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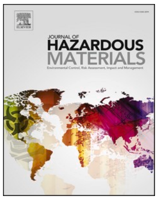

From molecules to organisms: A multi-level approach shows negative effects of trace elements from sewage sludge used as soil improver on honeybees

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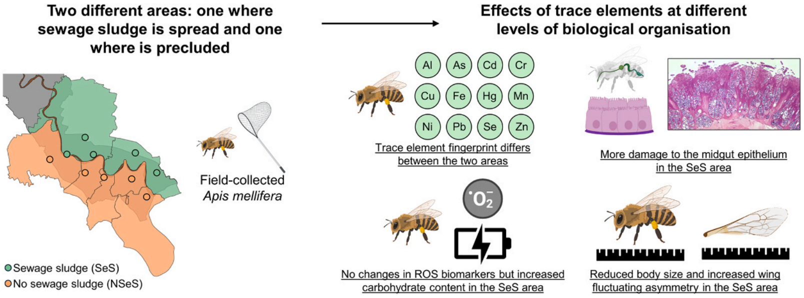
HIGHLIGHTS

- Sewage sludges used as soil improver in agroecosystems can contain trace elements.
- This is one of the first studies to use an integrated approach to investigate this issue.
- We sampled honeybees from two areas with different sewage sludge usage.
- We found negative effects of trace elements on morpho-physiological endpoints.
- First evidence of adverse effects of sewage sludge use under field conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of sewage sludge as a soil improver has been promoted in agroecosystems. However, sludges can contain toxic trace elements because of suboptimal wastewater treatment. Nonetheless, field studies investigating the negative effects of these practices on pollinators are lacking. We collected honeybees from an area where sewage

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Histology
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sludge use is widespread, and one where it is precluded. Trace elements in soils and bees were quantified. Cadmium, chromium, lead, mercury, and nickel were investigated because they were the least correlated elements to each other and are known to be toxic. Their levels were related to oxidative stress and energy biomarkers, midgut epithelial health, body size and wing asymmetry of honeybees. We found increased carbohydrate content in sites with higher cadmium levels, increased histological damage to the midgut epithelium in the sewage sludge area, and the presence of dark spherites in the epithelium of bees collected from the sites with the highest lead levels. Finally, we found that honeybees with the highest lead content were smaller, and that wing fluctuating asymmetry increased in sites with increasing levels of mercury. To the best of our knowledge, this is the first comprehensive study of the concentration and effects on honeybees of trace elements potentially deriving from soil amendment practices.

1. Introduction

The growth of the human population results in an increased demand for food resources, consequently driving the intensification of agroecosystems. In agricultural landscapes, policies have been implemented to reduce the use of synthetic products and to promote circular economy practices such as the use of sewage sludge [1], i.e. the fraction of solid matter extracted from wastewater during treatment in wastewater treatment plants (WWTPs). Indeed, due to its high content of organic matter and essential elements, sewage sludge is currently used as a soil improver [2] and this practice is strongly encouraged by the European Union and Italy following the Directive 86/278/EEC and the law 319/1976 [3,4]. One of the aims of these directives is to prevent and avoid harmful effects on soil and organisms due to the unmanaged use of sewage sludge. Despite this strong support, the use of sewage sludge as a soil improver has been debated because of the uncertainties about the nature, characteristics, and environmental effects of the trace elements it contains, which make the handling of sewage sludge complex [5]. Indeed, WWTPs are not adequately designed to remove trace element pollutants that accumulate in the sludge and, potentially, in the amended soils [6]. Trace elements are non-biodegradable contaminants that can be absorbed by crops, entering the trophic web where they can undergo processes of bioaccumulation and biomagnification, possibly leading to adverse effects on organisms [7,8]. As a result, there is a growing concern about the effects of sewage sludge application on crops [5], particularly concerning insect pollinators such as bees [9].

The honeybee *Apis mellifera* Linnaeus, 1758 is the most widespread managed insect pollinator in agroecosystems [10]. Honeybees provide several cultural, provisioning and regulating ecosystem services, including pollination [11]. Indeed, an estimated 35% of global food production comes from crops that depend on animal pollination [12] and, in the USA alone, honeybee pollination value is estimated at around 12 billion USD annually [13]. However, honeybees face exposure to numerous pollutants, including trace elements dispersed in agroecosystems [14]. Bees can encounter these trace elements through water, pollen, or nectar [15]; and thus being ingested [16]. In addition, these elements can both adhere directly to their bodies, such as on their wings [17]. Therefore, honeybees can act as bioindicators of environmental contamination [18], and hives can be used as passive sampling devices [19].

Not all trace elements are harmful to bees, though. For example, some of them have positive biological functions such as zinc and selenium that increase the antioxidant status and body weight of newly emerged bees [20], while others are toxic and mortal regardless of their concentration (e.g., cadmium, nickel, led, arsenic, chromium) [21].

Contamination from toxic metals can trigger different responses in bees, from sub-cellular to whole-body morphological alterations [22, 23]. For example, metal contamination was observed to alter superoxide dismutase and catalase activity in honeybees [24,25], which may lead to cellular damage and ultimately death [26,27]. Ingested metals can also affect tissues such as the midgut epithelium. Indeed, bees can sequester metals in spherites in the intestinal epithelium as a mechanism to isolate exogenous bodies, often causing histological damage [28]. Finally, exposure to metals may also induce external morphological variations. For example, a reduction in the larval growth rate that consequently affects the adult body size [29]. In addition, random deviations from perfect symmetry (i.e., fluctuating asymmetry, [30]), were found to increase in wild bees developing on a heavy metal pollution gradient [31].

Despite the evidence about the adverse effects of trace elements – and in particular toxic metals [32] – most of the studies carried out so far have been conducted in the laboratory [33]. Worryingly, the only biological test to approve the use of sludge is a growth test on *Lactuca sativa*, a common edible plant (DGR n. 12764). To date only one study has been carried out on the transfer of pollutants from sewage sludge to bees and it showed that caffeine, paraxanthine, cotinine, and acetaminophen were detected in the three bee species tested [34].

