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Assessing traffic-derived soil contamination: Insights from earthworm bioaccumulation and histomorphological responses

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ABSTRACT

This study aims to evaluate traffic-derived soil contamination using *Amynthas gracilis* (Kinberg, 1867) earthworms as bioindicators, with insights from bioaccumulation and histomorphological responses. Road pollution arises from multiple sources, including vehicle emissions, tire and brake wear, and stormwater runoff, with extensive environmental impacts. The negative impacts of heavy traffic pollution are well-recognized, but less information is available on that of moderate traffic road pollution. Besides serving as proxies for soil contamination, earthworms provide information on contaminant bioavailability and biological responses. Soil and *A. gracilis* earthworm samples were collected on either side of three roads in São Miguel Island, Azores, Portugal, with varying average annual daily traffic: Lagoa–Ponta Delgada (44.300 annual daily traffic), Lagoa–Ribeira Grande (9092 annual daily traffic), and Coroa da Mata–Santa Iria (<400 annual daily traffic), labelled MHT, MT, and LT sites. Soil and earthworm elemental concentrations were measured; soil elemental contamination and earthworm bioaccumulation factors were calculated. Earthworm histomorphometrical and histomorphological data were gathered to assess the impact of chronic exposure to moderate traffic road pollution on bioindicator tissues. Enrichment factors were elevated for Bi in MHT and MT sites, and Co in MT site; earthworms bioaccumulated As, Cd, Hg, Sb, Zn. Histological analyses revealed alterations in earthworm tissues indicative of physiological stress, namely thicker integument and peritrophic matrix (both of which reflect enhanced barrier function), thinner intestinal epithelium (reflecting impaired nutrient absorption and higher cellular turnover), and a higher proportion of mucous cells (reflecting enhanced barrier function) in the integument of earthworms from MHT and MT sites. The impact of moderate levels of traffic pollution is demonstrated by its contribution to soil contamination. The potential of using *A. gracilis* earthworms as sensitive bioindicators to moderate levels of road pollution is highlighted.

1. Introduction

Road pollution is a serious environmental and public health concern, with extensive impacts on air, soil, and water quality. Road pollution releases harmful substances like toxic gases, microplastics, and particulate matter (PM_{10} and $PM_{2.5}$) into the air. These pollutants circulate through nearby ecosystems, being associated with the decline of biodiversity, reduced agricultural productivity, and various health issues in humans, including respiratory and cardiovascular diseases, as well as developmental problems (Khan and Strand, 2018; Wang et al., 2018; Raymond et al., 2026). Several sources contribute to road pollution, ranging from vehicle emissions, tire and brake wear to road material degradation, road maintenance, and stormwater runoff (OECD, 2020). Road pollution results in the accumulation of elements in soil, many of which are harmful metals and metalloids (usually referred as potentially toxic elements, PTEs), such as arsenic (As), bismuth (Bi), cadmium (Cd),

Abbreviations: PM, particulate matter; NOx, nitrogen oxides; BEVs, battery electric vehicles; ICE, internal combustion engine; PTEs, potentially toxic elements; AADT, average annual daily traffic; MHT, moderately high traffic site; MT, moderate traffic site; LT, low traffic site; EF, enrichment factor; BAF, bioaccumulation factor; LC50, Lethal concentration 50; MeHg, methylmercury; EtHg, ethylmercury; ROS, reactive oxygen species; PAH, Polycyclic aromatic hydrocarbons.

