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Honey Bees (*Apis mellifera*, L.) as Active Samplers of Airborne Particulate Matter

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Abstract

Honey bees (*Apis mellifera* L.) are bioindicators of environmental pollution levels. During their wide-ranging foraging activity, these hymenopterans are exposed to pollutants, thus becoming a useful tool to trace the environmental contaminants as heavy metals, pesticides, radionuclides and volatile organic compounds. In the present work we demonstrate that bees can also be used as active samplers of airborne particulate matter. Worker bees were collected from hives located in a polluted postmining area in South West Sardinia (Italy) that is also exposed to dust emissions from industrial plants. The area is included in an official list of sites of national interest for environmental remediation, and has been characterized for the effects of pollutants on the health of the resident population. The head, wings, hind legs and alimentary canal of the bees were investigated with Scanning Electron Microscopy coupled with X-ray spectroscopy (SEM-EDX). The analyses pointed to specific morphological and chemical features of the particulate, and resulted into the identification of three categories of particles: industry -, postmining -, and soil –derived. With the exception of the gut, all the analyzed body districts displayed inorganic particles, mostly concentrated in specific areas of the body (i.e. along the costal margin of the fore wings, the medial plane of the head, and the inner surface of the hind legs). The role of both past mining activities and the industrial activity close to the study area as sources of the particulate matter is also discussed. We conclude that honey bees are able to collect samples of the main airborne particles emitted from different sources, therefore could be an ideal tool for monitoring such a kind of pollutants.

Introduction

Honey bees (*Apis mellifera* L.) are commonly used as bioindicators of the level of environmental contamination. During their wide-ranging foraging activity, these hymenopterans are exposed to pollutants present in the atmosphere, soil, vegetation, and water [1–3]. Depending on the type of environmental pollution, bee contamination may occur through adhesion of particles to the insect body hairs, inhalation of pollutants via spiracles of the tracheal system or ingestion of contaminated nectar, pollen and water. Contaminants are brought back to the hives and may also be found into the apiary products, such as honey and wax [4–6].

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Among environmental contaminants found in honey bees and bee products, the most commonly studied are heavy metals, pesticides, radionuclides and Volatile Organic Compounds (VOCs) [1, 4, 6–8]. Despite the well-known role of honey bees in environmental monitoring, studies using these hymenopterans as active samplers of airborne particulate matter (PM) are completely lacking, even if the morphological description and the physico-chemical characterization of PM collected by the bees would provide accurate information on both the emission source(s) and the potential health hazards [9–11]. Indeed, this is a key point for developing adequate control strategies in order to reduce the impact of pollutants on both the environment and public health.

Studies on atmospheric pollutants include the vast field of airborne particulate matter. PM is broadly defined as a complex mixture of airborne chemical components which are commonly classified by particle size. They include ultra-fine particles (up to 0.1 μm in diameter), fine particles or PM1 (up to 1 μm), PM 2.5 (up to 2.5 μm), coarse fraction or PM 10 (up to 10 μm). The airborne particles $\leq$100 μm in diameter are collectively referred as total suspended particulate (TSP).

PM can be directly emitted as primary compounds or formed as secondary compounds by chemical transformation or condensation of gases such as SOx, NOx, VOCs and ammonia. Primary sources comprise both natural sources, such as windblown dust, volcanic eruptions, forest fires and sea spray, and anthropogenic activities. The latter represent a broader domain, ranging from agricultural operations to industrial processes, mining and postmining activities, combustion of wood and fossil fuels, incineration of wastes and motor traffic (vehicles, aircrafts, ships, trains), etc. [12–15].

Over the years, several human diseases have been linked to PM exposure, which may be responsible for short-term, long-term and cumulative health effects [16–20]. Neonatal premature mortality, morbidity, cardiovascular and cardiopulmonary diseases, asthma and lung cancer are among the more frequent effects observed in patients exposed to airborne particles [16, 18, 19]. Toxicological researches have shown that, at a cellular level, PM may induce cytotoxicity, neurotoxicity, mutagenicity, stimulation of pro-inflammatory factors, and even epigenetic alterations of the DNA with consequences on gene expression [19, 21, 22].

Moreover, the size of the particles and their surface area determine the potential to elicit the adverse biological effects. Ultra-fine particles are of much concern, as they can penetrate deeper into the airways of the respiratory tract, enter blood circulation, and then distribute to most organs, including the brain [19, 21].

The aim of this work was to investigate the role of honey bees as active samplers of PM.

The study was carried out in a post-mining area of Sulcis-Iglesiente, in the municipality of Iglesias (Carbonia-Iglesias province, Sardinia, Italy). Sulcis-Iglesiente is included in an official list of sites of national interest for environmental remediation and has been characterized for the effects of pollutants (mostly metals and metalloids deriving from past mining activities) on the health of the resident population [23, 24]. The PM collected by the worker bees on the body was analyzed using a Scanning Electron Microscope (SEM) coupled with X-ray spectroscopy (EDX). The dissected alimentary canal of the hymenopterans was also investigated to detect inorganic particles potentially ingested during feeding.

