sand bath for 4 h, centrifuged, filtered and diluted to 100 ml with deionized water. The concentration of the elements was performed using an Optima 2000 Perkin Elmer Inductively Coupled Plasma (ICP) Optical Emission Spectrophotometry (OES) Dual Vision and expressed on a dry weight basis. The detection limits were (in $\mu g\ l^{-1}$): 2.3 (Cd), 3.6 (Cu), 0.93 (Mn), 10 (Ni), 28 (Pb) and 1.2 (Zn). Values below the lower detection limits were considered zero in statistical analyses. Certified reference materials (grade BCR, Fluka Analytical, Sigma–Aldrich) were used to verify the accuracy and the precision of the methods.

2.3. Micro-PIXE analysis

PIXE analysis was performed to obtain information on tissue-specific metals occurring within ant organs. Analyses (on samples maintained in air) were run at the external scanning microbeam facility [41,42] of the 3 MV Tandetron accelerator of the INFN LABEC Laboratory in Florence, widely used in cultural heritage studies [43,44] and geological investigations [45,46].

Measurements were carried out in a He atmosphere by means of 3 MeV proton beams with 0.5–2.0 nA typical intensities. Actual beam dimensions were ∼10 μm at the sample surface, yielding maps of elemental distribution with 10 μm spatial resolution. PIXE, is an X-ray spectrographic technique that can be used for the non-destructive, simultaneous quantitative determination of all the elements present in the samples, from Na onward [47]. When a sample is bombarded with MeV proton beams, collisions of the projectiles with target atoms can cause the expulsion of inner shell electrons which, in turn, determines X-ray emission. The energies of the emitted X-rays are characteristic of the emitting elements

and from the number of X-rays produced in the sample being analysed it is possible to obtain the concentration of the corresponding element. Obtaining quantitative measurement of metal concentration through PIXE is a complex issue requiring the knowledge of the matrix composition and the areal density of each sample (areal density = the mass per unit area, i.e. the product of the density and the thickness of the sample), which determine proton stopping power and X-rays absorption. Since this information was not available, the matrix was assumed to be constant across samples, with composition: C = 75%, H = 3%, O = 8% and Cl = 2% (mass). These figures were based on data obtained from mass spectrometry analysis performed on *C. scutellaris* (Ottonetti, personal communication), composition of the embedding resin (see below) and data from the literature [48–50]. Similarly, the density was assumed to be 1 g cm^{-3} and the thickness 17 ± 3 μm, from optical measurements detailed in Gramigni et al. [37]. However, because assuming different compositions or densities may lead to different estimations of metal content, direct comparisons with values obtained by other techniques (e.g. the whole-body values obtained from ICP) or data from the literature could be misleading and thus not performed.

Four ants were sampled from an urban site ("Repubblica") and left to starve for 48 h, to allow emptying their guts. Analyses were restricted to abdomens, which were carefully excised from the body. Available information on insects and other arthropods [11,51] showed that metals accumulate preferentially in the organs contained in this body area. Furthermore, our preliminary observations on *Crematogaster* samples showed little or no metal accumulation both in the head and in the thorax. Samples were prepared according to standard histological methods to obtain sections to be used either for histological/anatomical or PIXE analyses. The first step consisted of cryo-cutting: to facilitate the penetration of fixative and embedding medium 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 formic aldehyde for 3 h, then rinsing in deionized water and successively dehydrating in ethanol series at low temperature (~3 °C), according to routine procedure. Samples were soaked in propylene oxide and embedded in Spurr resin (Spurr Low Viscosity Embedding Kit, Polysciences Inc.). Chemical fixation using aldehydes has proven to be suitable for preserving metal deposits, such as those occurring in discrete granules, in a non-soluble form [52]. Sections were obtained using glass knives in an ultramicrotome (LKB, type: 4801A); 2 μm and 10 μm thick sections were cut in sequence. The thinner sections (2 μm) were stained with buffered toluidine blue and used for organ mapping under the light microscope; thicker sections were used for micro-PIXE analysis. Further details on the micro-PIXE setup, spectra acquisition and analysis can be found in Gramigni et al. [37].

2.4. Data analysis

Differences in metal concentrations in both ants and soil sampled in the four sites were assessed using ANOVA using a nested design with two factors: (1) treatment, fixed factor (urban vs. control) and (2) site, random factor nested within treatment. *F* ratios for the treatment and the site factor were computed as $F_{treat} = MS_{treat}/MS_{site}$ and $F_{site} = MS_{site}/MS_{residual}$, respectively, where MS is mean square [53]. When the nested factor site was not significant (with $P > 0.25$) pooling was performed to achieve a more powerful test for the treatment factor [54]. Linear discriminant analysis was used to investigate multivariate differences in ant metal content between the control and urban sites [55]. The correlation between metal ant content and amount in the soil was assessed using Pearson's correlation coefficient after $\log(x + 0.0000001)$ transform of data [53].