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copper (Cu), chromium (Cr), mercury (Hg), nickel (Ni), lead (Pb), palladium (Pd), and antimony (Sb), which explains the negative effects of road traffic on soils and living organisms (Hjortenkrans et al., 2006; Johansson et al., 2009). Vehicles are the primary source of road pollution, responsible for over 70% of global transport-related emissions (Jandacka et al., 2017; IEA, 2025; Wang et al., 2026). It is known that the size and type of a vehicle dictate its overall emissions, with larger, heavier vehicles – like trucks, car carriers, concrete mixers, among others – generally exhibiting larger amounts of tailpipe emissions (exhaust fumes) and shedding of microplastics from tires and brakes. Characteristics like engine and fuel type also differently impact road pollution – e.g., diesel engines emit higher levels of nitrogen oxides (NOx) than gasoline engines; on the other hand, battery electric vehicles (BEVs) tend to be heavier than internal combustion engine (ICE) vehicles, resulting in more tire and brake wear in spite of the absence of tailpipe emissions (Opetnik et al., 2024; Wilson et al., 2025).

The investigation into the broader effects of road pollution has engaged researchers for an extended period, leading to the development of various strategies aimed at addressing this issue (Roorda-Knape et al., 1998; Perez et al., 2013; Alengebawy et al., 2021; Jaroń and Borucka, 2024). Earthworms are soil-dwelling terrestrial invertebrates of the Annelida phylum that are very sensitive to environmental stimuli – such as touch, vibrations, light, and chemical signals –, which helps them survive and quickly adapt to their habitats (Singh et al., 2019; Al-Maliki et al., 2021). Considering this, earthworms have been used as bioindicators for both natural and anthropogenic sources of pollution, including volcanic soils, agricultural land use, and heavy traffic road pollution (Fründ et al., 2011; Parelho et al., 2017; Bhowmik et al., 2022; Feng et al., 2026). However, comparatively less information is available on the overall impacts of moderate traffic road pollution, and little is known about the use of earthworm bioindicators in such situations.

The collection and interpretation of data from animals inhabiting contaminated areas is vital for the assessment of human and environmental health risks, as it highlights hazardous effects throughout ecosystems and food chains. Moreover, field studies using animal models yield essential ecotoxicological insights under conditions that are difficult to replicate in laboratorial settings (Bocedi et al., 2022; Bancel et al., 2024). Earthworms are recognized as fundamental and effective contributors to food production, with estimates suggesting they account for roughly 6.5% of global grain production (wheat, rice, maize, barley) and 2.3% of legume yields, totaling an output of 140 million metric tons of food annually (Fonte et al., 2023). Agricultural soil health could be impacted by nearby roads via multiple source traffic-related pollutants (Kibblewhite, 2018; Bacior et al., 2022). As ecosystem engineers, there has been an ever-growing interest in the use of earthworms as agents of bioremediation of soil PTE contamination – achieved through their burrowing, feeding, and digestive processes, which naturally accelerate the breakdown of pollutants and restore soil health (Xiao et al., 2022; Tagliabue et al., 2023; Stephen et al., 2026). Earthworms are terrestrial annelids with segmented bodies, possessing a mouth located anteriorly, followed by a reproductive structure called clitellum located anteriorly, and an anus located posteriorly. Although externally they look homogenous safe for the anteriorly-located clitellum and setae on the ventral region of each segment, their internal anatomy vastly differs antero-posteriorly (Walia and Kaur, 2024) (Fig. 1). The earthworm integument is composed of a protective, yet permeable outer layer called cuticle, and an inner layer called epidermis, where there are single-celled glands that produce mucus for lubrication and protection from external hazardous particles. Histologically, earthworm mucous cells are classified as metachromatic when they stain pink, and orthochromatic when they stain blue, exhibiting distinct distribution on the ventral and dorsal regions, and being especially abundant in the clitellum (Çinar et al., 2014). The internal protection of the earthworm from hazardous particles in ingested soil is conferred by a non-cellular, chitin microfibrils layer lining the intestinal epithelium called peritrophic matrix (Hodžić et al., 2025). Earthworms integrate exposure over time and reflect the bioavailable fraction of contaminants, thereby providing insight into the potential biological consequences of pollution. As such, it is possible to assess not only elemental accumulation in their tissue but also histomorphological responses, which can reveal organism-level effects of exposure. Alterations in epidermis thickness, cuticle thickness, intestinal epithelium thickness, and peritrophic matrix thickness represent sensitive effect biomarkers, given they reflect situations of stress and adaptation by the earthworms in response to environmental pressures – e.g., exposure to toxicants (Cunha et al., 2011; Briones and Álvarez-Otero, 2018; Yang et al., 2022; Briones et al., 2024).