Materials and Methods

Study area

The town of Iglesias is located in South West Sardinia, about 50 km West of Cagliari (Fig 1A). Its surroundings are known for the baryte and Pb-Zn ore deposits, extensively exploited during the Nineteenth Century and until recent times through dozens of mines. Among them, the Pb-Zn mines of Monteponi, Campo Pisano and San Giovanni are certainly the most famous

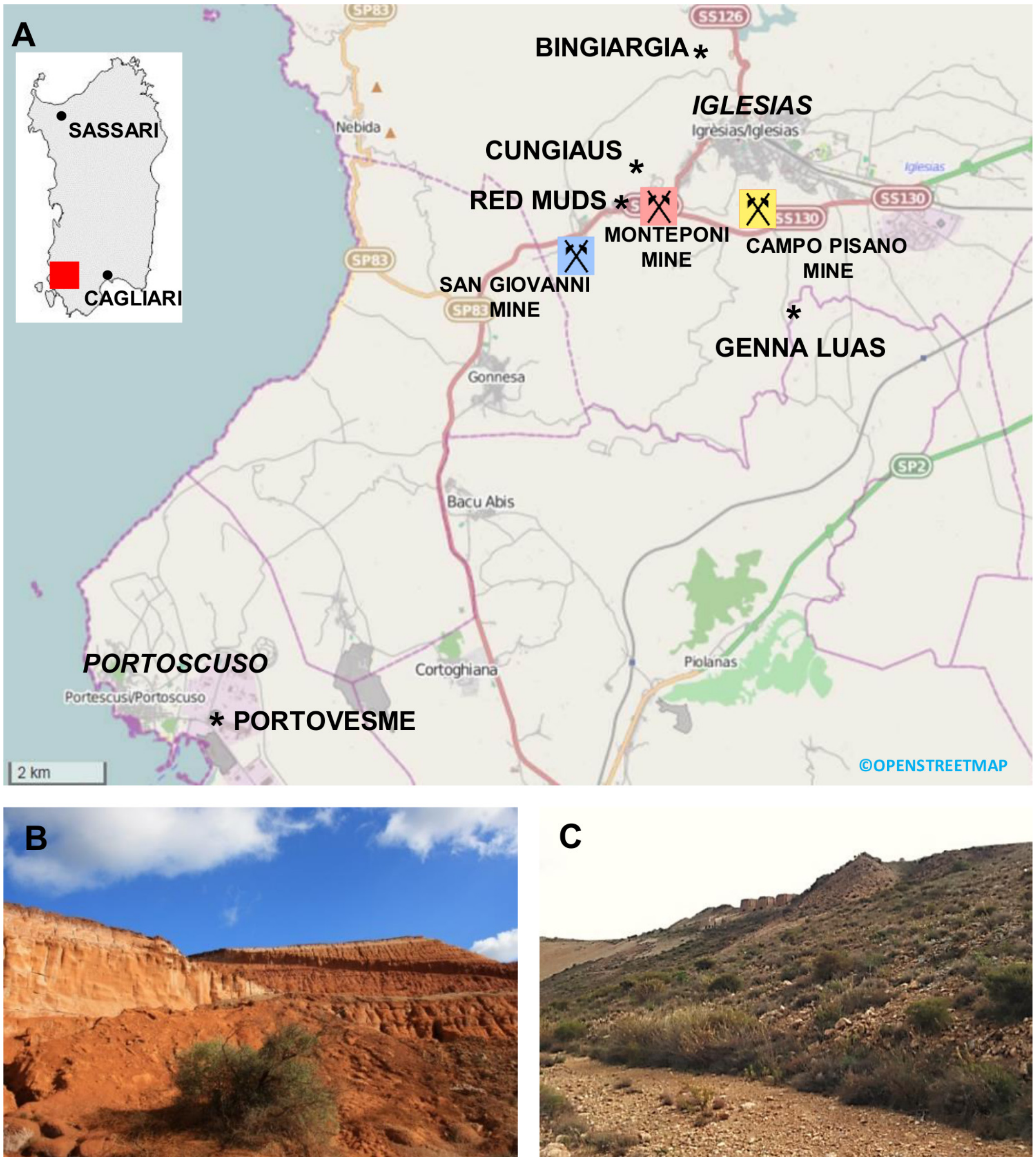

Fig 1. (A) Study area; (B) view of the Red Muds, and (C) the mine dumps of Cungiaus.

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(Fig 1A) [25] and, together with about 40 mines—spread out over an area of 150 km^2 –they exploited the deposits of the so called "Metalliferous Ring" [25, 26].

As a result of those intense mining operations, several million metric tons of ore material were extracted, leaving to broad daylight extensive tailings. This is especially the case of the Monteponi mining complex, located immediately West of Iglesias. In particular, the treatment of the oxidized Zn-ores (calamines) was carried out in an electrolytic plant, which involved fine grinding (typically <40 μm grains) of calamines and then their treatment with sulphuric acid, $FeSO_4$ and MnO_2 for the enrichment of the Zn concentrate. The wastes from this process were deposited downstream of the industrial complex and nowadays constitute the hill of the Red Muds ("Fanghi Rossi" in Italian; RM in this work) (Fig 1B). The hill—which is subjected to preservation regulations as an industrial archaeology site—mainly contains iron oxy-hydroxides associated with Zn-silicates and carbonates, gypsum, and toxic elements such as Cd, Pb, As, Hg, Mn, and Ba [26–27]. In total, the RM cover an area of about 15 ha with a volume of 500,000 m^3, occupying a major part of the lowest, West—South West flank of the mined hill of Monteponi. At the present state, the RM sediments are only contained by wooden bulkheads. The steep slopes, along with the fine grain size of the material, promote intense weathering of the postmining materials. During rain events, runoff transfers the sediment load to the surrounding areas via the San Giorgio creek, which lies immediately downhill [26–27].

North of Monteponi, the open pit of Cungiaus is the largest of Sardinia, covering more than 10 ha of surface. Past mining activities exploited an extensive mass (about one million m^3) of calamines since 1869 (the year of discovery) to the first half of the Twentieth Century [25, 28].

Besides the past intensive mining activity, Sulcis-Iglesiente is also exposed to emissions by industrial plants, mostly located along the South West seashore (industrial district of Portovesme). Portovesme is approximately 8 Km South West of Iglesias, in the municipality of Portoscuso (Fig 1A). The main industrial plants include different units: a sector for the production of alumina from bauxite and the production of aluminum by electrolysis of alumina (currently in standby); electric power stations, composed by a coal-powered generation plant and an oil-powered plant; and a Pb-Zn smelter that uses steelwork dusts for Zn extraction. This type of smelter is known to produce post-processing atmospheric fall-out impacting on the immediate surroundings [29].