To fill these gaps, we investigated the multi-level effects of trace elements typically present in sewage sludge on *A. mellifera* foragers collected from two adjacent areas, one where sewage sludge is used as a soil amendment (hereafter SeS area) and one where its spreading is precluded (NSeS area). First, we quantified the levels of twelve trace elements (among which some toxic metals) in the bees and soils providing a contamination fingerprint of the two study areas. Then we tested the effects of trace elements at the biochemical, histological, and morphological levels. We hypothesised that bees collected from more contaminated areas would have higher levels of stress-related biomarkers (superoxide dismutase, catalase and glutathione peroxidase), lipid peroxidation and energy-related biomarkers (protein, carbohydrate, lipid content and total caloric content) due to detoxification activities. We also expect that bees from more contaminated areas would show alterations such as the presence of spherites or compromised midgut epithelial integrity due to the absorption of trace elements. Finally, we hypothesise a possible reduction in body and wing size and an increase in fluctuating asymmetry in bees collected from more contaminated areas.

2. Materials and methods

2.1. Study area and honeybee sampling

The study was carried out in the Parco Adda Sud (Lombardy, Northern Italy, approximate centre of the study area: 45°09’22.8" N 9°49’38.0" E) where the river Adda divides the plain into two areas. The use of sewage sludge as a soil amendment is widespread on the western side of the river and prohibited on its eastern side. The rivers thus act as a physical barrier between the SeS and NSeS areas. The study area is the same as the paper by Costanzo et al. [35], which has already shown different levels of trace elements in birds collected from these areas.

In both the SeS and NSeS areas the soils are defined as loamy, mixed, superactive, and mesic (USDA classification, [36]); while the land use is defined as “arable land”. In both areas, the first 40 cm of soil have a pH between 5.5 and 11 and a Cation exchange capacity (CSC) > 8 meg/100 g soil, which make these soils compliant for sludge spreading according to the local regulation (https://losan.ersaflombardia.it/oss/oss_P9_32.html for NSeS area, https://losan.ersaflombardia.it/oss/oss_P15_32.html for SES area). As set by DGR 2031/2014 (Lombardy, Italy), limits for metals and metalloids concentration values in sewage sludge are: cadmium ≤ 20 mg/kg dry matter (DM); copper ≤ 1000 mg/kg (DM); nickel ≤ 300 mg/kg (DM); lead ≤ 750 mg/kg (DM); zinc ≤ 2500 mg/kg (DM); mercury ≤ 10 mg/kg (DM); arsenic ≤ 20 mg/kg (DM); selenium ≤ 10 mg/kg (DM). Organic carbon, nitrogen and phosphorus are > 20, 1.5, and 0.4 mg/kg (DM), respectively.

In both areas, 5 sites in proximity to apiaries with about 2 km between each other were selected (Fig. 1). The sampling sites were characterised in terms of land use, temperature, and Normalised Difference Vegetation Index (NDVI) to verify that the only environmental difference was the use of sewage sludge ([37], see supporting information).

At each site, a total of 53 honeybees were sampled during foraging trips with entomological nets, between 20 April and 24 May 2022. Foragers are highly exposed to environmental pollution during their foraging bouts and likely have the same age since honeybees perform an age-dependent division of labour [38], thus they likely experienced comparable durations of exposure to contaminants. Each bee was stored in a 25 mL tube and placed in a cool bag in the field to anaesthetise them and bring them back alive to the laboratory. Then, 30 sampled foragers (3 bees/site) were immediately processed as described below for histological analyses, while the others (50 bees/site) were killed and stored at −80 °C. These were divided between the different analyses: 300 bees (30 bees/site) were used for external morphology and quantification of trace elements, 200 bees (20 bees/site) were used for biochemical analyses.

2.2. Chemical analyses

The following reagents and apparatus were used to analyse trace element contents in soil samples and honeybees. Certified multi-standard solution Merck VI for ICP-MS, Trace-SELECT® Ultra ultrapure HNO_3 (65 % w/w), HF (40 % w/w), and H_2O_2 (30 % w/w) were purchased by Merck (Milan, Italy), and Ultrapure water by Carlo Erba Reagents (Cornaredo, Milan, Italy). A microwave oven (Mars 5, CEM s.r.l., Cologno al Serio, Italy) equipped with 8 PFA polytetrafluoroethylene modified vessels (PTFE-PFA, 10 mL, Xpress) and 3 TFM polytetrafluoroethylene modified vessels (PTFE-TFM, 100 mL, EasyPrep) was used for trace elements extraction from honeybees and soil samples. A single quadrupole inductively coupled plasma mass spectrometry (SQ-ICP-MS, iCAP RQ Thermo Fisher Scientific), equipped with a quartz cyclonic chamber cooled at 3 °C, a MicroMist nebulizer (400 μL / min), a quartz torch, a Ni sampler and skimmer cones, and a QCell pressurized with helium to remove undesirable molecule ions by kinetic energy discrimination (3 V, KED mode), was used for trace elements analysis in the honeybees and Hg determination in soil samples. An inductively coupled plasma optical emission spectroscopy (ICP-OES iCAP 7400, Thermo Fisher Scientific) equipped with a concentric nebulizer, cyclonic spray chamber, and ceramic duo-torch, was used for trace elements analysis in soil samples.

2.3. Experimental procedures

2.3.1. Analytical procedure for trace elements content in soil samples

Soil samples were collected around the honeybee sampling areas to analyse the trace elements in the soil. Four soil samples of 100 g were collected twice (mid-May and mid-June 2021) from five sites for each area (SeS *vs* NSeS). Soil samples were collected with a plastic scoop after removing the topsoil layer and placed into sealed plastic bags. Samples were then stored at −20° for the chemical analyses (EPA Method 3052 as in [39-42]; see supporting information). Concentrations of eleven trace elements, arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), selenium (Se), and zinc (Zn) were determined by external calibration curves according to the procedure above. Results are reported as μg/g dry weight.

2.3.2. Analytical procedure for trace elements content in the honeybees

Bees that underwent this procedure were stored dry at −80 °C and were processed similarly to the soil samples (see supporting information). Concentrations of the same trace elements as above plus aluminium (Al), were determined by external calibration curves (0.25 – 1000 μg/L). Results are reported as μg/g dry weight.

