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Wild rats as urban detectives for latent sources of asbestos contamination

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TITLE: Wild rats as urban detectives for latent sources of asbestos contamination

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HIGHLIGHTS

Abstract

1. INTRODUCTION

According to international laws 'asbestos' is a set of six asbestiform silicates, five belonging to the mineralogical group of amphiboles (actinolite, amosite or grunerite asbestos, anthophyllite, crocidolite and tremolite) and one to serpentine group (chrysotile) (Dir. 2009/148/EC).

In the past, nearly all over the world, asbestos have been widely employed in many composite materials for their chemical-physical and technological properties: resistance to abrasion, heat (non-flammable even at high temperatures) and chemicals, low sound-transmission coefficients, low thermal conductivity and low density, flexibility (Gualtieri, 2012). Chrysotile, crocidolite and amosite were broadly employed in the building field to produce asbestos-cement.

The asbestiform habit and surface activity are responsible of adverse effects on human health, when the fibres from asbestos and asbestos containing material (ACM) are inhaled, especially at high doses. ACM may potentially release respirable fibres (Stanton, 1981) especially if the ACM is deteriorated or constituted by a friable matrix. At present, there is a large body of scientific literature on the presence of asbestos fibres in human lungs (and other tissues and organs) and its association with pathological changes (*e.g.*, lung cancer, pleural and peritoneal mesothelioma) both in humans and in animals (IARC, 2012). In Italy there were from 2001 to 2007 about 1,000 cases of deaths per year for pleural mesothelioma, 3,000 estimated cases of lung cancer and 560 asbestosis per year from 2003 to 2007 (Marinaccio *et al.*, 2008); between 1993 and 2015 the Italian National Register of Malignant Mesotheliomas collected 27,356 cases (Marinaccio *et al.*, 2018).

From 1907 to 1986 the most important Italian manufacturing plant of asbestos-cement (Eternit S.p.A.) was active in the municipality of Casale Monferrato (Piedmont Region, north-western Italy). Corrugated and plane sheets, pipes and pressure pipes and other artefacts were produced, reaching a peak consumption of 15,000 metric tons of raw asbestos in 1981. Chrysotile represented 90% of all asbestos used that year and crocidolite the remaining 10% (Maule et al, 2007). Chrysotile was provided by the Italian mine in Balangero but was also imported, mainly from Russia. It is worth noting that Russian chrysotile was contaminated by tremolite asbestos (REF?). Today Casale Monferrato is known for the great impact that occupational exposure to asbestos had on the health of its citizens, in terms of incidence of asbestos related diseases (*i.e.*, asbestosis, mesothelioma, lung cancer, etc.) (Magnani *et al.*, 2001; Magnani *et al.*, 2007; Comba *et al.*, 2018). However, most mesothelioma cases occurring among residents of Casale Monferrato had never been employed at the asbestos-cement factory. Epidemiological studies carried out in Casale Monferrato have shown that the asbestos related diseases can not only depend on occupational and para-occupational exposure but also on passive exposure in asbestos containing buildings, such as public offices or schools, where

the people involved have no awareness of direct physical contact with asbestos-containing material (Mirabelli *et al.*, 2010; Comba *et al.*, 2018). The main sources of exposure were traced to residential exposure to fall-out from the factory, sharing home with Eternit workers, and reuse of waste materials that were made freely available to the local population. These materials included broken products, reduced by people to the dimension of small pebbles and used as substitutes of gravel in road and courtyard pavements, and fine dust resulting from the grinding of asbestos-cement pipes extremities, used as thermal insulation in attics or remixed with cement to produce pavements (Magnani *et al*., 2001; Maule *et al.*, 2007). In 1992 the Italian legislation forbade the extraction, import, processing, marketing, use and sales of asbestos and ACM and required the issue of information about remediation measures and controlled disposal. The widespread uses of asbestos-cement waste materials were the target of remediation projects promoted over the years by the Casale Monferrato municipality, but no census existed and there is concern that potential sources of asbestos pollution still exist in the city, unrecognised because either undeclared or forgotten, perhaps decades after their installation.