3. Results

3.1. Metal body burdens

The metal concentrations in soil and ants are shown in Fig. 1 and the results of the ANOVA are reported in Table 1. In the soil of the urban sites greater amounts of Ni and Pb were found, while greater concentrations of Mn and Cd were surprisingly found in the control sites. The latter, in particular was always below the detection limits in all the urban sites and in 3 out of 8 of control sites. There was also a difference among sites (plots) within each treatment level (urban vs. control). For example, Mn was significantly higher in only one of the control sites ("Travalle") while all the other sites had comparable concentrations. Similarly, Zn, although being globally more abundant in urban soils (under detection limits in 6 out of 8 control sites), was much more concentrated in one of these ("Repubblica"). A similar behaviour was shown by copper, which was, however, always well above the detection limits.

Similar results were obtained from ant samples. Greater amounts of Zn, Ni and Pb were observed in ants from urban than those from control sites. Pb and Ni were almost absent in ants from the control sites (Pb was never detected, Ni detected only once). Cd concentrations were, in contrast, higher in control than urban sites, and Mn showed a pattern similar to that obtained from soil samples, with greater concentration in the "Travalle" site (control). No significant difference between urban and control samples was found for Cu.

When comparing ant and soil metal content, the concentration within *Crematogaster* bodies was always significantly correlated with the amount in the soil, with the exception of Cu (Table 2). Amounts of Zn and Cd in ants were higher than in the soil, while the reverse was true for other metals (Fig. 1).

Discriminant analysis clearly separated urban and control sites (Fig. 2). The first two discriminant functions explained 99.8% of total variance, although this was almost entirely due to the first function (98.8%). Prediction success was 100% for treatments (i.e. all the observations were correctly assigned to urban or control sites), and the two groups segregated at the extremes of the first discriminant function. There was also 100% discrimination success within control sites (i.e. "Travalle" samples were separated from those taken at "Le Croci") along the second discriminant function, whereas it was impossible to discriminate between the two urban sites.

3.2. Tissue metal localization

Examples of PIXE maps, together with their corresponding histological images, are shown in Fig. 3. Not all metals could be detected, due to concentrations lower than the detection limits (10–30 μg g^{-1} for all metals depending on the sample and on the statistics gathered). The clearest pattern was observed for Zn, whose greatest amounts were found in the gut and particularly in the midgut (Fig. 3A): in this tract a mean (±SE) concentration of 260 ± 80 μg g^{-1} was detected. Lower amounts were found in fat bodies (90 ± 25 μg g^{-1}). Zn accumulation was also observed in Malpighian tubules in all the samples (Fig. 3A). Cu was widespread throughout several tissues, with less obvious organ-specific deposits. Mean content in both the midgut and fat bodies was around 60 ± 25 μg g^{-1}. Mn was concentrated in the gut (90 ± 20 μg g^{-1}), whereas it was detected only once in the fat bodies, with a concentration of about 20 μg g^{-1}. In one sample, manganese deposits were also observed in Malpighian tubules (Fig. 3A). Other elements