2.3.3. Biochemical analysis

A battery of oxidative stress (superoxide dismutase - SOD; catalase - CAT and glutathione peroxidase - GPx), lipid peroxidation (LPO) and

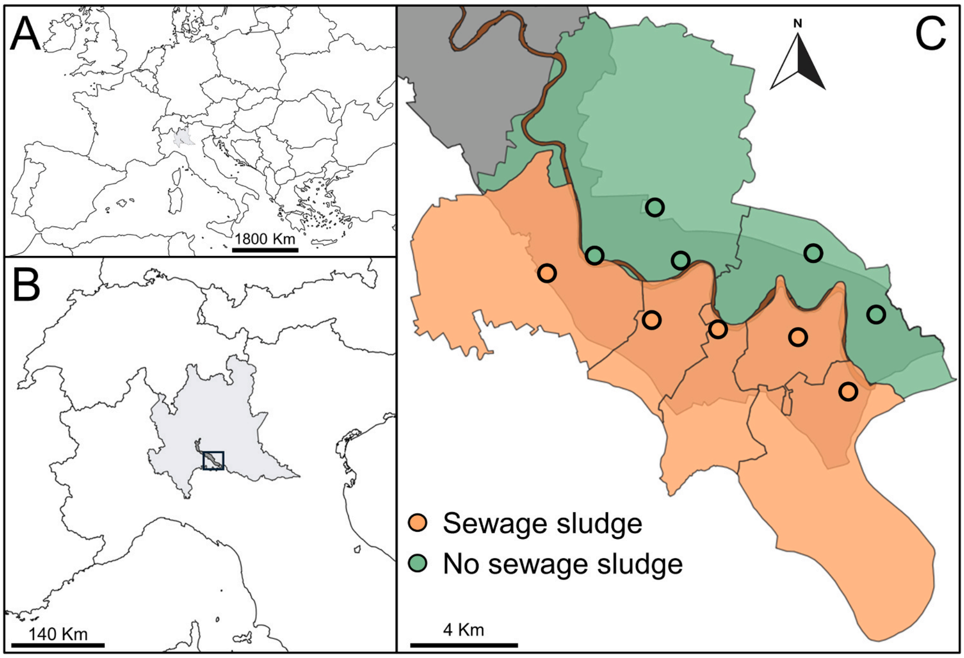

Fig. 1. A) Position of Lombardy and the study area in Europe and B) in northern Italy (the black box indicates the study area). C) Map of the sampling sites (dots) in the two areas. Green: no sewage sludge area; Orange: sewage sludge area; in brown the Adda river. In A and B, the light-grey area is the region Lombardy, and the dark-grey portion is the Parco Adda Sud.

energetic biomarkers (protein, carbohydrate, lipid content, and the total caloric content) were investigated following an established protocol used for different invertebrates among which honeybees [43-51] and further adjusted for our purposes (see supporting information). The biomarkers analyses were conducted on fifty pools of the posterior part (abdomen) of bees. Five independent replicates (i.e., 5 pools of 4 bees each for a total of 20 bees) were analysed for each of the 10 sampling sites.

2.3.4. *Histology of midgut*

Upon arriving at the laboratory, bees were anesthetised for around 20 min in the fridge (4 °C). They were then placed on a plastic Petri dish and the intestines were gently removed from the bee by pulling the sting using forceps and processed for Epoxy resin embedding, ultramicrotome sectioning (0.99 μm), and staining (crystal violet 1 % w/v and basic fuchsine 1 % w/v) ([52-55], see supporting information).

The level of histological damage was assessed both qualitatively, with a general description of the appearance of the epithelium, and semiquantitatively [53,56]. To quantify damages, a final *damage score* was calculated by taking into account the severity of the histological damage and its frequency (see supporting information). Image analyses were performed using ImageJ [57].

2.3.5. *External morphology*

For each bee we measured the fresh weight, intertegular distance (ITD, used as a proxy for body size, [58]), the total area of the front and hind wings (fWA + hWA = WA), and the length of the front wing (fWL) (i.e., the linear span between the base and the tip). We thus calculated the aspect ratio as $AR = (fWL)^2 / 4 * (fWA)$ and the wing loading as $WL = \text{Fresh weight} / WA$ ([37,59], see supporting information).

For the evaluation of fluctuating asymmetry (hereafter, FA) we followed a widely used procedure for bees ([60,37,61,62], see supporting information). We selected 11 landmarks on the front wing and 5 on the hind wing (Fig. A1, Table A2) and calculated the asymmetry for 7 linear traits on the front wing and 3 on the hind wing (see supporting information).

2.4. *Statistical analysis*

First, the differences in the environmental variables between the two areas (5 sampling sites per area) were tested with a PERMANOVA using Euclidean distance [63] applied to standardised values (i.e. by subtracting the mean and dividing by the standard deviation) to avoid giving too much importance to those environmental variables with very high values over those with lower ones. The results were then visualised with a Principal Component Analysis (PCA).

Then, differences in soil and honeybee trace element composition between the two areas (for both soil and honeybees) and among the ten sampling sites (for honeybees only) were tested with redundancy analyses (RDAs). As before, we standardised the concentrations of each element and used the Euclidean distance. Samples that, for technical issues, did not have the complete trace element profile were excluded from this analysis. We also tested for differences in the levels of each trace element in honeybees among sites with ANOVA and between the two areas using linear mixed models (including the sampling sites as a random effect to account for intra-site replication).

Biological endpoints (i.e. morphology and results of histological and biochemical analyses) were related to trace element concentrations using linear mixed models. Since the concentrations of elements were collinear, they could not be used simultaneously as predictors in these linear mixed models, so a correlation analysis was performed (Fig. A2), and correlated elements were excluded (Pearson's $r > 0.3$). Only five (possibly toxic) elements were retained as "representative": Cd, Cr, Hg, Ni, and Pb (Table A6) [19,64].

For the morphological analysis, each bee was assigned its individual concentration of the selected five metals. However, due to methodological incompatibility, it was not possible to quantify trace elements for the bees used in the histological and biochemical analyses. Therefore, for these analyses, each bee was assigned a mean concentration of the selected metals calculated from the bees collected in the same sampling site.

The biochemical (protein, carbohydrates, lipids and total caloric content as well as for SOD, CAT and GPX activities, and LPO) and the histological endpoints (damage score, number of vacuoles and spherites, loss of brush border, and cell contact) were analysed with linear mixed models using Cd, Cr, Hg, Ni and Pb, and the two areas (SeS vs NSeS) as fixed effects, and the sampling site as a random effect. For histological data recorded as presence/absence, a binomial distribution was used. The same model was built also to analyse the morphological endpoints (body and wing size, wing loading, aspect ratio, and fluctuating asymmetry). In this case, however, the body size was added as a covariate to the models of wing loading and aspect ratio to assess size-dependent effects. Finally, the number of traits showing fluctuating asymmetry in each site was correlated (Spearman) with the mean concentration of the five selected trace metals. P-values were corrected with a false discovery rate procedure [65].

All the analyses were performed with the R Statistical Software (*v. 4.2.2.* [66]) with the following packages: *multcomp* [67], *vegan* [68], *glmmTMB* [69] and *ggplot2* [70]. In the following sections, results are presented as mean ± standard error.