This study aims to evaluate the impact of moderate traffic road pollution on soils using *Amynthas gracilis* (Kinberg, 1867) (Annelida, Oligochaeta) earthworms as bioindicators, considering data on soil and earthworm elemental concentrations, and histological techniques to assess earthworm tissue alterations. The objective was to answer the following questions: (i) Does moderate traffic road pollution lead to the accumulation of specific elements in the nearby soil? (ii) If so, does chronic exposure to moderate traffic road pollution lead to the accumulation of such elements in earthworms and consequent alterations in their tissues? The hypothesis was that chronic exposure to moderate levels of road pollution leads to soil elemental contamination and visible histological changes in earthworm tissue.

2. Methodology

2.1. Study sites

The Azores Archipelago comprises nine volcanic islands belonging to Portugal, located in the Atlantic Ocean somewhere between 36°45′–39°45′N and 24°45′–31°17′W (Fig. 1A and B). This study was conducted in São Miguel Island, namely in Lagoa–Ponta Delgada road (zone 1, located in the Picos volcanic complex), Lagoa–Ribeira Grande road (zone 2, located in the Picos volcanic complex), and Coroa da Mata–Santa Iria road (zone 3, located in the Fogo volcanic complex) (Fig. 1C). The fact that the soils of the study sites are all of volcanic origin and located in the same geological complex (Picos Fissural Volcanic System, except for zone 3) means they possess similar bedrock and pedological conditions (Pacheco et al., 2013), ensuring their distinction is based mostly on the occurrence and amount of traffic.

Measurements of the average annual daily traffic (AADT) (2023) per study site were obtained via automatic calculation by EUROSCUT, the concessionaire company that manages the roads included in the study. An AADT of 50.000 and above is generally considered heavy traffic (high-volume roadway), whereas an AADT of 400 and below is generally considerate low traffic (low-volume roadway) (FHWA, 2009; FHWA, 2014). In 2023, Lagoa–Ponta Delgada registered 44.300 AADT (21.779 light vehicles per direction, per day, plus 371 heavy vehicles per direction, per day), followed by Lagoa–Ribeira Grande road with 9092 AADT (4467 light vehicles per direction, per day, plus 79 heavy vehicles per direction, per day), and finally Coroa da Mata–Santa Iria road registering less than 400 AADT (less than 200 vehicles per direction, per day). Considering these measurements, within the context of this study, each site was respectively labelled “moderately high traffic” (MHT), “moderate traffic” (MT), and “low traffic” (LT).

In the past, the MHT site supported the operations of an alcohol factory in Lagoa and its economic connections with Ponta Delgada, serving as a logistical route. The road facilitated the transport of raw agricultural products from Ponta Delgada for sugarcane-based operations and from nearby fields to Lagoa for the production of alcohol. It still has an impact on regional economic planning and urban reconstruction today, making it a significant axis in the post-industrial landscape of São Miguel Island.

The MT site is located in the context of a larger industrial liability that has existed for decades, long before the construction of the road. Within a radius of 2.5 km, on either side of the road, there are several aggregate mining operations (Marques, Tecnovia, Vieiras quarries, etc.;