In recent decades, it was found that the identification and monitoring of a wide variety of hazardous environmental pollutants on human health can be done through surveys of animal populations defined as "sentinel animals" systems (Reif, 2011). Specific experiences have been gained on the animal exposure to asbestos (Dumortier *et al.*, 2002; De Nardo *et al.*, 2004; Bellis *et al.*, 2005; Belluso *et al.*, 2006; Fornero *et al.*, 2009; Ben-Shlomo *et al.*, 2011; Capella *et al.* 2017).

In 2011, our research team has conducted a pilot study to develop and assess procedures to capture wild rats and analyse them to make possible the monitoring of an urban area (Ardizzone *et al.*, 2014). Casale Monferrato had been selected as study area based on the known historical and diffuse asbestos contamination and the choice of the rats was based on their adaptation in urbanized environments, the extensive and widespread presence and the demonstrated susceptibility to asbestos fibres.

The aim of this study was to uncover sites with the greatest potential of non-occupational exposure to asbestos within the urban area of Casale Monferrato by quantifying the amount of asbestos fibres in lung tissue of captured rats used as sentinel animals.

2. MATERIAL AND METHODS

2.1. Design of the study and sampling

Rats (*i.e.*, *Rattus rattus* and *R. norvegicus)* have been chosen as sentinel animals to uncover asbestos contamination sites in the urban area of Casale Monferrato.

The sample design was initially set as follows:

a) A regular grid of squares of 200 meters per side was placed on the map of the city (Fig. 1, OpenStreetMap® cartographic image processed with QGIS System). The length of the side of the squares was defined considering the rat behaviour and the home range usually covered around a permanent den (Gardner-Santana *et al.*, 2009). Initially the priority was given to areas with buildings built in 60's and 70's ("*60/70 area*"), perimeter area located S and SW of the old town, *i.e.* dating back to the period of greater asbestos production and high densities of population. A control location, to check the ability of rat capturing of the research team, has been added in a suburban area (~2.5 km from Casale Monferrato) where the presence of rats had been reported by private citizens.

b) Multiple capture traps (Ekomille, Ekommerce SRL, Atessa, Italy) were selected as sampling tools. These devices allow multiple and continuous catches, until 10-12 wild rats are captured discouraging other animal species to enter the trap. Traps were used following the instructions of the construction companies using the appropriate personal protective equipment. It was planned that the available traps (1 to 3) should have been placed as close as possible to the centre of squares for about 3 weeks or less if the number of captured rats had been satisfactory (initially a target of 5 rats per trap had been fixed). After the maximum 3-week period the traps were moved to other squares in the grid. Each sampling point was localized using a GPS. The traps were named with the name of the quadrant (letter-number, Fig. 1) plus a consecutive number per site and activation period (mid-April 2013 to end-June 2015).

c) The rats had to be individually subjected to necropsy and histopathology. Later, the lung material at each trapping sites was to be pooled and analysed, keeping separate the material from rats weighing 100 g or more (*heavy rats*: H) from that of smaller rats (*light rats*: L), regardless of sex or species. The "100 g" cut-off was then used as a proxy for age at exposure to asbestos fibres: the assumption was that younger animals (body-weight < 100 g), given their shorter lifespan, would have had fewer opportunities to inhale and accumulate asbestos.

After the first year of sampling, the number of sampling points with successful captures and the number of captured rats were much lower than expected. To improve capture performance, advice from rodent control companies was obtained and an enhanced cooperation was searched from citizens reporting rats. Therefore, the revised procedures about the traps' management were: site selection based on information from residents, new types of bait, multiple traps per sampling point. Additional 30 individual-capture traps (hereinafter called as "snap traps") were incorporated into the sampling. These traps (Trapper® T-Rex, Bell Laboratories Inc., Madison, US) differed in the working features (snap spring), in the number of catchable rats (one animal at time) and in the management of the captured animals. Moreover, as a result of citizen reporting, the area was extended to the Casale's old town (Fig. 1) where inhabitants were complaining about the presence of many rodents.