3. Results

The two areas did not significantly differ for environmental variables (PERMANOVA, $F_{1,\ 8} = 0.856$, $P = 0.633$, Fig. A3A). All sites were characterised mainly by arable land (ranging between 73–99 %, Fig. A3B). Ambient temperature was constant throughout the sampling period and sites (16.86–17.61 °C, Fig. A3C), as well as NDVI (0.39–0.60, Fig. A3D).

3.1. *Trace elements content in the soil samples and honeybees*

The trace elements content was analysed in 40 samples of soils. In descending order of abundance (μg /g), the metals were: Fe (2581 ± 97), Zn (98 ± 4), Cr (97 ± 7), Mn (66 ± 3), Ni (58 ± 6), Pb (29 ± 2), Cu (26 ± 1), As (14 ± 1), Se (6.8 ± 0.8), Cd (0.38 ± 0.04), Hg (0.37 ± 0.06). The RDA showed that the trace element concentrations in the soils significantly differed between the two areas ($F_{1,\ 40} = 9.737$, $P < 0.001$, Fig. A4). As was significantly higher in the NSeS area (Table A7; Fig. A5A), while Cr (Table A7, Fig. A5C) and Ni (Table A7, Fig. A5H) were higher in the SeS area.

Regarding the trace element concentration in the bees, for one sample the Al, Cr, Fe and Ni concentrations did not differ from the blanks of the method. For two other samples, the concentration of Pb did not differ from the blanks, and finally for two other samples Ni and As concentrations did not differ from the blanks. These samples were consequently excluded from the RDA. In descending order of abundance (μg/g), the concentrations of the trace elements were: Fe (312 ± 5), Mn (178 ± 4), Zn (110 ± 2), Al (32 ± 1), Cu (21.7 ± 0.3), Cd (1.09 ± 0.05), Pb (0.66 ± 0.06), Se (0.53 ± 0.02), Ni (0.47 ± 0.01), As (0.23 ± 0.01), Cr (0.22 ± 0.01), and Hg (0.025 ± 0.001). The RDA showed that the trace element fingerprint of honeybees significantly differed between the two areas ($F_{1,\ 283} = 8.628$, $P < 0.001$, Fig. A6) as well as among the sampling sites ($F_{8,\ 283} = 9.902$, $P < 0.001$). Ni was significantly higher in the SeS area (Table A8, Fig. A7). Differences were larger when the comparisons were done at the site level (Table A9, Fig. A8).

3.2. *Biochemical analyses*

No significant difference between the sampling areas was found for any of the endpoints analysed (Table 1). However, bees had a significantly higher content of carbohydrates in sites with a higher level of Cd

Table 1
Summary statistics of the linear mixed models used to test for the variation of the energetic content and the biomarkers with the concentrations of the five selected heavy metals and between the two areas. SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, LPO: lipid peroxidation, N: number of individuals, S.E.: standard error of the estimate. For the predictor “Area”, positive estimates mean higher values in the SeS area. Statistically significant results ($P < 0.05$) are boldfaced.

Trait	N	Predictors	Estimate	S.E.	z	P
CAT	50	Cd	5.726	15.040	0.381	0.703
		Cr	−93.451	59.963	1.558	0.119
		Hg	940.934	726.155	1.296	0.195
		Ni	−105.652	61.968	1.705	0.088
		Pb	−12.037	12.742	0.945	0.345
		Area	15.574	12.172	1.279	0.200
GPx	50	Cd	12.221	7.496	1.630	0.103
		Cr	−21.117	29.885	0.707	0.480
		Hg	583.041	361.910	1.611	0.107
		Ni	42.838	30.884	1.387	0.165
		Pb	−7.661	6.351	1.206	0.228
		Area	−4.077	6.066	0.672	0.502
SOD	50	Cd	−0.715	0.546	1.309	0.191
		Cr	0.995	2.177	0.457	0.648
		Hg	−7.285	26.365	0.276	0.782
		Ni	0.371	2.250	0.165	0.869
		Pb	0.639	0.463	1.381	0.167
		Area	0.379	0.442	0.857	0.392
LPO	45	Cd	−23.832	19.125	1.246	0.213
		Cr	−2.984	75.923	0.039	0.969
		Hg	3.082	909.706	0.003	0.997
		Ni	5.151	78.532	0.066	0.948
		Pb	5.073	16.403	0.309	0.757
		Area	2.875	15.257	0.188	0.851
Carbohydrates	49	**Cd**	**46.036**	**23.159**	**1.988**	**0.047**
		Cr	−29.105	92.719	0.314	0.754
		Hg	1429.857	1122.221	1.274	0.203
		Ni	−174.921	96.835	1.806	0.071
		Pb	−6.001	19.703	0.305	0.761
		Area	−4.345	19.356	0.224	0.822
Lipids	50	Cd	−0.116	22.083	0.005	0.996
		Cr	−55.912	88.041	0.635	0.525
		Hg	1128.003	1066.190	1.058	0.290
		Ni	−160.435	90.985	1.763	0.078
		Pb	−5.762	18.709	0.308	0.758
		Area	7.715	17.872	0.432	0.666
Proteins	50	Cd	−6.668	4.205	1.586	0.113
		Cr	−20.641	16.767	1.231	0.218
		Hg	86.909	203.049	0.428	0.669
		Ni	−13.415	17.328	0.774	0.439
		Pb	−0.857	3.563	0.241	0.810
		Area	5.584	3.404	1.641	0.101
Total caloric content	50	Cd	−0.009	2.087	0.004	0.996
		Cr	−5.286	8.322	0.635	0.525
		Hg	106.664	100.781	1.058	0.290
		Ni	−15.170	8.600	1.764	0.078
		Pb	−0.545	1.768	0.308	0.758
		Area	0.729	1.689	0.432	0.666

(Table 1, Fig. 2A).

3.3. *Histology of midgut*

In the NSeS area, the epithelium was often intact and thick, with a clear presence of the brush border (Fig. 3A, C, E). It showed signs of epithelial turnover activity, such as the presence of vacuoles and some cells in the lumen (Fig. 3A). Conversely, in bees from the SeS area, the epithelial cell layer was often very thin and disaggregated, with high vacuolisation. The brush border was sometimes not visible (Fig. 3B, D). In addition, we found the presence of a great number of thick dark spherites, especially in bees from site #07 of the SeS area – the richest in Pb (Fig. 3F) – whereas they were practically absent in the bees from the NSeS area (Fig. 3A, C, E). The damage score (Table 2, Fig. 2B) and the number of vacuoles (Table 2, Fig. 2D) were significantly higher in the SeS area, and both decreased with increasing Cd levels (Table 2, Fig. 2C, E). Finally, the number of vacuoles also significantly decreased with increasing Hg levels (Table 2, Fig. 2F).