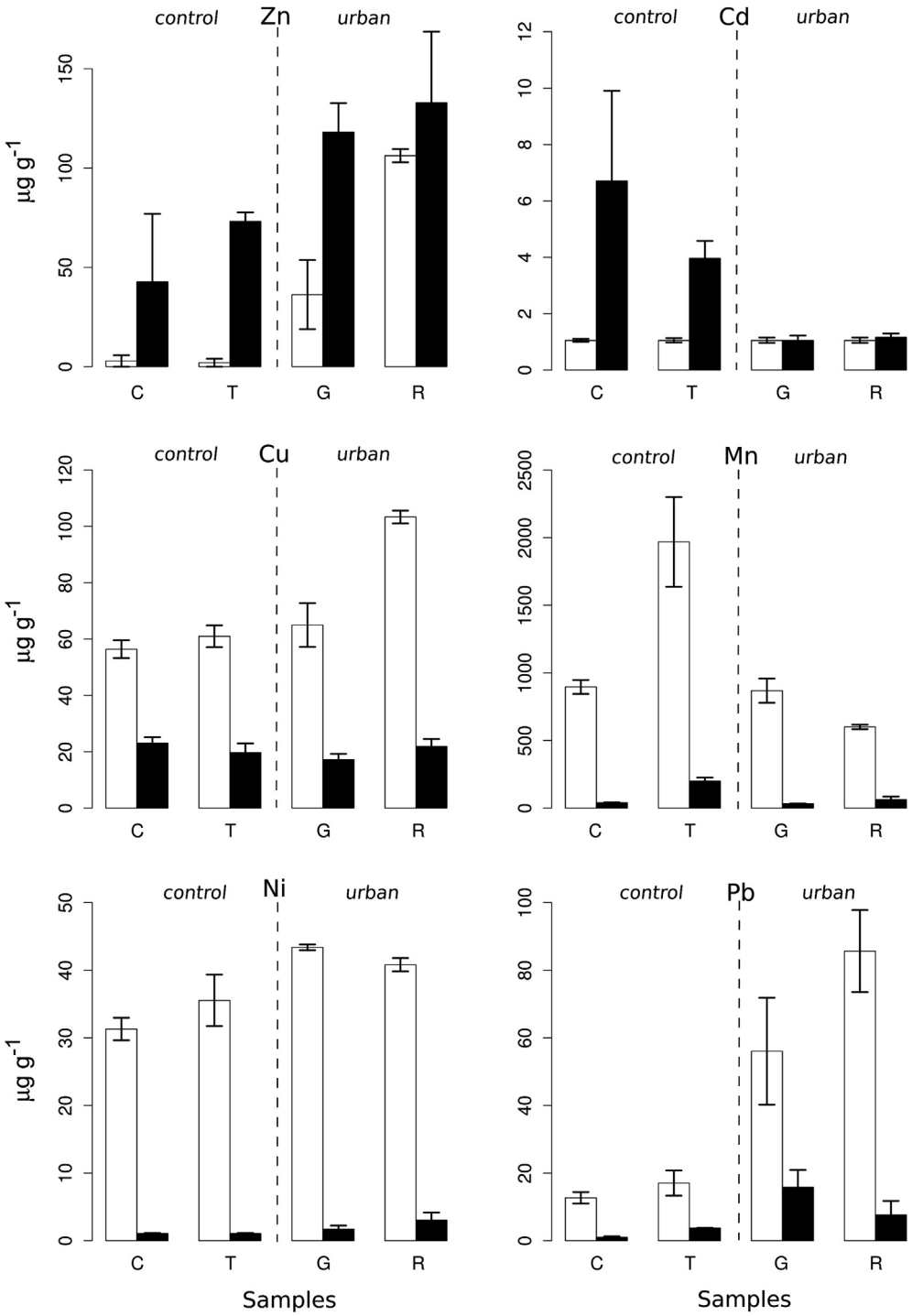

Fig. 1. Metal concentrations in the soil and ants from the four sampling sites. Mean (±SE) of metal concentrations in soil (white bars) and ants (*Crematogaster scutellaris*) (black bars) from four sites. Vertical dashed line in each graph separates control (left) from urban sites (right). C = "Le Croci", T = "Travalle", G = "Galilei", R = "Repubblica".

considered in this paper (Ni, Pb, Cd) were either found in much smaller amounts – with no clear distribution pattern – or not detected at all. PIXE analysis also showed the occurrence of other elements, not considered in whole-body analyses. Among these, of greatest interest in the context of the present study were the results for Fe and Sr. In detail, the latter was detected mainly in Malpighian tubules (only in two samples), following a distribution similar to that of Zn. More interesting was the noticeable and consistent occurrence of Fe, detected mainly in fat bodies (Fig. 3B), where a mean concentration of about 370 ± 20 µg g^{-1} was found. Fe was also detected in both the midgut and Malpighian tubules.

4. Discussion

The results of this study provide the first comprehensive evidence of metal accumulation in *C. scutellaris*, one of the commonest and most important ant species throughout the Mediterranean basin [31,35]. In general, the metal pool content of ants closely

Table 1
F-values of ANOVA for both soil and *Crematogaster scutellaris* samples. Tr = treatment (urban, control), Si(Tr) = site nested within treatment, Res = residuals, df = degrees of freedom. Sample size is $n = 16$ for all metals.

Metal	Source	df	Soil *F*	Ants *F*
Zn	Tr	1	3.87^{ns}	7.43^{*}
	Si(Tr)	2	15.03^{***}	0.42^{ns}
	Res	12		
Cu	Tr	1	1.74^{ns}	0.5^{ns}
	Si(Tr)	2	16.47^{***}	1.29^{ns}
	Res	12		
Ni	Tr	1	15.51^{***}	15.26^{***}
	Si(Tr)	2	1.32^{ns}	1.23^{ns}
	Res	12		
Mn	Tr	1	1.59^{ns}	0.80^{ns}
	Si(Tr)	2	10.10^{**}	21.37^{***}
	Res	12		
Cd	Tr	1	7.80^{*}	7.53^{*}
	Si(Tr)	2	1.04^{ns}	0.73^{ns}
	Res	12		
Pb	Tr	1	29.14^{***}	9.47^{**}
	Si(Tr)	2	1.46^{ns}	0.47^{ns}
	Res	12		

^{ns}Not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

reflected that of soil samples. Samples' origin (urban vs. controls) could be easily discriminated on the basis of their pool metal content by linear discriminant analysis. Multivariate results paralleled those obtained from univariate measures, where a correlation between soil and ant body burdens was found for most metals.

Zn and Cd clearly accumulated within the ant body with respect to soil concentrations. Interestingly, ant body burdens of these metals were detectable even when their environmental counterparts were very low and close to instrumental (ICP) detection limits (as in Cd urban samples). Despite this finding, however, our results indicate that some sort of regulation in Zn uptake or accumulation may operate in *C. scutellaris*, at least at the higher concentrations observed. In the most polluted sites (urban) the Zn body content was in fact 1.3 and 3.3 times greater than that in the soil, whilst this ratio increased to 15 and 37 times for the two control sites ("Le Croci" and "Travalle", respectively), where ambient zinc availability was considerably lower. Whether this pattern is due to a general ability to regulate the uptake at higher concentrations or instead is a feature developed locally by populations subject to long-term pollution is unknown and deserves further investigation [56,57]. When we compared our results with those of previous authors, similarities, but also several differences, emerged. High body concentrations of Zn and Cd have been documented in other species [10,11,21,58,59]. More recently, Grześ [20,60] investigated Zn and Cd uptake in two Formicinae ants (*Formica cunicularia* and *Lasius flavus*) and the Myrmycine *Myrmica rubra*. The former two accumulated Zn with respect to its environmental availability, although a clear limit at higher concentrations was evident. In contrast, *M. rubra* was able to regulate Zn uptake efficiently, and its body contents did not vary with environmental availability. For all species studied, Grześ [20] reported very weak relationships between environmental and body Cd concentrations, suggesting some Cd regulatory ability, at least at higher concentrations. Cd regulation at high concentrations has also been shown in *Lasius niger* [26].