2.2. Necropsy

The captured rats were sexed and weighed. A necropsy was performed according to a standardised protocol in order to detect the presence of any lesions referable to zoonotic diseases (as post-capture safeguarding of the health of the research team) and to collect tissue portions for further examination. Spleen, liver and kidneys were collected and tested for *Francisella tularensis* and *Leptospira* spp. by PCR (Forsman *et al.*, 1994; …). Lungs were sampled and fixed in 4% buffered formaldehyde solution. The left lung was subjected to histopathology whereas the right lung was subjected to electron microscopy. Heart, spleen, liver and kidneys were also sampled for histopathological examination to evaluate the general health status of the animals.

2.3. Histopathology

Histological examination of the lung tissue was performed in order to highlight the so-called asbestos corpuscles, golden-brown rounded or handlebar formations with a thin and translucent fibre in the core, or any histopathological changes related to the inhalation of asbestos fibres. The corpuscles are typically described around fibres of amphiboles, while rarely around those of chrysotile and are usually located within the fibrous tissue or can be placed within the alveolar spaces or intracytoplasmic in macrophages or in multinucleated giant cells.

Each sampled organ-was fixed in 10% neutral buffered formalin and routinely processed: paraffin inclusion and cut into 3-5 μm sections and stained with haematoxylin-eosin (HE).

Serial sections of the lung were also stained by two histochemical stainings: Perls (method for ferric iron, Bio Optica Milano S.p.A, Milan, Italy) and Masson (Masson trichrome with aniline blue, Bio Optica Milano S.p.A, Milan, Italy), in order to easily detect, respectively, the presence of ferruginous corpuscles due to asbestos and the presence and the extent of pulmonary fibrosis.

The Perls staining is suitable to stain in blue iron-ferric on tissue sections but is not able to stain the iron-ferrous and the iron bound to haemoglobin, ferritin and pigments due to use of acid formalin. As positive control for the Perls staining was used a sample of lung tissue from rats subjected to inhalation of amosite. The result of Perls staining was expressed as positive or negative.

The Masson staining allows the visualization of muscular fibres in red, collagenous tissue in blue and erythrocytes in yellow. An *ad hoc* grading score was used for the evaluation of presence/absence of pulmonary fibrosis and its extent, performing a semi-quantitative evaluation: Negative (absence of appreciable pulmonary fibrosis), Positive with grade I (minor fibrosis), Positive with grade II (moderate fibrosis), and Positive with grade III (severe fibrosis).

164 **2.4. Scanning electron microscopy**

165 The pools of lung samples were prepared for scanning electron microscopy with energy dispersive
166 spectrometry (SEM-EDS) and examined to evaluate the presence of asbestos fibres and their
167 concentration according the protocol described by Belluso *et al.* (2006).

168 Each preparation was investigated by SEM (StereoScan-360, Cambridge Instruments, Cambridge,
169 UK). Only inorganic particles having aspect ratio > 3:1 have been considered. Their chemical
170 composition was measured by EDS (INCA Energy 2000, Oxford Instruments, Abingdon, UK). The
171 detected inorganic fibres were identified by comparing their EDS spectrum with those collected in
172 the available laboratory database. The inorganic fibres detected in rat lungs have been classified in
173 'asbestos fibres' and 'non-asbestos fibres', and in turn the 'asbestos fibres' were divided based on
174 dimension into 'short asbestos fibres' (SAF: $L < 5$ μm, $d < 3$ μm and $L/d > 3$) or 'long asbestos fibres'
175 (LAF: $L > 5$ μm, $d < 3$ μm and $L/d > 3$) according to the criteria defined in the European directive
176 (Dir. 2009/148/EC).