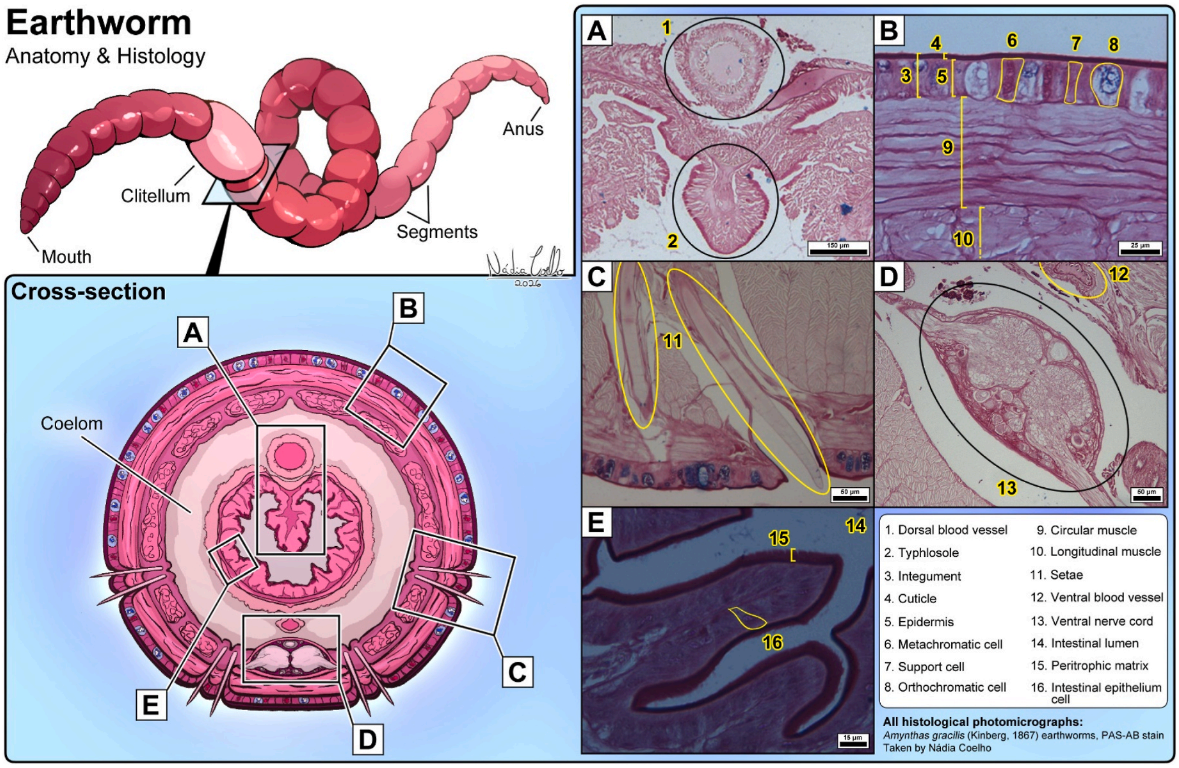

Fig. 1. Diagram of the anatomy and histology of earthworms. Earthworms possess a mouth in anterior position, followed by a thickened set of segments called clitellum (reproductive structure), and an anus in posterior position. This study focused on in immediate post-clitellum cross-sections, namely on: (B) the integument (composed of the non-cellular cuticle and the epidermis, with single-celled glands, called meta- and orthochromatic cells, that produce mucins for lubrication) and the circular and longitudinal muscle layers, and (E) the peritrophic matrix (a non-cellular chitinous layer that protects the fragile intestinal cells against food particle abrasion) and intestinal epithelium. *Amynthas gracilis* (Kinberg, 1867) histological sections are shown; PAS-AB stain (all photomicrographs); diagram and photomicrographs by Nádia M. P. Coelho.

removal of all the bagasse from Monte das Feiticeiras with thousands of trucks that transported these materials to a location about 3 km away, crossing the road), two industrial parks, and the entire civil construction and precast concrete industry of Marques, Tecnovia, and Vieiras. The materials for major works (marine ports, airport, roads) in São Miguel Island are produced and processed in this area. All this activity is carried out using heavy machinery – trucks, backhoe loaders, among others – with diesel engines.

About 20 years ago, the LT site (Regional Ecological Station) was the old regional road before the construction of the Ribeira Grande–Nordeste road. Today, this road is surrounded by woods and some pastures, mainly serving as access to the pastures, with some agricultural tractors and the occasional tourist passing through.