Hives and honey bees

Eleven hives were located in Bingiargia (39°19’31”N–08°31’07”E), a Mediterranean scrub area just outside the town of Iglesias (Fig 1A). At the beginning of November 2013, twenty worker bees were sampled alive with a butterfly net, while returning to their hives. The climate was characterized by warm and sunny weather. Honey bees were collected at 11 a.m. with a temperature of 23°C. The bees were immediately put in soda glass capped vials (Chromacol Limited), stored on ice in order to keep them inactive, and quickly brought to lab for sample preparation.

After a few hours at -20°C, heads, wings and hind legs were cut under a stereoscope with scalpels and ophthalmological scissors, and mounted onto SEM stubs using double adhesive carbon tape.

We excluded from the analyses the other two pairs of legs because preliminary SEM observations demonstrated that PM almost predominantly concentrates on the hind legs, in particular on their inner surface, following the antero-posterior “handling” of the pollen [30].

In order to analyze the gut content and the intestinal wall, the remaining body (thorax and abdomen) was put in sterile saline solution and the alimentary canal dissected. After dehydration through 70%, 80%, 90% (one passage for 20min) and 100% (two passages for 20min) ethanol series, the entire alimentary canal was mounted onto SEM stubs. Later, honey stomach, ventriculum and rectum were longitudinally cut, gently opened and air-dried, in order to preserve the gut content.

A few days later, ten worker-bees were collected (as control samples) in a rural area 10 km South of Parma (Northern Italy), near the bed of Parma creek and close to the foothills of the Apennine Mountains (44°41’24.7”N–10°20’9.9”E). Worker bees were sampled at 1 p.m. with a temperature of 19°C. The weather was partly cloudy. This control site (CS) was far from any known emitting sources of PM (i.e. vehicular traffic, incinerators, cement plants, industries, etc.). The hives were placed along the creek floodplain, and the surrounding hills consisted mainly of sandstones, marls and calcarenites, with noticeable clayey layers [31].

The preparation technique applied to the control bees was identical to the Sardinian ones.

All honey bees used in this study were collected in the presence of the beekeepers and with the permission of the owners of the private land were the hives were located.

No endangered or protected species are involved in this research.

Sediment samples

In order to identify the potential source(s) of the PM detected on the Sardinian honey bees, specific candidate sites were selected: Bingiargia (BNG; private land sampled with the owner permission), Monteponi Red Muds (RM; 39°17'52.8"N–08°30'25.2"E) and Cungiaus (CUN; 39°18'29"N–08°30'27"E) (Fig 1). The authors are not aware of any restriction regarding the sampling of soil sediments in the RM and CUN sites.

Main choice criterion of the sites was the exposure to wind uptake. BNG samples were collected up to 10 m from the hives. While BNG site undoubtedly fell within the foraging range of the apiary, CUN and RM possibly did not because they were quite distant from the apiary, i.e. about 3 and 3.5 Km far, respectively, and field studies have demonstrated that in autumn the average foraging distance achieved by the honey bees is less than 1.5 Km, reaching a maximum in the summer of about 2.2 Km [32]. In addition, foraging bees were exclusively collecting honeydew from holm oaks (*Quercus ilex*), which were absent in both sites.

With the aim of making a direct comparison between the mineral particles detected on the honey bees and the wind-available fraction of the soil, topsoil/exposed sediment samples were collected and then investigated with the same analytical technique (SEM-EDX).

In each candidate site, 3 to 5 samples were collected: shallow pits were excavated (down to max 5 cm) and up to 0.5 kg of material was recovered per pit. Samples were air-dried and an aliquot (about 1 g) was mixed with all other samples from the same site in order to obtain a representative group sample. The obtained mixture was then poured and mounted onto SEM stubs using double adhesive carbon tape. Despite the obvious grain size heterogeneity, only particles <100 μm in diameter (i.e. the fraction which can account for the TSP) were investigated for this study. Five replicates were analyzed for each soil sample.

The CS soil composition was not measured, but derived by literature [31, 33]. Soils of the area are mostly developed over fluvial sediments of various grain sizes, ranging from clays to conglomerate of sedimentary origin (Holocene–Upper Pleistocene, and older) [31, 33].

SEM-EDX analysis

SEM-EDX measurements were carried out on both the dissected portions (wings, head, hind legs, alimentary canal) of the honey bees and sediment samples. No coatings or other treatments were applied. Charging artifacts were largely suppressed using the low vacuum mode (100 Pa water vapor) at room temperature in a SEM FEI Quanta 200 FEG, equipped with an Ametek-EDAX ApolloX analytical system. Secondary Electrons (SE) and BackScattered Electrons (BSE) images, as well as EDX point analyses, were acquired in alternating sequence at the same conditions of 20 kV with a nominal beam current of about 1 nA, in order to provide the chemical composition, morphology, surface characteristics and size of the particles.

Each EDX spectrum was then interpreted according to a mineralogical point of view, also taking into account both the simultaneous presence of multiple phases and mineral content of the surrounding geological formations.