At the opposite extreme, copper appeared to be well regulated . Again, inspection of the available literature shows contrasting observations. Rabitsch [10,28] reported that in several ant species, including the myrmicine *Myrmica sabuleti*, Cu body burdens did not vary from polluted to unpolluted sites, and similar findings have been reported more recently by Nummelin et al. [7] for *Formica lugubris*, *Formica rufa* and *L. niger*. Contrasting results have, however, been obtained by Migula and Głowacka [21] and Eeva et al. [25] for *Formica polyctena* and *Formica aquilonia*, respectively, in which species body Cu content increased with its environmental availability. However, if accumulation depends on exposure levels these variable results in literature may not be contrasting but just represent different exposure levels. Under this view, the lack of correlation in your study may just reflect relatively similar soil levels among the sites.

Finally, the remaining elements (Ni, Mn and Pb) were in an intermediate position: lower amounts of these metals were found in ants than in the soil, but ant body burdens were always positively correlated with the amount in the soil, making a ranking of sites' pollution from biological content potentially possible. However, since ants from only two supposedly polluted and two unpolluted sites were analysed and the gradients of metals in the soil used were relatively short, further samples, spanning a greater range of metal contents, should be analysed before this ant is considered a reliable biomonitor of metal contamination.

A few final remarks are needed on the tissue-specific localization of elements within the ants. Although reports exist on metal concentrations in the tissues of many insects [61–63], this information is rare for ants. To our knowledge, the only reports on tissue-specific accumulation patterns of trace metals in ants are those of Jeantet et al. [64], Jeantet [65] (quoted in Marchal-Ségault et al. [66]) for *F. polyctena* and Rabitsch [11] for two *Formica* spp. and *Camponotus ligniperda*. Results from our study showed that several metals (Zn,

Table 2
Correlation between metal concentration within *Crematogaster scutellaris* body and the concentration in the soil. r = Pearson correlation coefficients. P = probability level. Sample size is $n = 16$ for all metals.

Element	*r*	*P*
Zn	0.603	0.0093
Cu	0.055	0.8928
Ni	0.562	0.0013
Mn	0.600	0.0011
Cd	0.626	0.0087
Pb	0.608	0.0042

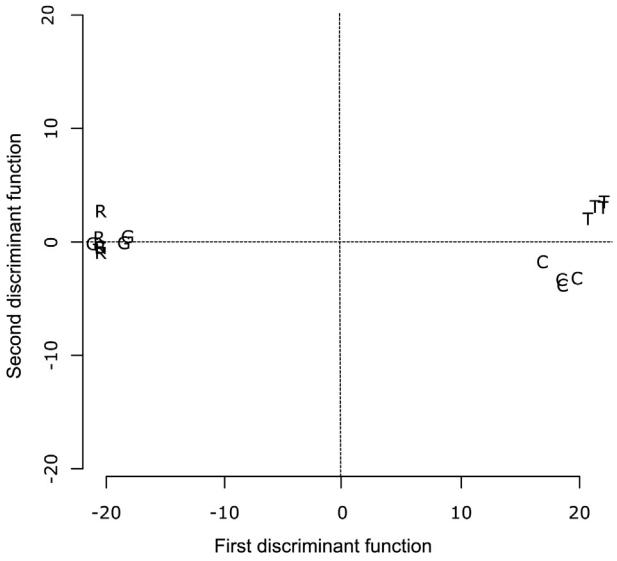

Fig. 2. Results of linear discriminant analysis on *Crematogaster scutellaris* samples. Each letter indicates a site: C = "Le Croci", T = "Travalle" (control), G = "Galilei", R = "Repubblica" (urban).