Soil and earthworm samples were collected on either side of each of the roads in the study in flat areas of the road – to avoid biases related to ascents and descents (variable vehicle strain impacting the amounts of tailpipe emissions and brake wear) –, at distances spanning approximately 1–5 m from the main road and 9–10 m from the main road.

2.2. Soil sampling and elemental analyses

Soil samples were collected using the same methodology commonly used for the analysis of agricultural soils. Two composite soil samples (with two sub-samples each – one for physicochemical properties, and another elemental concentration analysis) from the topsoil layer (0–20 cm) were collected with PVC core barrels from the NR and FR areas of the roads from the study sites.

Soil physicochemical properties (pH, electrical conductivity, organic matter content, and granulometry) were analyzed following nationally recommended procedures and the Portuguese official methods (LNEC, 1967a; LNEC, 1967b). Soil subsamples were thoroughly mixed and oven-dried at 60 °C until constant weight, prior to the determination of physicochemical properties. Three replicates were performed per sample. Table 1 displays information regarding the physicochemical properties of the soil from each of the study sites.

Soil subsamples for elemental concentration analysis were sieved to <2 mm and oven-dried (60 °C until constant weight) prior to analysis. Based on information about common road pollution contaminants (Hjortenkrans et al., 2006; Johansson et al., 2009), the concentration of the elements silver (Ag), aluminium (Al), arsenic (As), gold (Au), boron (B), barium (Ba), bismuth (Bi), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), gallium (Ga), mercury (Hg), potassium (K), lanthanum (La), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), sulfur (S), antimony (Sb), scandium (Sc), selenium (Se), strontium (Sr), tellurium (Te), thorium (Th), titanium (Ti), thallium (Tl), vanadium (V), tungsten (W), and zinc (Zn) in the soil of each of the study sites was measured using aqua regia mass spectrometry (AR-MS) following QOP Hydrogeo Rev. 6.6, carried out in a certified and accredited laboratory (ActLabs, Activation Laboratories Ltd., Canada; ISO 17025 and ISO 9001:2015). Quality control was assured by the analysis of duplicate samples, blanks, and reference materials (GXR-1, GXR-4, GXR-6, and USGS SAR-M).

2.3. Soil elemental enrichment factor (EF)

The enrichment factor (EF) of soils is a geochemistry index used to gauge the degree to which a given element is concentrated in a sample in relation to a baseline or background composition. It allows the isolation of non-lithogenic influences – such as anthropogenic sources – from the natural variability in soil elemental profiles – e.g., the influence of pH, organic matter, and grain size. This measurement determines the