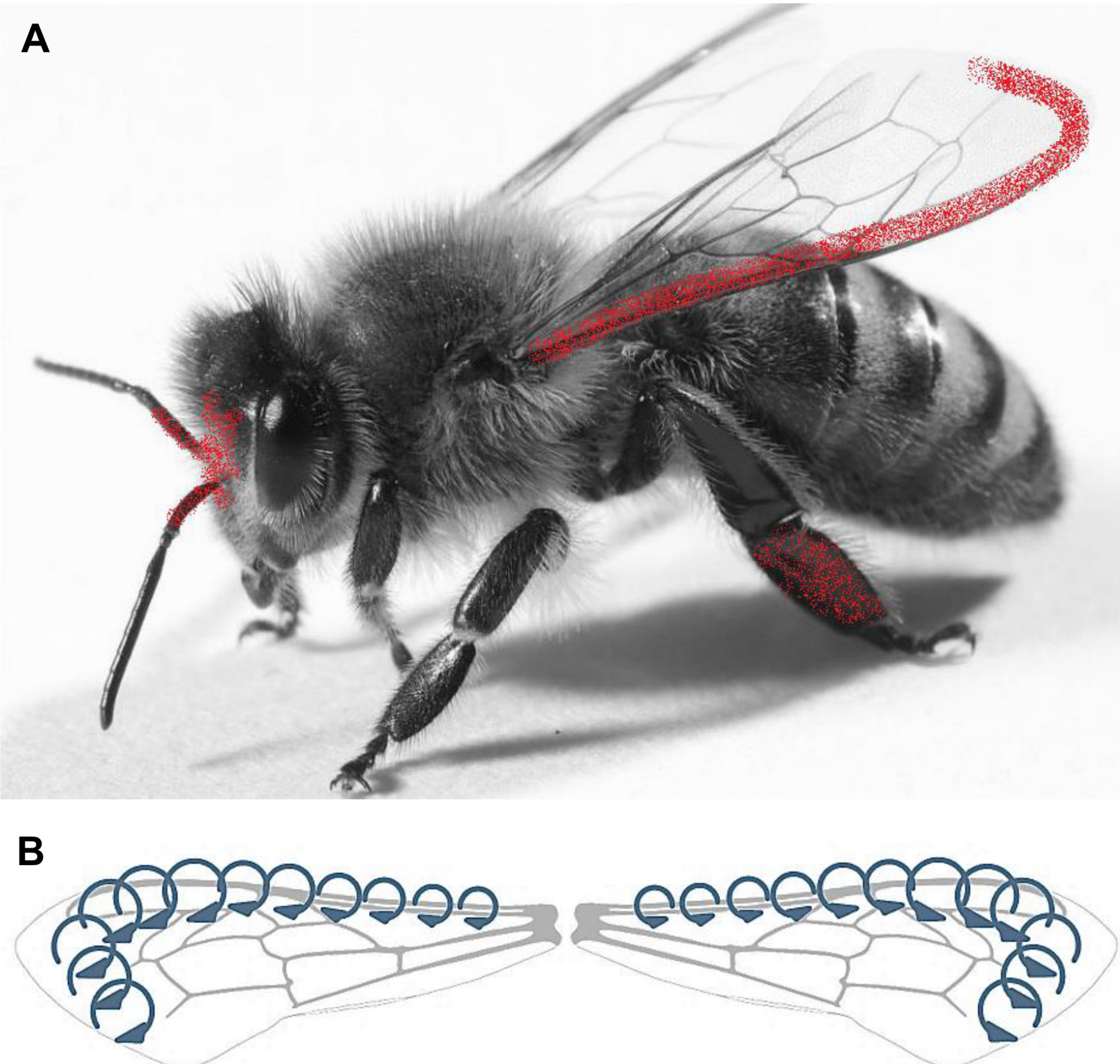

Fig 2. (A) Airborne PM (red) on the honey bees is mostly concentrated along the costal margin of the fore wings, the medial plane of the head, and the inner surface of the hind legs. (B) The Leading Edge Vortex (LEV) formed at the leading edge of the fore wings during the insect flight.

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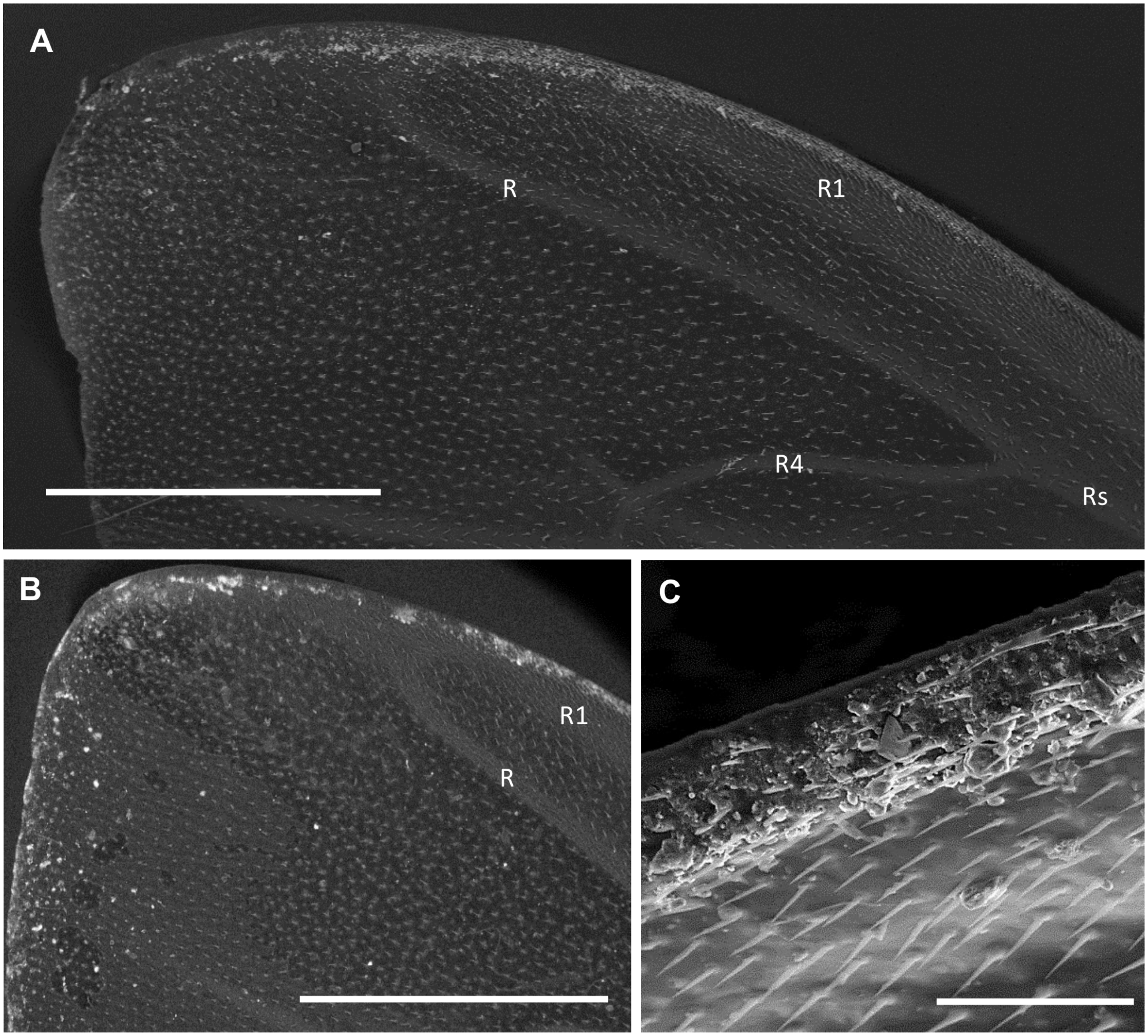