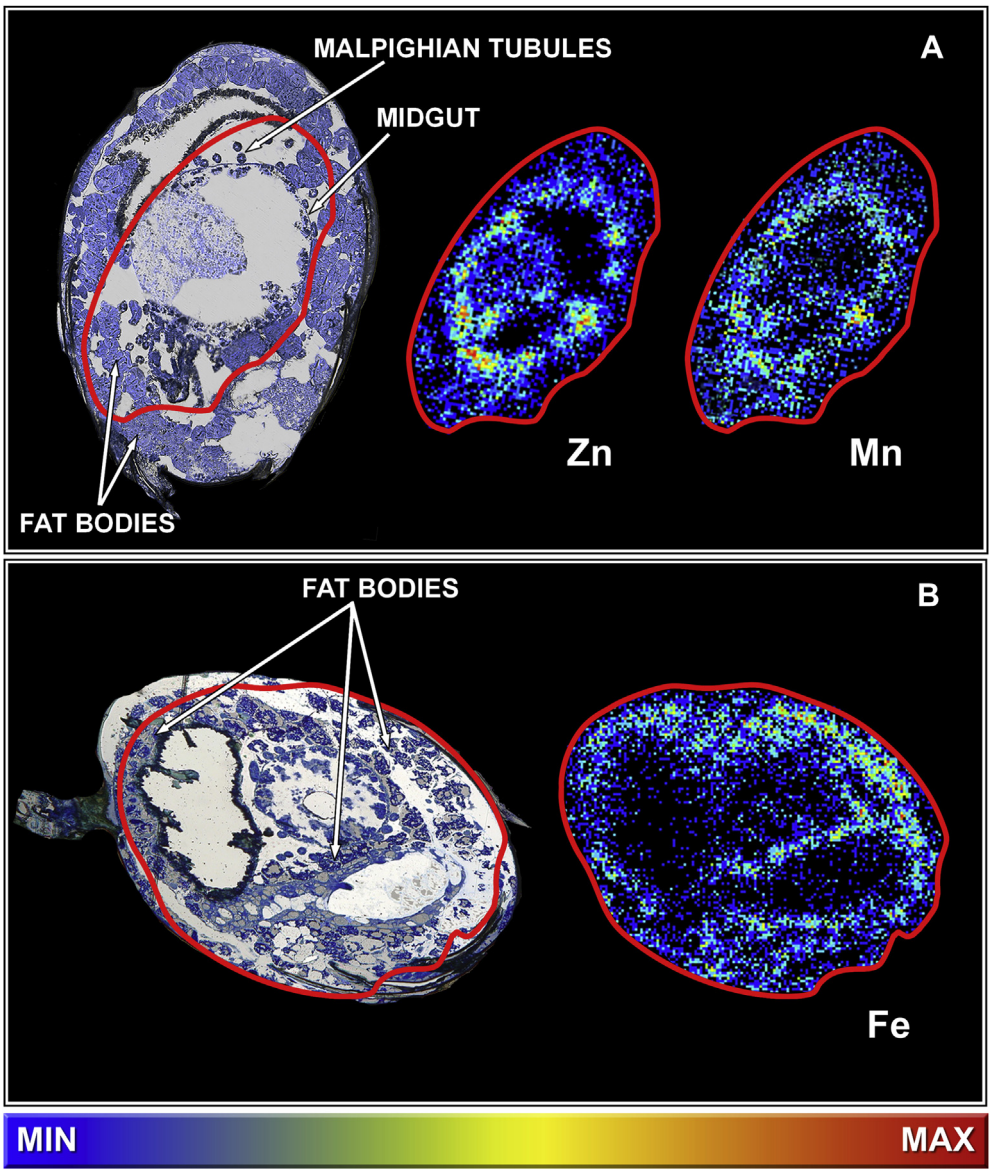

Fig. 3. Examples of PIXE maps for Zn, Mn and Fe (the lighter the colour, the higher the concentration) in *Crematogaster scutellaris* with the corresponding ordinary light microscopy images. PIXE: analysis was restricted to the area within the insets. (A) Detail of an abdomen section. The midgut, Malpighian tubules and fat bodies are recognizable. Zn and Mn deposits can be identified in the gut walls. (B) Abdomen section with fat bodies containing Fe deposits.

Mn, Fe and less regularly Sr and Cu) accumulated in the midgut and the Malpighian tubules, supporting the role of these organs as primary sites of metal storage or immobilization. The major difference to previous investigations is in the high Fe content of fat bodies; Rabitsch [11] failed to find any evidence of this element within this metabolically active tissue, suggesting a difference in metal processing between *C. scutellaris* and the Formicinae species analysed in those studies. More recently Carneiro et al. [29] analysed the presence of metals within amorphous mineral granules in four Brazilian species (*Camponotus abdominalis*, *Camponotus* sp., *Acromyrmex subterraneus* and *Pachycondyla marginata*). This study demonstrated the presence of several metals, including Mg, Mn, Zn, Fe and Sr, deposited in amorphous mineral granules in abdomen isolates of these species, but the technique used did not allow to differentiate among the different tissues/organs within the abdomen.

The tissue-specific analysis of the metals may be extended in several different directions. First, different body regions could be more finely examined. Although preliminary observation did not reveal significant metal amounts either in the head or the thorax, a more careful inspection of these parts could be done. In particular, a detailed scanning of the external cuticle could be performed since it is known that ants (and other arthropods) may accumulate metals such as Zn and Mn in their mandibles (or even tarsi), where they contribute to increase the hardness of these body appendages [67]. Second, although the fixation procedure employed here is suitable for identifying metals in a non-soluble form, such as those deposited in membrane-bound granules, the process may not be sufficiently fast to prevent fully the loss of those metals bound to water-soluble proteins such as metallothioneins [52]. Therefore, a further refinement of the technique or its coupling with chemical estimation of the soluble component would enhance our knowledge.

5. Conclusions

C. scutellaris accumulates some of the most relevant contaminants in its organs with concentrations greater than or at least

proportional to their environmental availability. This behaviour, coupled with several of its biological traits, such as the long-term persistence of nests excavated in tree trunks, and its commonness and abundance in both natural and urbanized/industrial habitats, make this species a potential candidate as a tool for the biomonitoring of metal pollution in anthropized habitats. However, the usefulness of this ant species in biomonitoring is to be treated with extreme caution until repeated measures from samples collected along a longer contamination gradient will not be available. Further ad-hoc investigations to include other metals in the analysis [68] and to clarify how metals from soil are transferred to ants are also needed. Metal uptake may in fact occur through complex pathways (e.g. from plants to parasites/herbivores and from these to ants) and observed differences among ants collected may partly reflect differences in habitat composition (e.g. tree stands) other than pollution. Finally, a better definition of the fate of the elements within the different regions of the ant body is also a mandatory step before *C. scutellaris* is accepted as a biomonitor of metal pollution. Showing tissue-specific accumulation of some of these metals is, in fact, only a first step towards a fuller understanding of the mechanism of their activity and toxicity in ecosystems.