3.4. *External morphology*

No significant effect of the sampling area was found for any of the parameters analysed (Tables 3 and 4, Fig. 4). Bees with the highest Pb levels were significantly smaller (Fig. 4A) and, when correcting for body size, had also smaller wings (Fig. 4B). Wing loading significantly decreased with increasing Cd concentrations (Fig. 4C). Finally, bees had a higher number of traits showing fluctuating asymmetry in sites with higher Hg levels (Fig. 4D). When analysed individually, the asymmetry value of the trait F6 significantly increased with Hg levels, as did H2 with Cd, and H3 with Ni (Table A10).

4. Discussion

In this study, we found that honeybees, the most economically important pollinator of agroecosystems, have different trace element fingerprints in areas where sewage sludge is used or prohibited as a soil amendment (SeS *vs* NSeS), and that some of these elements alter

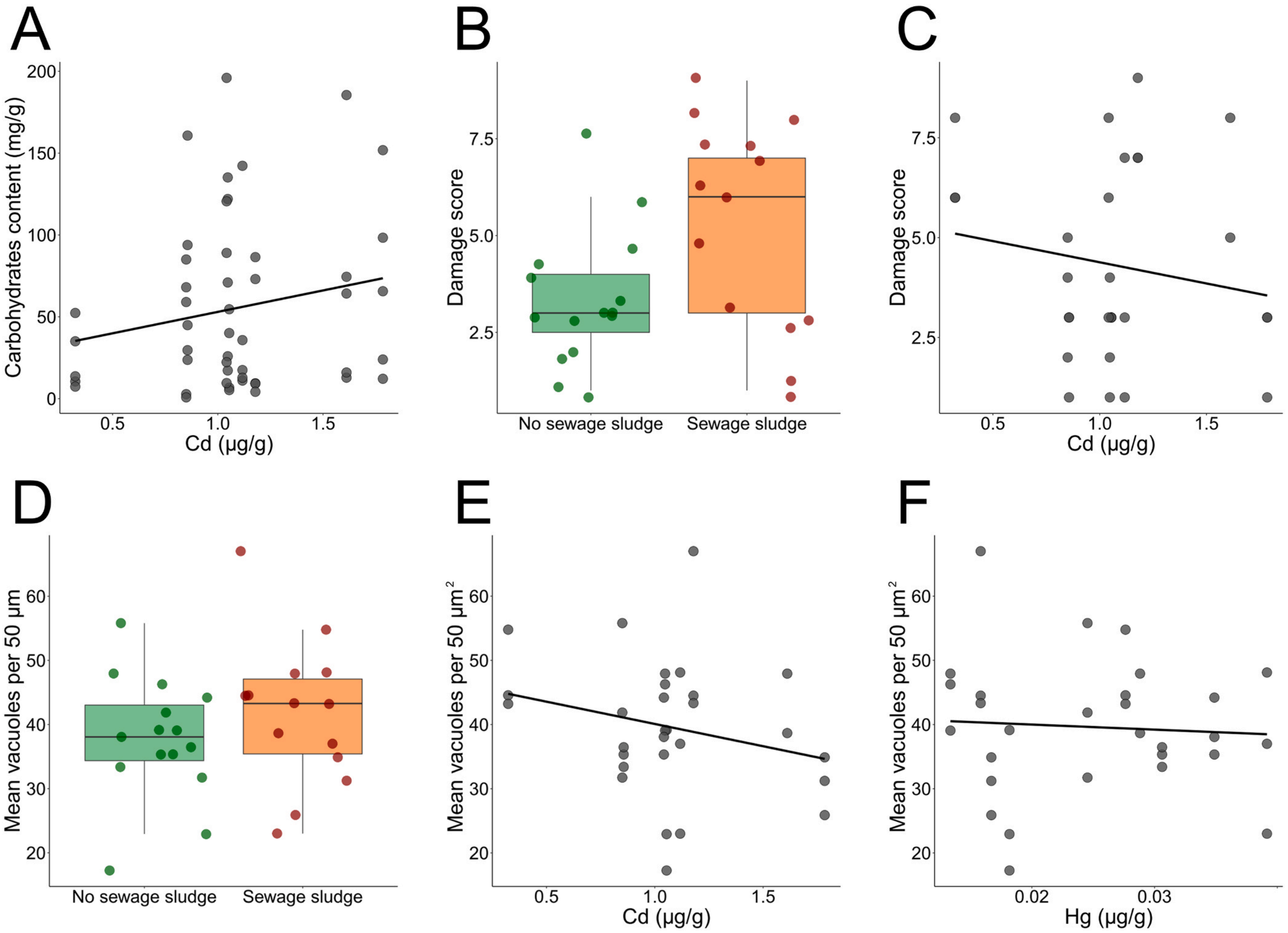

Fig. 2. Graphical representation of the statistically significant effects found in the linear mixed models investigating the variations in the energy biomarkers (A) and histology of the midgut (B-F). In all the plots, points are the measured values. In the scatterplots, the line shows a simple linear regression; in the boxplot the horizontal line is the median. Abbreviations of the elements: Cd (cadmium), Hg (mercury).

biochemical, histological, and morphological endpoints of individuals. To the best of our knowledge, this is the first comprehensive study of the effects of sewage sludge use as a soil amendment on morphological, histological, and biochemical endpoints in honeybees. However, we also acknowledge a possible shortcoming of our work, which is the impossibility of knowing the exact locations and times of sludge use. In addition, field-based studies often suffer from the uncontrolled effects of other environmental variables not considered. On the other hand, studies like the present one have the main strength of providing a snapshot of the true health status of the organisms analysed in that study area.

4.1. Trace elements content in the soil samples and honeybees

Trace elements are increasingly studied as environmental pollutants as they may contain toxic metals [64,71]. Overall, the trace element fingerprint of honeybees differed significantly between the two areas. This suggests that the element patterns may reflect local environmental concentrations of trace elements [35]. However, only Ni had a significantly higher level in the honeybees of the SeS area. This may be due to the limited statistical power of our univariate analyses where we also accounted for multiple statistical tests. Future analyses on larger sample sizes may thus identify differences in other trace elements that have remained undetected in the present study.