177 By the SEM-EDS it is difficult to distinguish correctly between chrysotile and asbestiform antigorite
178 (a non-asbestos), both species belonging to the serpentine group (Fornero *et al*., 2009). When it was
179 not possible to classify the fibre as chrysotile *sensu stricto*, in conservative mode and considering that
180 the final objective is to locate residual sources of asbestos to sanitize the area, for our analysis we
181 assumed that these fibres belonging to the asbestos group chrysotile. Besides, our assumption is
182 supported by the fact that there is no deposit or natural outcrops of these minerals were reported near
183 Casale Monferrato; therefore, it was reasonable to assume that these fibres derive from anthropogenic
184 manufacturing linked to asbestos.

185 Since mineral species tremolite and actinolite (both asbestos) do differ only in their chemical
186 composition (amount of Mg and Fe), it is not possible to distinguish them by SEM-EDS, are hereafter
187 considered one group (indicated tremolite/actinolite) (Capella *et al.*, 2017).The number of detected
188 inorganic fibres was normalized to 1 g of dry tissue as indicated by international guidelines (De Vuyst
189 *et al.*, 1998) reporting the concentration in terms of load of asbestos fibres per gram of dry lung tissue
190 weight: ff/gdw.

191

192 **2.5. Statistical analysis**

193 The concentration of inorganic fibres (ff/gdw) detected in each pool was described by sampling point,
194 fibre category and weight group (*i.e.*, L: < 100 g or H: ≥ 100 g) of sampled animals.

195 A statistical analysis was aimed at exploring the existence of geographical differences in the
196 concentration of asbestos fibres and to detect the potential for outliers associated to specific sites. The
197 outliers identified were considered as probable hot-spot points. For this purpose, since the fibre

concentration in the different pools analysed did not fit a Gaussian distribution, a MAD (median absolute deviation) method was used (Leys *et al.*, 2013; Iglewicz and Hoaglin, 1993). Iglewicz and Hoaglin (1993) suggested that observations could be labelled outliers when |MAD-score|>3.5. Statistical analysis was performed using Stata 14.1 (StataCorp, 2015).

Furthermore, to identify the most likely area where putative unrecognized local sources of asbestos contamination were present, we delimited a circular area with a 100 m radius ("buffer area") around sampling points with outliers in the lung fibre burden. One hundred meters is a plausible distance covered by wild rats around a permanent den (Byers *et al.*, 2019). The buffer area was processed with QGIS System (GNU) and drawn on an OpenStreetMap® cartographic image (CC BY-SA).

3. RESULTS

3.1. Rodent trapping

Based on the operational capacity of the research team and the reporting of murine presence by the citizens, over the study period, the traps have been placed in (or just outside) 29 squares of the grid (17 from the 60/70 area and 12 from the old town, Fig. 1). It was not possible to obtain captures in all the sampled squares, although the presence of rodents was demonstrated by the large consumption of bait in all traps. In total 40 rats (37 *R. norvegicu*s and 3 *R. rattus*) were caught from 15 sampling points (11 and 4 respectively from the old town and the 60/70 area; Fig. 1, Table 1) and from the control location outside urban area. Thirty animals were trapped by multiple capture traps (20, 7 and 3 from respectively the old town, the 60/70 area, and the control location), 8 by snap traps and 2 caught by a cat near a multiple capture trap placed in the old town. Both H and L rats were captured in five sampling points.

3.2. Necropsy and PCR

During the necropsy, no macroscopic lesions were found in any captured rat. All PCR analyses conducted on the samples were negative for *F. tularensis* and *Leptospira spp*. It was not possible to perform any laboratory analysis on two of the captured rats since, at a first macroscopic evaluation, they were in very poor conditions of conservation.

3.3. Histopathology

Out of the 38 rats (22 classified as H) available for histopathological analysis, in 12 it was possible to detect pneumonia or bronchopneumonia with different degree of inflammatory lesions, and in one rat an area of fibrotic pleural thickening was apparent. No pathological lesions directly attributable to

the inhalation of asbestos fibres were identified in hearts, spleens, livers, and kidneys. Perls staining was positive in 17 animals, even if only in 5 of them it was possible to identify the presence of ferruginous bodies compatible with asbestos corpuscles. Masson staining allowed the detection of severe, moderate, and minor fibrosis in 5, 14 and 3 samples respectively; no appreciable fibrosis was found in 16 samples.