Acknowledgements

This work was partly funded by the Ente Cassa di Risparmio di Firenze (Grant number 2008.0732) to GS. The authors thank C. Gonnelli, P. Pollovio, M. Falorsi, A. Catelani, R. Ciaranfi, M. Montecchi, M. Manetti, G. Tobia, and G. Tanteri for their assistance.

References

[1] S.N. Luoma, P.S. Rainbow, Metal Contamination in Aquatic Environments: Science and Lateral Management, Cambridge University Press, Cambridge, 2008.
[2] B. Markert, S. Wuenschmann, S. Fraenzle, A.M. Graciana Figueiredo, A.P. Ribeiro, M. Wang, Bioindication of atmospheric trace metals – with special references to megacities, Environ. Pollut. 159 (2011) 1991–1995.
[3] J.M. Pacyna, Monitoring and assessment of metal contaminants in the air, in: L.W. Chang (Ed.), Toxicology of Metals, CRC Press, Boca Raton, 1996, pp. 9–28.
[4] L. Järup, Hazards of heavy metal contamination, Brit. Med. Bull. 68 (2003) 167–182.
[5] S. Tong, Y.E. von Schirnding, T. Prapamontol, Environmental lead exposure: a public health problem of global dimensions, Bull. World Health Organ. 78 (2000) 1068–1077.
[6] A. Heikens, W.J. Peijnenburg, A.J. Hendriks, Bioaccumulation of heavy metals in terrestrial invertebrates, Environ. Pollut. 113 (2001) 385–393.
[7] M. Nummelin, M. Lodenius, E. Tulisalo, H. Hirvonen, T. Alanko, Predatory insects as bioindicators of heavy metal pollution, Environ. Pollut. 145 (2007) 339–347.
[8] P.J. Folgarait, Ant biodiversity and its relationship to ecosystem functioning: a review, Biodivers. Conserv. 7 (1998) 1221–1244.
[9] I.M. Grze&sacute, Ants and heavy metal pollution – a review, Eur. J. Soil Biol. 46 (2010) 350–355.
[10] W.B. Rabitsch, Seasonal metal accumulation patterns in the red wood ant *Formica pratensis* (Hymenoptera) at contaminated and reference sites, J. Appl. Ecol. 34 (1997) 1455–1461.
[11] W.B. Rabitsch, Tissue-specific accumulation patterns of Pb, Cd, Cu, Zn, Fe, and Mn in workers of three ant species (Formicidae Hymenoptera) from a metal-polluted site, Arch. Environ. Contam. Toxicol. 32 (1997) 172–177.
[12] B.L. Fisher, Biogeography, in: L. Lach, C. Parr, K. Abbot (Eds.), Ant Ecology, Oxford University Press, Oxford, 2009, pp. 18–37.
[13] L. Ottonetti, L. Tucci, G. Santini, Recolonization patterns of ants in a rehabilitated lignite mine in Central Italy: potential for the use of Mediterranean ants as indicators of restoration processes, Restor. Ecol. 14 (2006) 60–66.
[14] E. Underwood, B. Fisher, The role of ants in conservation monitoring: if, when, and how, Biol. Conserv. 132 (2006) 166–182.
[15] N. Blüthgen, G. Gebauer, K. Fiedler, Disentangling a rainforest food web using stable isotopes: dietary diversity in a species-rich ant community, Oecologia 137 (2003) 426–435.
[16] C.V. Tillberg, D.P. McCarthy, A.G. Dolezal, A.V. Suarez, Measuring the trophic ecology of ants using stable isotopes, Insect. Soc. 53 (2006) 65–69.
[17] M.R. Quinn, X. Feng, C.L. Folt, C.P. Chamberlain, Analyzing trophic transfer of metals in stream food webs using nitrogen isotopes, Sci. Total Environ. 317 (2003) 73–89.
[18] B. Hölldobler, O. Wilson, The Ants, Springer-Verlag, Berlin, 1990.
[19] I.M. Grze&sacute, Cadmium regulation by *Lasius niger*: a contribution to understanding high metal levels in ants, Insect Sci. 16 (2009) 89–92.
[20] I.M. Grze&sacute, Zinc and cadmium regulation efficiency in three ant species originating from a metal pollution gradient, Bull. Environ. Contam. Toxicol. 84 (2010) 61–65.
[21] P. Migula, E. Głowacka, Heavy metals as stressing factors in the red wood ants (*Formica polyctena*) from industrially polluted forests, Fresen. J. Anal. Chem. 354 (1996) 653–659.
[22] A.B. Mukherjee, P. Nuorteva, Toxic metals in forest biota around steel works of Rautaruukki Oy, Raahe, Finland, Sci. Total Environ. 151 (1994) 191–204.
[23] J. Sorvari, T. Eeva, Pollution diminishes intra-specific aggressiveness between wood ant colonies, Sci. Total Environ. 408 (2010) 3189–3192.
[24] J. Sorvari, L.M. Rantala, M.J. Rantala, H. Hakkarainen, T. Eeva, Heavy metal pollution disturbs immune response in wild ant populations, Environ. Pollut. 145 (2006) 324–328.
[25] T. Eeva, J. Sorvari, V. Koivunen, Effects of heavy metal pollution on red wood ant (Formica s. str.) populations, Environ. Pollut. 132 (2004) 533–539.
[26] I.M. Grze&sacute, Ant species richness and evenness increase along a metal pollution gradient in the Bolesław zinc smelter area, Pedobiologia 53 (2009) 65–73.