Fig 3. SEM images of the fore wings partially covered with PM. (A, B) Fore wings of Sardinian worker bees displaying PM (bright spots) most concentrated along the costal margin lining the first branch of the radial vein and the apex. BSE images. Bar = 1 mm. (C) A detail of particles gathered along the first branch of the radial vein. SE image. Bar = 100 μm. R = radial vein; R1 = first branch of the radial vein; Rs = second branch or radial sector; R4 = fourth branch.

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Results

Honey bees: wing and body surface

A detailed investigation of all the worker bees sampled in Sardinia revealed high contamination due to thousands of inorganic particles on the external body districts (i.e. head, hind legs and wings), mostly concentrated in specific areas (Fig 2A).

In all specimens, a large amount of particles was observed on the fore wings (upper surface), along the costal margin lining the first branch of the radial vein and the apex (Figs 2A and 3).

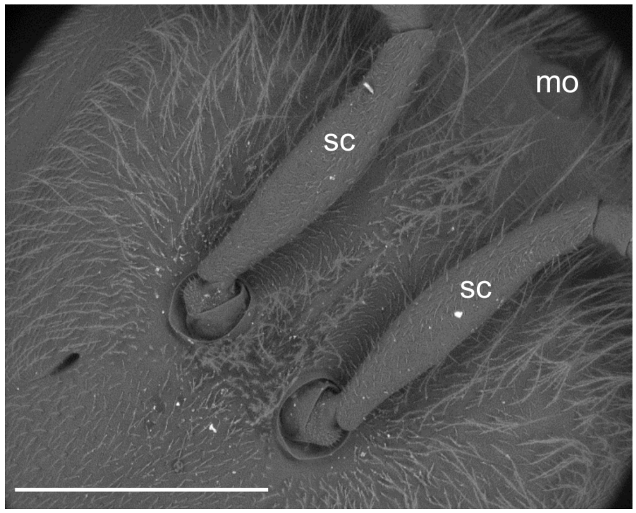

Fig 4. Honey bee head displaying PM (bright spots) almost exclusively along the medial plane, nearly between the median ocellus (mo) and the antennae, including the scapus (sc). BSE image. Bar = 1 mm.

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Fewer particles were dispersed on the remaining sector of the fore wings (Fig 3A and 3B) and on the hind wings (S1 Fig).

Heads showed particles almost exclusively along the medial plane, in a narrow area nearly between the bases of the antennae and the median ocellus (Figs 2A and 4); PM was also observed on the scape of each antenna (Fig 4).

On the third pairs of legs, the coverage of inorganic particles was always rather diffused along the most distal segments of the inner surface, and involved the structures dedicated to the body grooming, pollen collection, and wax handling (e.g. the pecten on the lower end of the tibia and the pollen comb on the metatarsus) (Figs 2A and 5).

Particle size was rather various and ranged from a few nm to 50 μm. Where present, the ultra-fine and fine particles were uniformly spread across the scanned surfaces (Fig 6); EDX analysis revealed that they were always fragments of baryte.

Finer particles were often observed adhering to and covering the bigger ones, thus forming complex, multi-grain aggregates of different mineral phases (Fig 7).

Frequently, the particles were embedded in organic matrix (only C and O detected), (Fig 8).

SEM observation and X-ray spectroscopy pointed out specific morphological and chemical features of the grains. On Sardinian bees natural mineralogical phases and anthropogenic compounds have been identified (Table 1).

Among natural phases, we were able to detect calcite/aragonite (S2A Fig) dolomite, phyllosilicates (Fig 7A, 7B and S2B Fig), and Na-rich plagioclases Fig 7D). Moreover, two mineral phases containing Ba and Pb, i.e. baryte (Figs 6 and 7) and galena (Fig 9A), were found.

In addition, on the honey bee body, rare cubic crystals of salt (halite) were observed (Fig 10).

The honey bees also collected anthropogenic particles, which generally displayed a subspherical morphology, sometimes with a scaly surface, ranging from about 500 nm up to 10 μm in diameter (Figs 9 and 11). Characterization with EDX defined their chemistry as either Fe-rich particles or alumino-silicate (Fig 11).

Other anthropogenic particles showed irregular shapes and consisted of Fe or Fe combined with Zn (Fig 12).