Typically, ingested trace elements pass through the digestive tract and are partly metabolised in the bees' fat bodies, while their residues are excreted [72]. This may explain why we did not find any major differences in the levels of other metals in the bees between the SeS and the NSeS areas. Alternatively, this lack of differences may simply reflect the lack of differences in the trace element concentrations in the soils.

Remarkably, the levels of these toxic elements in honeybees vary widely in the literature and there is no consensus on which trace elements should be measured [73-76,16,77,19,78-80]. The most analysed metals are Cd, Cr and Pb with values (µg/g) ranging, respectively, between 0.004–0.750, 0.0001–1.030 and 0.001–1.670. The levels we found for these three elements are within these ranges but often approach the upper limit. This means that contamination in honeybees in our study area – at least for these three elements – is comparable to that of other honeybees from different altered landscapes (e.g., heavily urbanised areas).

4.2. Biochemical analysis

Antioxidant enzymes are widely used as biomarkers of xenobiotic exposure as they play an important role in protecting the organism against reactive oxygen species (ROS) [81]. We observed no statistically significant changes in SOD and GPx activities, and in lipid peroxidation levels, suggesting that exposure to sewage sludge may not cause an overproduction of ROS [82]. Nonetheless, we found a marginally non-significant decrease of CAT activity with increasing Ni levels. This is consistent with the published literature. For instance, in honeybees fed with sucrose syrup containing 0.1, 0.01, 0.001 mg/L of Cd, a decrease in

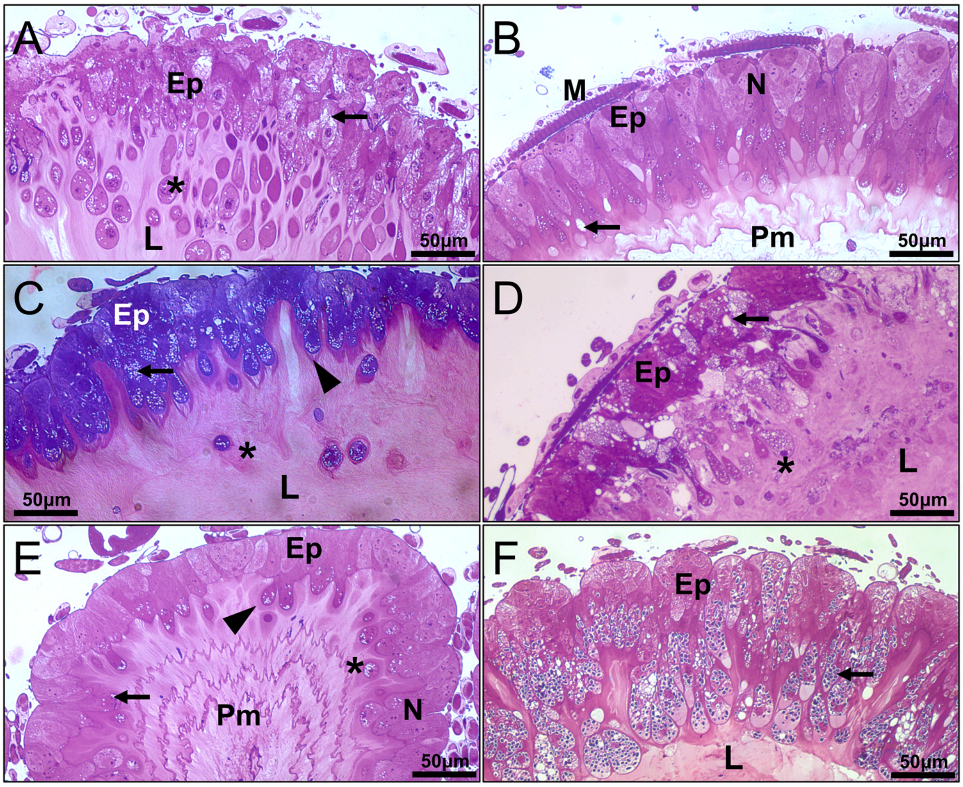

Fig. 3. Comparison between histological sections of the midgut epithelium from the no sewage sludge area (A, C, E) and the sewage sludge area (B, D, F). Ep: epithelium, L: lumen, N: nuclei, Pm: peritrophic membrane. Asterisks: cells in the lumen, black arrows: vacuoles (either empty or presenting black spherites), arrowheads: epithelial brush border.

CAT activity was observed, but no change in SOD activity or lipid peroxidation levels [25]. Conversely, a disrupt redox balance and oxidative stress was found in *A. cerana* fed with sucrose solution with up to 800 mg/L of Cd [27].

Exposure to a contaminant often causes the organism to defend itself by up-regulating detoxification enzymes which result in higher energy consumption [83]. This may result in a modulation of energy metabolism, a physiological function that helps insects cope with heavy metal exposure [83]. We only found a significant increase in carbohydrates content with increasing levels of Cd. Evidence from the literature about protein and glycogen content is contrasting. For example, an increase in total protein and glycogen content was observed in *Cryptolaemus montrouzieri* Mulsant, 1850 (Coleoptera) fed with heavy metals solutions [84]. On the other hand, it was found that *Poecilus cupreus* (Linnaeus, 1758) (Coleoptera) had reduced energy reserves when fed on metal-contaminated food [85]. Therefore, we can hypothesise that Cd contamination has increased the energy production – in terms of carbohydrate content – of honeybees. However, we also found a marginally non-significant decrease in carbohydrate, lipid content, and total caloric content with increasing Ni levels.

Overall, our results suggest that some of the available energy could be diverted to detoxification, such as CAT activity, but we argue that this indicates that the bees are not much stressed. This leads us to hypothesise that the actual levels of contamination found in the study area are not that biochemically stressful to honeybees, but they could be if contamination levels were to increase.

4.3. Histology of midgut

In insects, trace elements reach their higher concentrations in the gut. We found that the damage score and the vacuolisation frequency were significantly higher in the SeS area and decreased with increasing Cd concentrations. Vacuoles also decreased with increasing Hg levels. Although the first result is somehow easy to interpret, the other two are less trivial. Nevertheless, as expected, honeybees in the SeS area have a higher damage score, which considers both the frequency and severity of histological damage to the midgut epithelium and a higher vacuolisation rate. In addition, albeit marginally non-significant, vacuoles were also more frequent with increasing Cr levels, a known toxic trace element. Altogether, this is consistent with some studies that found increased levels of damage in honeybees exposed to the fungicide pyraclostrobin [86,87], but contrasts what was found in other studies about heavy metal exposure to honeybees [28].