[27] C.R. Ribas, R.R.C. Solar, R.B.F. Campos, F.A. Schmidt, C.L. Valentim, J.H. Schoereder, Can ants be used as indicators of environmental impacts caused by arsenic? J. Insect Conserv. 16 (2011) 413–421.
[28] W.B. Rabitsch, Metal accumulation in arthropods near e lead/zinc smelter in Arnoldstein, Austria. II. Formicidae, Environ. Pollut. 90 (1995) 239–247.
[29] F.G. Carneiro, C.N. Keim, D. Acosta-Avalos, M. Farina, Elemental composition of biomineralized amorphous mineral granules isolated from ants: correlation with ingested mineral particles from the soil, Micron 44 (2013) 120–124.
[30] R. Vecchi, M. Chiari, A. D'Alessandro, P. Fermo, F. Lucarelli, F. Mazzei, S. Nava, A. Piazzalunga, P. Prati, F. Silvani, G. Valli, A mass closure and PMF source apportionment study on the sub-micron sized aerosol fraction at urban sites in Italy, Atmos. Environ. 42 (2008) 2240–2253.
[31] F. Bernard, Les Fourmis d'Europe Occidentale et Septentrionale, Masson, Paris, 1968.
[32] J. Casevitz-Weulersse, Habitat et comportement de *Crematogaster scutellaris* Olivier (Hymenoptera, Formicidae), Bull. Soc. Entomol. Fr. 77 (1972) 12–19.
[33] Ottonetti, L. Tucci, G. Chelazzi, G. Santini, Stable isotopes analysis to assess the trophic role of ants in a Mediterranean agroecosystem, Agr. Forest Entomol. 10 (2008) 29–36.
[34] G. Santini, L. Tucci, L. Ottonetti, F. Frizzi, Competition trade-offs in the organisation of a Mediterranean ant assemblage, Ecol. Entomol. 32 (2007) 319–326.
[35] G. Santini, P.M. Ramsay, L. Tucci, L. Ottonetti, F. Frizzi, Spatial patterns of the ant *Crematogaster scutellaris* in a model ecosystem, Ecol. Entomol. 36 (2011) 625–634.
[36] C. Ballan-Dufrancais, Localization of metals in cells of pterygote insects, Microsc. Res. Techniq. 56 (2002) 403–420.
[37] E. Gramigni, S. Calusi, G. Chelazzi, F. Del Greco, G. Delfino, N. Gelli, L. Giuntini, M. Massi, G. Santini, Analysis of metal deposit distribution in ants (*Crematogaster scutellaris*) at the Florence external scanning microbeam, X-Ray Spectrom. 40 (2011) 186–190.
[38] M. Simon, P. Barberet, P. Moretto, D. Bacqueville, A. Mavon, H. Seznec, The skin barrier function: a micro-PIXE study, X-Ray Spectrom. 38 (2009) 132–137.
[39] G. Pratesi, M. Zoppi, T. Vaiani, F. Calastrini, A morphometric and compositional approach to the study of ambient aerosol in a Medium Industrial Town of Italy, Water Air Soil Pollut. 179 (2007) 283–296.
[40] V. Maavara, A. Martin, A. Oja, P. Nuorteva, Sampling of different social categories of red wood ants (Formica s. str.) for biomonitoring, in: B. Market (Ed.), Environmental Sampling for Trace Analysis, VCH, Weinheim, 1994, pp. 465–489.
[41] S. Calusi, E. Colombo, L. Giuntini, A. Lo Giudice, C. Manfredotti, M. Massi, G. Pratesi, E. Vittone, The ionoluminescence apparatus at the LABEC external microbeam facility, Nucl. Instrum. Meth. B 266 (2008) 2306–2310.
[42] L. Giuntini, M. Massi, S. Calusi, The external scanning proton microprobe of Firenze: a comprehensive description, Nucl. Instrum. Meth. A 576 (2007) 266–273.
[43] S. Calusi, The external ion microbeam of the LABEC laboratory in florence: some applications to cultural heritage, Microsc. Microanal. 17 (2011) 661–666.
[44] L. Giuntini, F. Lucarelli, P.A. Mandò, W. Hooper, P.H. Barker, Galileo's writings: chronology by PIXE, Nucl. Instrum. Meth. B 95 (1995) 389–392.
[45] M. Massi, The ion microbeam facility of Florence: a versatile instrument for the analysis and modification of materials, Nuovo Cimento C 34 (2011) 91–102.
[46] G. Vaggelli, A. Borghi, S. Calusi, R. Cossio, L. Giuntini, M. Massi, Micro-PIXE determination of Zr in rutile: an application to geothermometry of high-P rocks from the western Alps (Italy), X-Ray Spectrom. 37 (2008) 146.
[47] L. Giuntini, A review of external microbeams for ion beam analyses, Anal. Bioanal. Chem. 401 (2011) 785–793.
[48] S.M. Bertram, M. Bowen, M. Kyle, J.D. Schade, Extensive natural intraspecific variation in stoichiometric (C:N:P) composition in two terrestrial insect species, J. Insect Sci. 8 (2008) 1–7.
[49] A.D. Kay, S. Rostampour, R.W. Sterner, Ant stoichiometry: elemental homeostasis in stage-structured colonies, Funct. Ecol. 20 (2006) 1037–1044.
[50] H.A. Woods, W.F. Fagan, J.J. Elser, J.F. Harrison, Allometric and phylogenetic variation in insect phosphorus, Funct. Ecol. 18 (2004) 103–109.