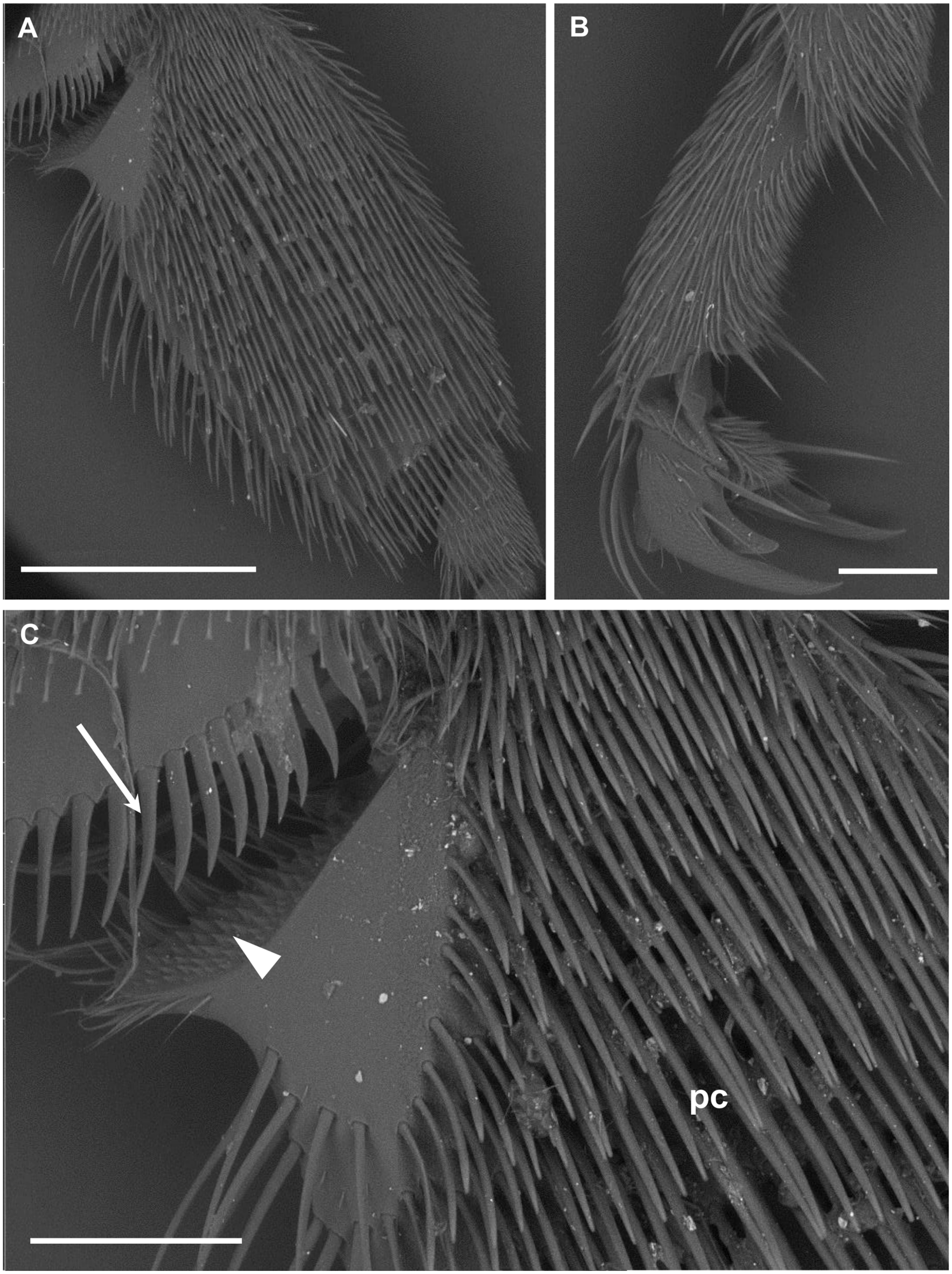
A
B
C
pc

Fig 5. BSE images of PM (bright spots) on the hind legs. (A) Metatarsus. Bar = 1 mm. (B) Distal tarsal segments. Bar = 150 μm. (C) Detail of the structures involved in the grooming behavior and pollen collection. The pecten spines (arrow) and the pyramidal spines of the auricle (arrowhead) convey and pack the pollen into the pollen basket located on the outer surface of the leg. The pollen comb (pc), composed by transverse rows of stiff spines, brush off pollen from the lateral surface of the body and collect wax scales from the abdomen. Bar = 300 μm.

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On control bees, electronic scan detected very few PM (compared to Sardinian bees), generally located along the costal and apical margins of the fore wings (S3 Fig). Particulate, ranging from about 400 nm to 30 μm in size, was without exception composed by natural mineral phases. EDX analyses showed that singular grains (with sharp edges) or multi-grain conglomerates belonged to calcite/aragonite (S4A Fig), quartz (S4B Fig) and clay minerals.