Vacuolisation is a natural process, but when it becomes intense, it may signal autophagic cell death [56]. This was also found in other studies on insects exposed either to heavy metals [62] or to insecticides [88,89]. We also found a general deterioration of the integrity of the midgut epithelium and of the brush border of the bees collected from the SeS area. Both changes are symptoms of cellular and tissue stress. In fact, a loss of the brush border and a disorganised epithelium were found in honeybees exposed to neonicotinoids [89,90] or fed with toxic metals such as Cd and Pb [28]. A degradation of the brush border can damage the epithelial cells since it protects them from physical damage [56]. We also found a massive presence of thick, dark spherites in bees collected from the site with the higher concentrations of Pb. We hypothesise that honeybees may sequester toxic substances in these midgut spherites, a defence mechanism that has been shown also in wasps where the spherites contained Pb [62,91]. These spherites play a key role in preventing intoxication and facilitating osmoregulation within the midgut, ultimately constituting a compensatory mechanism to prevent cell intoxication [92].

We may therefore conclude that higher vacuolisation and damage score underlines a stressed condition of the midgut in the bees caused by trace elements in the two study areas.

Table 2
Summary statistics of the linear mixed models used to test for variation in histological alterations with the concentrations of the five selected heavy metals and between the two areas. N: number of individuals, S.E.: standard error of the estimate. For the predictor “Area”, positive estimates mean higher values in the SeS area. Statistically significant results ($P < 0.05$) are boldfaced.

Trait	N	Predictors	Estimate	S.E.	z	P
Damage score	29	**Cd**	−3.166	**1.397**	**2.266**	**0.024**
		Cr	8.754	5.570	1.572	0.116
		Hg	−71.549	67.475	1.060	0.289
		Ni	1.910	5.760	0.332	0.740
		Pb	1.503	1.276	1.178	0.239
		Area	**2.686**	**1.131**	**2.375**	**0.018**
Vacuoles	29	**Cd**	−15.632	**5.388**	**2.901**	**0.004**
		Cr	39.793	21.482	1.852	0.064
		Hg	−522.344	**260.259**	**2.007**	**0.045**
		Ni	−1.545	22.225	0.069	0.945
		Pb	4.862	5.146	0.945	0.345
		Area	**9.777**	**4.363**	**2.241**	**0.025**
Spherites	29	Cd	−7.840	9.889	0.793	0.428
		Cr	−5.659	45.372	0.125	0.901
		Hg	79.943	622.240	0.128	0.898
		Ni	−0.727	54.571	0.013	0.989
		Pb	12.318	8.594	1.433	0.152
		Area	5.614	10.031	0.560	0.576
Loss of cell contact (presence/ absence)	29	Cd	−0.262	4.150	0.063	0.950
		Cr	24.760	41.842	0.592	0.554
		Hg	−80.208	296.884	0.270	0.787
		Ni	10.232	15.790	0.648	0.517
		Pb	−4.745	9.947	0.477	0.633
		Area	4.900	8.080	0.607	0.544
Loss of brush border (presence/ absence)	29	Cd	−2.627	1.732	1.517	0.129
		Cr	4.493	5.654	0.795	0.427
		Hg	−44.026	71.358	0.617	0.537
		Ni	10.377	6.571	1.579	0.114
		Pb	1.898	1.982	0.958	0.338
		Area	0.757	1.360	0.557	0.578

4.4. External morphology

Body size and wing asymmetry are two common morphological parameters measured in insects under stressful conditions. We found reduced body and wing size in bees with higher levels of Pb. Reduced body mass was also found in ants reared in industrial sites with trace elements contamination [93] and in bees grown along a trace metal gradient [94]; while bees and wasps with smaller wings were also found in metal contaminated sites [31] and in agriculturally intensively managed habitats [95].

Under stable environmental conditions, the body size of insects should be relatively constant [96,97], and it may change in the presence of stressors [98]. Most insects show slower growth or development when exposed to Pb and Cd [29,99,100]. Wings develop proportionally to the size of the entire insect and can follow similar patterns to that of body size [101]. Therefore, we may hypothesise that a possible reduction in growth and development due to Pb toxic effects produced smaller adult bees with smaller wings [102]. Body size should increase with colony size [103], therefore we may also hypothesise that these contaminants negatively influence colony size and consequently the body size of workers. This may in turn possibly affects foraging efficiency [104] and the pollination service as well [105].

In addition, we found a reduction in wing loading in bees with higher levels of Cd, and the same tendency was observed also with Ni, albeit this latter effect was marginally non-significant. Reduced wing loading improves flight manoeuvrability [106], however, we believe that this is not an adaptive mechanism.

Table 3
Summary statistics of the linear mixed models used to test for variation in the external morphological traits with the concentrations of the five selected heavy metals and between the two areas. N: number of individuals, S.E.: standard error of the estimate. For the predictor “Area”, positive estimates mean higher values in the SeS area. Statistically significant results ($P < 0.05$) are boldfaced.

Trait	N	Predictors	Estimate	S.E.	z	P
Intertegular distance	294	Cd	0.004	0.010	0.380	0.707
		Cr	0.053	0.032	1.630	0.104
		Hg	−0.950	0.635	1.500	0.135
		Ni	−0.038	0.032	1.200	0.228
		Pb	−0.016	**0.007**	**2.280**	**0.022**
		Area	−0.115	0.082	1.400	0.160
Wing area	294	Cd	−0.353	0.209	1.689	0.091
		Cr	−0.038	0.730	0.051	0.959
		Hg	−22.385	13.906	1.610	0.107
		Ni	0.573	0.711	0.805	0.421
		Pb	−0.578	**0.160**	**3.622**	**< 0.001**
		Area	0.592	0.770	0.769	0.442
		ITD	**6.020**	**1.356**	**4.440**	**< 0.001**
Wing loading	294	**Cd**	−0.028	**0.011**	**2.670**	**0.008**
		Cr	0.047	0.036	1.325	0.185
		Hg	−0.980	0.695	1.410	0.158
		Ni	−0.067	0.035	1.925	0.054
		Pb	−0.006	0.008	0.783	0.433
		Area	−0.006	0.053	0.122	0.903
		ITD	**0.142**	**0.065**	**2.194**	**0.028**
Aspect ratio	294	Cd	−0.007	0.025	0.280	0.780
		Cr	0.050	0.084	0.598	0.550
		Hg	0.779	1.528	0.510	0.610
		Ni	0.105	0.081	1.296	0.195
		Pb	0.013	0.018	0.678	0.498
		Area	−0.097	0.058	1.672	0.094
		ITD	0.066	0.133	0.497	0.620

Table 4
Spearman’s correlations between the number of traits presenting fluctuating asymmetry (FA) in each sampling site and the mean value of the five selected heavy metals Statistically significant results ($P < 0.05$) are boldfaced. All p-values (Adj. P) were adjusted with the false discovery rate procedure.