A histopathological diagnosis of asbestosis could be done in 2 animals from one sampling point (F1_1), where both corpuscles and severe and moderate fibrosis were simultaneously detected. In 9 rats (7 sampling points), asbestosis could be hypothesized since Perls staining was positive and associated with fibrosis detected by Masson staining; however, they didn't show any ferruginous body.

3.4. Scanning electron microscopy

In total lung tissues from 36 wild rats were suitable for SEM-EDS investigation and have been analysed in 19 pools (7 from L rats); the remaining four rats were excluded due to very poor lungs condition.

Inorganic fibres have been detected in 15 pools. In total, 13 types of fibrous inorganic species have been identified, among which asbestos tremolite *s.s.* (or tremolite/actinolite), amosite, and chrysotile *s.s.* (or chrysotile/antigorite). Eleven positive pools (8 pools from H rats) contained asbestos fibres; in one case only SAF were detectable whereas in 6 only LAF.

Among the 11 pools positive to asbestos, one contained the 2 rats with histopathological diagnosis for asbestosis; one rat with probable asbestosis (*i.e.*, Perls and Masson stains positive but without identifying ferruginous bodies) was present in each of other 3 pools.

The mean concentration of asbestos fibres was 30,136 ff/gdw (n = 11 pools, sd = 27,126 ff/gdw). LAF showed higher concentration (n = 10, mean = 30,600, sd = 26,336 ff/gdw) compared with SAF (n = 5, mean = 5,100, sd = 0 ff/gdw).

Regardless of the length of the asbestos fibres, tremolite/actinolite (including tremolite *s.s.*) was the most common species of asbestos found in the lungs of rats, both in absolute number of fibres (43 fibres in 9 pools, 16 rats) and per gram of dry tissue (mean = 24,367 ff/gdw), followed by amosite (9 fibres in 2 pools, 7 rats; 22,950 ff/gdw) and chrysotile/asbestiform antigorite (including those properly identified as chrysotile: 9 fibres in 6 pools, 12 rats; 11,050 ff/gdw).

The highest asbestos load has been detected in the pools of the sampling points F2_1 (2 pools, 1 H and 1 L, mean = 58,650 ff/gdw) and F2_3 (81,600 ff/gdw) (Fig. 2).

Outlier concentrations was detected at the sampling point F2_3 (MAD-score = 4.38); a high MAD-score (2.87), but not significant, was obtained for the sampling point F2_1. Furthermore, was

observed an overlap of the respective buffer areas (*i.e.*, hypothetical home-range around the sampling point), consistent with the hypothesis of an asbestos hot-spot in this area (Fig. 3).

4. DISCUSSION AND CONCLUSIONS

Our study confirms the rat (*R. norvegicus* and *R. rattus*) as a helpful sentinel of remaining asbestos; moreover, our results suggest that at least one geographically defined location in the urban area of Casale Monferrato could represent a probable hot-spot. Our approach is based on the observation that the murine lung tissue acts as a filter for mineral fibres, among which the asbestos fibres are distinguishable and can be also classified by species.

The heterogeneity of the mineral species found and the marked differences between the concentrations detected make it possible to process the data and search for the causes (sources) of these differences, valid for territorial realities even diverse from Casale Monferrato.

Three types of asbestos were detected in the lung tissue of the captured rats: chrysotile (the most used asbestos at the Eternit plant), tremolite/actinolite, and amosite. Interesting to note is the high load of tremolite/actinolite asbestos, which were not commercially used in ACM to a significant extent (*e.g.*, in the preparation of fibre cement), and the absence of crocidolite fibres.