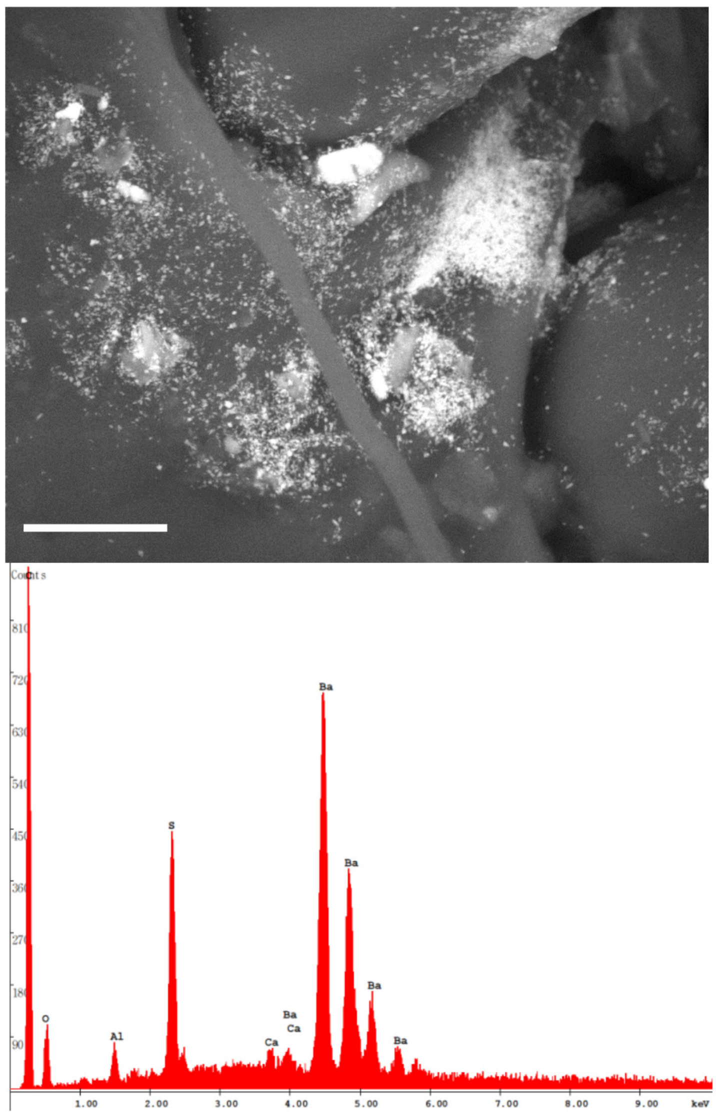

Fig 6. Fine and ultra-fine particles of baryte evenly spread across the honey bee wing. BSE image. Bar = 10 μm.

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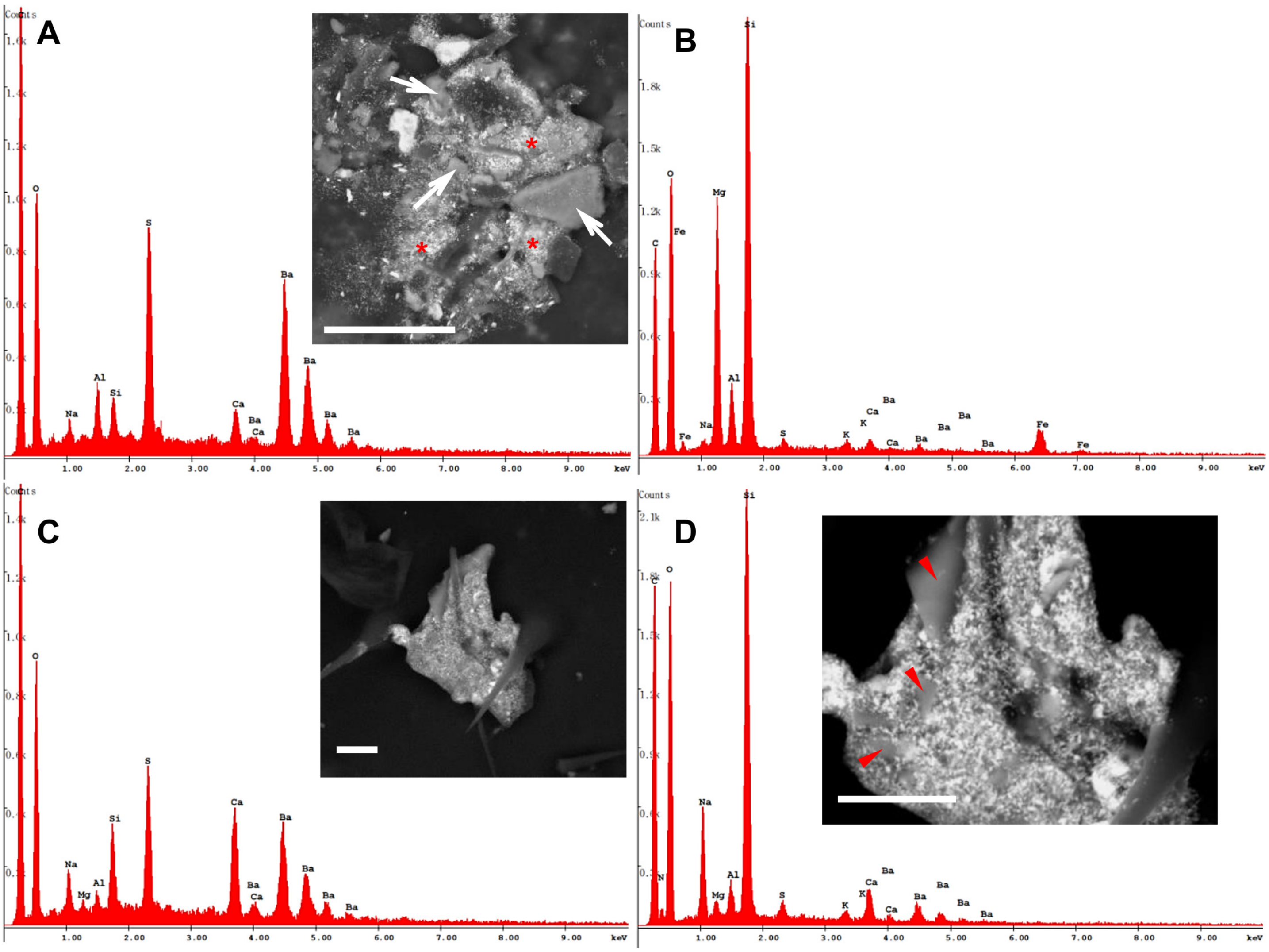

Fig 7. Aggregates of different mineral grains on the honey bee wings. BSE images. (A) Multi-grain aggregate of diverse mineral phases, including fine and ultra-fine grains of baryte (asterisks; EDX spectrum), and bigger particles of a phyllosilicate (arrows), whose EDX spectrum is shown in (B). Bar = 30 μm. (C) A multi-grain aggregate mainly composed of baryte. Bar = 10 μm. (D) A detail showing fragments of Na-rich plagioclase (arrowheads) and its EDX spectrum (note the contamination of baryte and possibly dolomite). Bar = 10 μm.