Trait	Predictors	ρ	S	Adj. P
N° traits FA	Cd	−0.169	192.950	0.850
	Cr	0.690	51.139	0.068
	Hg	**0.778**	**36.648**	**0.040**
	Ni	0.069	153.610	0.850
	Pb	0.132	143.260	0.850

We also analysed fluctuating asymmetry, which may be used as a proxy to assess the impact of environmental stress on organisms. We found that the number of traits showing fluctuating asymmetry at each sampling site increased with increasing Hg concentration and, albeit marginally non-significant, with Cr. These results are consistent with what was found in wasps [101] exposed to heavy metals and confirms our hypothesis that higher Hg – and possibly Cr – concentrations likely increased stress during honeybee development.

5. Conclusions

We have shown how trace element contamination can affect honeybees at biochemical, cellular, tissue and external morphological levels. We found that Cd, Pb, and Hg (some of the most toxic heavy metals) affected the morpho-physiology of bees. In addition, we found clear differences in the levels of damage in honeybee midgut epithelium between the areas where sewage sludge was used or not as soil amendment. We argue that the local contamination (site level) may play a role in producing the effects here presented. This highlights the importance of studies based on organisms collected in the field and including analyses at different levels of biological organisation, which

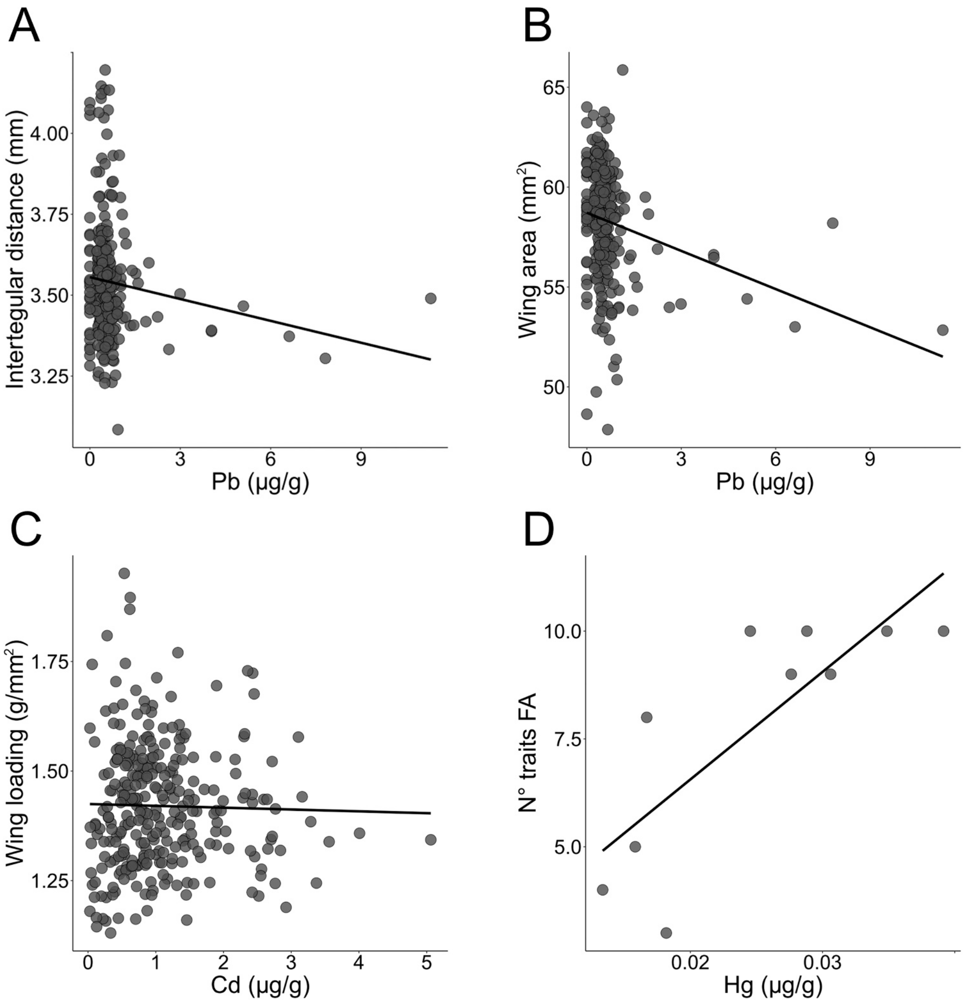

Fig. 4. Graphical representation of the statistically significant effects found in the linear mixed models investigating the variations in morphological traits. Points are the measured values; the line shows a simple linear regression. Abbreviations of the elements: Cd (cadmium), Hg (mercury), Pb (lead).

are still largely lacking. This is particularly important in Italy, because the only biological test to approve the use of sewage treatment is a growth test on *Lactuca sativa*. In our opinion, it would be necessary to carry out physiological tests on an animal model to authorise the use of sewage sludge. In addition, future studies should aim at investigating the effects of sewage sludge use on wild bees as they may differ in their susceptibility to this anthropogenic disturbance [107,108].

Environmental implication

In agricultural landscapes, the use of sewage sludge as a soil improver has been promoted to recycle wastewater as part of circular economy practices. However, sewage sludge contains hazardous materials such as heavy metals, known to be harmful to pollinators. Currently, field-based studies investigating the effects of sludge on bees are lacking. With a multi-level approach, we highlighted the adverse effects of some heavy metals typically contained in the sludge on *Apis mellifera*. By increasing the knowledge about the effects of sewage sludge use in agroecosystems, we could provide the basis for a risk assessment analysis for pollinators.

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CRediT authorship contribution statement

Andrea Ferrari: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Michela Sturini**: Writing – review & editing, Methodology. **Beatrice De Felice**: Writing – review & editing, Methodology, Formal analysis. **Francesco Bonasoro**: Writing – review & editing, Supervision, Methodology. **Chiara Francesca Trisoglio**: Writing – review & editing, Methodology. **Marco Parolini**: Writing – review & editing, Methodology. **Roberto Ambrosini**: Writing – review & editing, Formal analysis. **Luca Canova**: Writing – review & editing. **Antonella Profumo**: Resources. **Federica Maraschi**: Writing – review & editing, Methodology. **Carlo Polidori**: Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Alessandra Costanzo**: Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135497.

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