The high load of tremolite/actinolite asbestos in the rat lungs could be explained by the following reasons: the presence of natural sources (Capella *et al.*, 2017), their high persistence in biological tissues (Ref?), and the contamination of crocidolite imported by Eternit from Russia by tremolite asbestos (Ref?). On the other hand, given the great distance from the hypothetical home-ranges of asbestos-positive rats to the railway track (Fig. 3B), it would not seem likely that the railway ballast are sources of asbestos tremolite/actinolite.

The non-detection of crocidolite (fibres that were commonly used at the plant) could be the result of the remediation effort carried out in recent decades in our study area. On the contrary, amosite was rarely used in the productive process of Eternit, but it was widely used for the insulation of the boiler bodies, in the central heating pipes and for the spray coating of walls and ceilings of electrical control units. Then the quite unexpected finding of high load of amosite in two close sites could be explained by a specific source of asbestos.

Significant rat capture difficulties arose during the study: despite of large consumption of bait in all traps, the number of caught rats was less than expected. The reasons for this can be found in the innate ability of rats to warn of the danger of traps, in the periodic rodent control activities carried out in the urban area, and in the low effectiveness of traps. Multiple capture traps allow for long-term monitoring with acceptable management costs (*i.e.*, personnel effort to check the traps) and it is very effective when the spread of rats is epidemic. However, given that periodic rodent control activities

have reduced the murine population in the urban area, it is reasonable to assume that the presence of wild rats is widespread but without very large colonies, except in certain areas.

In contrast, snap traps appeared to be more effective, but these require higher management costs to ensure daily, or twice-daily, controls.

Therefore, the skill and the effectiveness in the capture are crucial to successfully complete the study and were a main stone in the planning of the study. Our experience suggests to considering all the information about the territory, the density of murine population and to place several and different kind of trap devices. For example, we suggest using the snap traps for short intensive periods and the multiple capture traps for long periods, especially with high murine population density.

The methodology proposed by this study represents a preliminary screening step that, in situations where the presence of latent sources of asbestos is suspected, significantly reduces the geographic magnitude of the area to be investigated. Given a radius of 100 m around the sampling point, the exact identification of the latent source requires a further and more detailed search. At this stage, sentinel animals may still be useful, *e.g.,* other traps could be placed at a shorter distance and additional research strategies applied in the suspect area, such as the administration to local inhabitants of an *ad hoc* questionnaire to help in the detection of the putative latent source. Furthermore, the search could be guided by the type of asbestos found in the lungs of locally captured rats, that is, particularly by looking at the ACM that might contain them or the sites where they were known to be located. Finally, to improve the search, the assistance of rodent experts could help to track the footpaths and burrows of rats, which would make it possible to better determinate the rodents' home-range and thus the search area.

As conclusion, we can say that the approach presented in this study can be used in the framework of public health campaigns to address asbestos removal activity.

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Table 1: number of rats captured and analysed by sampling point, capture method, species, weight class (L: < 100 g or H: ≥ 100 g).

Sampling point	Species	Capture method	Captures			SEM-EDS	
			Total	L	H	L	H
00_1	*R. norvegicus*	Multiple capture trap	3		3		**2****
D9_1			1		1		1
F7_1			4	1	3	1	**3**
F6_2			1		1		**1**
E6_2			1		1		**1**
H3_1			2	2		2	
E3_1			7	6	1	4*	**1**
F2_1			6	5	1	**5**	**1**
F1_1			3		3		**3**
H3_1		Snap trap	1		1		1
E3_4			1		1		**
H2_1			2	1	1	**1**	1[#]
E1_2			2	1	1	**1**	1[§]
E1_3			1		1		1[§]
E2_3		Preyed by cat	2	2		2	
F2_3	*R. rattus*	Multiple capture trap	2		2		**2**
H2_1		Snap trap	1		1		1[#]
Total			40	18	22	16	20

SEM numbers in bold were positive pools for asbestos fibres.

*: two rats have been excluded by the histopathologic and SEM-EDS investigations since they were in poor conditions of conservation at a first macroscopic evaluation.

**: one rat has been excluded by the SEM-EDS investigations because the quality of the sample did not allow it.

[#] and [§]: analysed in the same pool.

Figure in altro file

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