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Honey bees: alimentary canal

Surprisingly, the dissected alimentary canal of the hymenopterans both from Sardinia and control sites did not feature any apparent PM like that carried on the body surface. Inside the ventriculum and in the Malpighian tubules wall (close to gut) of all bees, only spherocrystals or spherites (i.e. spherical mineral concretions commonly found in many invertebrates, including insects) were detected (Fig 13).

These granules were usually grouped in grape-shaped clusters, and ranged between about 500 nm and 1.5 μm in diameter (Fig 13). EDX analyses on spherytes of both the gut and the Malpighian tubules of the Sardinian bees and control site revealed the presence of C, Ca, K, Mg, Mn, N, Na, O, P, S and Zn (S5 Fig).

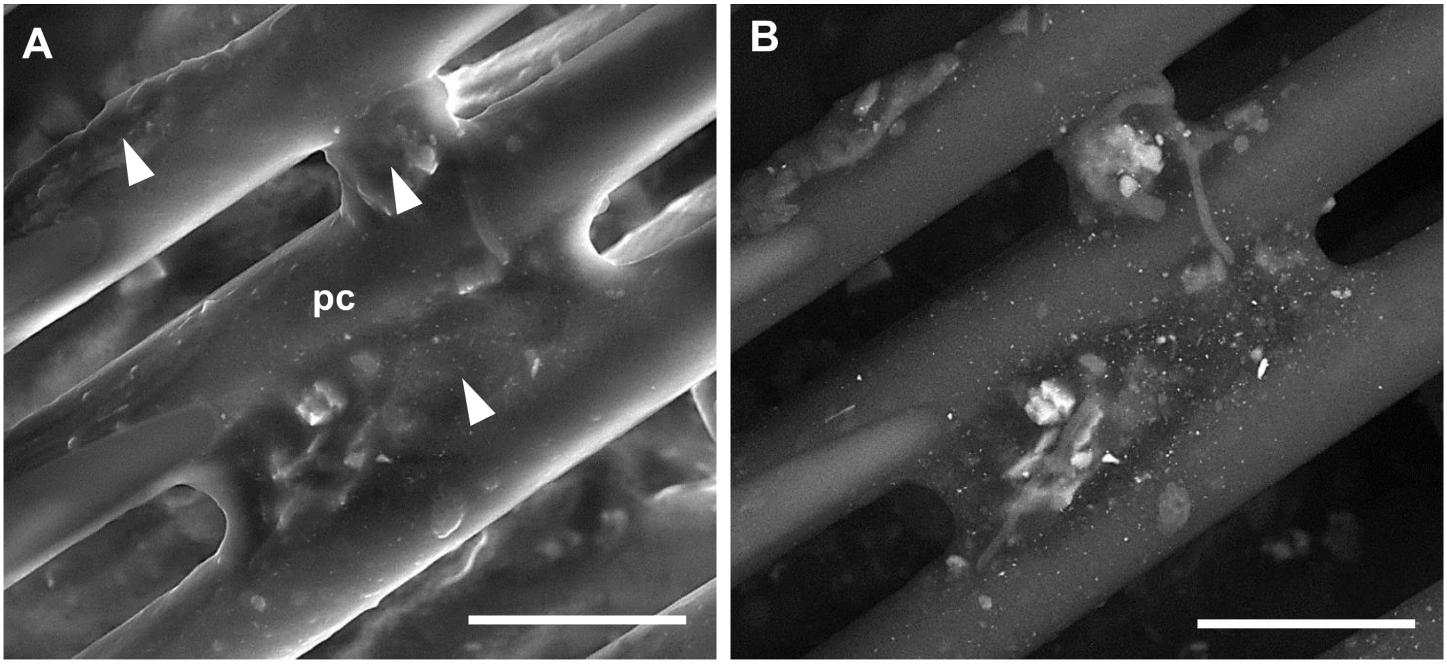

Fig 8. (A) SE and (B) BSE images of PM embedded in organic matrix (arrowheads) on the hind legs. pc = pollen comb. Bars = 30 μm.

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Mineralogy of sediments

Sediment analyses deliberately focused on the mineralogical composition of the wind available topsoil and top sediment cover.

The RM sites were characterized by absent soil coverage, with few exceptions (i.e. wind-repaired trenches with accumulated organic matter and litter; not sampled). The mineralogy of RM samples was distinct, with observed particle size up to 50 μm in diameter of baryte (S6A Fig), hemimorphite (S6B Fig), and smithsonite (S6C Fig). More rarely, gypsum was detected (S6D Fig). General grain habitus was subangular, reflecting cleavage and lattice structure of the individual mineral phases. Most grains were covered by a Fe-oxide layer: this attributes to the Red Muds hill a typical yellow-reddish appearance.

CUN sampling sites were located on an abandoned Pb-Zn mine dump. A portion of the dump was being colonized by shrubs and it was characterized by a thin layer of weakly, patchy developed topsoil, featuring small amounts of organic matter (not quantified). Mineral particle size was typically up to 60 μm in diameter, and the grains were frequently aggregates of smaller (<1 μm) particles. The mineralogical composition was mainly given by galena, even in tiny euhedral crystals (S7A and S7B Fig), and by fine/ultrafine grains of baryte, but it also included secondary Pb and Zn phases (e.g. cerussite, smithsonite and hemimorphite), calcite/aragonite and dolomite. Some specimens featured a partial coating by Fe- and Mn-oxides (S7B Fig).

BNG sites were the most developed, soil-wise. The sampled topsoils contained visible dark organic matter (field observation). Mineral grains were rather sporadic in this topsoil, due to relative dilution within litter and organic matter, and often appearing under the form of aggregates up to 100 μm. Main minerals detected were Na-rich plagioclase (possibly albite), calcite/aragonite, phyllosilicates and, occasionally, zircon, namely subangular to euedrally shaped (S7C Fig). Other phases, like quartz and baryte, were only observed as part of the aggregates. In BNG soil only a few subspherical anthropogenic particles were detected, and they were basically composed of Si and Al (S7D Fig).