[51] G. Pigino, M. Migliorini, E. Paccagnini, F. Bernini, Localisation of heavy metals in the midgut epithelial cells of *Xenillus tegeocranus* (Hermann, 1804) (Acari: Oribatida), Ecotoxicol. Environ. Safety 64 (2006) 257–263.
[52] T. Nagata, X-ray microanalysis of biological specimens by high voltage electron microscopy, Prog. Histochem. Cytochem. 39 (2004) 185–319.
[53] R.R. Sokal, F.J. Rohlf, Biometry: The Principles and Practice of Statistics in Biological Research, Freeman, New York, 1995.
[54] A.J. Underwood, Experiments in Ecology, their Logical Design and Interpretation Using Analysis of Variance, Cambridge University Press, Cambridge, 1997.
[55] B.F.J. Manly, Multivariate Statistical Methods: A Primer, second ed., Chapman and Hall, New York, 1994.
[56] A.J. Morgan, P. Kille, S.R. Sturzenbaum, Microevolution and ecotoxicology of metals in invertebrates, Environ. Sci. Technol. 41 (2007) 1085–1096.
[57] L. Posthuma, N.M. van Straalen, Heavy-metal adaptation in terrestrial invertebrates: a review of occurrence, genetics, physiology and ecological consequences, Comp. Biochem. Physiol. 106 (1993) 11–38.
[58] P. Stary, J. Kubiznakova, Content and transfer of heavy metal air pollutants in populations of *Formica* spp. wood ants (Hym., Formicidae), J. Appl. Entomol. 104 (1987) 1–10.
[59] P. Nuorteva, Metal Distribution Patterns and Forest Decline. Seeking Achilles' Heels for Metal in Finnish Forest Biocoenoses, Department of Environmental Conservation at the University of Helsinki, vol. 11, 1990, pp. 1–77.
[60] I.M. Grze&sacute, Zinc kinetics in the ant *Myrmica rubra* originating from a metal pollution gradient, Chemosphere 88 (2012) 1015–1018.
[61] P.J. Hensbergen, M.J. van Velzen, R.A. Nugroho, M.H. Donker, N.M. van Straalen, Metallothionein-bound cadmium in the gut of the insect *Orchesella cincta* (Collembola) in relation to dietary cadmium exposure, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 125 (2000) 17–24.
[62] E.M. Leonard, L.M. Pierce, P.L. Gillis, C.M. Wood, M.J. O'Donnell, Cadmium transport by the gut and Malpighian tubules of *Chironomus riparius*, Aquat. Toxicol. 92 (2009) 179–186.
[63] R.S. Sohal, R.E. Lamb, Intracellular deposition of metals in the midgut of the adult housefly *Musca domestica*, J. Insect Physiol. 23 (1977) 1349–1354.
[64] A.Y. Jeantet, R. Martoja, M. Truchet, Role des spherocristaux de l'epithelium intestinal dans la resistance d'un insecte aux pollutions minerales. Donnees experimentales obtenues par utilisation de ia microsonde electronique et du microanaiyseur par emission ionique secondaire, C. R. Acad. Sci. Hebd. Seances Acad. Sci. D 278 (1974) 1441–1444.
[65] A.Y. Jeantet, Principaux aspects de l'accumulation de composes mineraux et de reserves organiques par un insecte social, *Formica polyctena* F. Implications physiologiques et ecotoxicologiques, These de Doctorat d'etat, Paris, 1981.
[66] D. Marchal-Ségault, C. Briançon, S. Halpern, P. Fragu, G. Laugé, Secondary ion mass spectrometry analysis of the copper distribution in *Drosophila melanogaster* chronically intoxicated with Bordeaux mixture, Biol. Cell 70 (1990) 129–132.
[67] R.M.S. Schofield, M.H. Nesson, K.A. Richardson, Tooth hardness increases with zinc-content in mandibles of young adult leaf-cutter ants, Naturwissenschaften 89 (2002) 579–583.
[68] F.G. Fujiwara, D.R. Gómez, L. Dawidowski, P. Perelman, A. Faggi, Metals associated with airborne particulate matter in road dust and tree bark collected in a megacity (Buenos Aires, Argentina), Ecol. Indic. 11 (2011) 240–247.