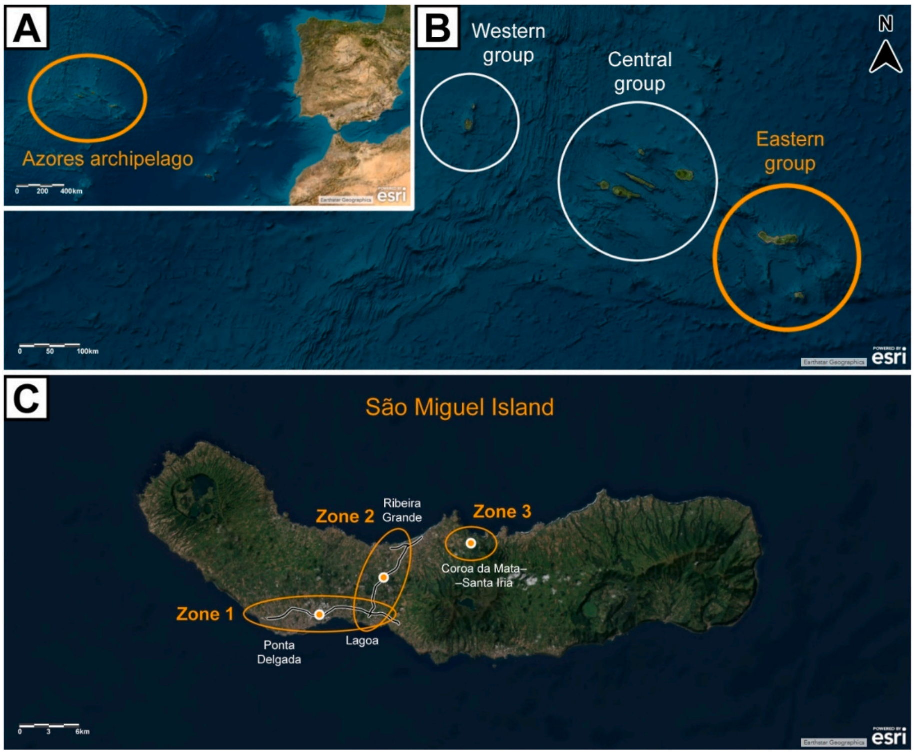

Fig. 2. Map of the Azores Archipelago showcasing: (A) its location in the Atlantic Ocean, (B) its geographical groups, and (C) São Miguel Island and study sites [zone 1 = Lagoa–Ponta Delgada road (37°44′52.05″N, 25°38′35.48″W), "moderately high traffic", MHT site; zone 2 = Lagoa–Ribeira Grande road (37°46′56.64″N, 25°34′18.56″W), "moderate traffic", MT site; zone 3 = Coroa da Mata–Santa Iria road (37°48′43.75″N, 25°28′13.84″W), "low traffic", LT site]. Basemap aerial view backgrounds obtained from ESRI ArcGIS online: "World Imagery" [basemap], "World Imagery Map". Attribution to ESRI and other data providers is present in the figure. URL: https://www.arcgis.com/home/item.html?id=10df2279f9684e4a9f6a7f08febac2a9 (last accessed on 10 December 2024).

Table 1
Physicochemical characteristics of soil sampled from "moderately high traffic" (MHT), "moderate traffic" (MT), and "low traffic" (LT) sites. Per parameter, between each site, values marked in bold indicate the highest mean, whereas underlined values indicate the lowest mean.

Site	Mean pH	Mean electrical conductivity (µS/cm)	Organic matter content (%)	Gravel (>2 mm)	Granulometry (%)	Clay-silt (<0.062 mm)
					Sand (<2 and >0.62 mm)	
MHT	**6.84 ± 0.28**	**94.33 ± 50.22**	2.67 ± 1.75	20.05 ± 4.18	**75.98 ± 5.29**	**3.99 ± 1.13**
MT	6.75 ± 0.13	38.33 ± 11.96	**2.81 ± 0.76**	**37.22 ± 0.87**	61.89 ± 1.90	0.88 ± 1.03
LT	6.77 ± 0.14	35.17 ± 10.89	1.73 ± 0.18	28.57 ± 4.82	70.80 ± 5.42	0.66 ± 0.59

contribution of human activity to how much the presence of an element has increased relative to its natural average abundance in the studied samples (Barbieri, 2016; Agyeman et al., 2023).

The EF is calculated as EF = (C_x/C_{ref})sample/(C_x/C_{ref})background, corresponding to the ratio between the concentrations of the element of interest (C_x) and those of the normalizer or reference element (C_{ref}), both for the studied sample and for nearby non-polluted, similarly-textured samples (Zoller et al., 1974). Calcium (Ca) is commonly used as reference element as it is one of the main components of Earth's crust (Loska et al., 2004; Abrahim and Parker, 2008; Parelho et al., 2014). Soil from LT site and the reference element Ca were considered for EF calculation, since, among the study areas, LT was the least prone to anthropogenic influence on the concentration of this element. It is accepted that an EF = 2 defines the limit above which the studied sample is significantly enriched by anthropogenic contamination (Hernandez et al., 2003). In this study, the scales were defined as: significant, when EF > 5; moderate, when EF 2–5, and not significant, when EF < 2.

2.4. Earthworm sampling and elemental analyses

Amynthas gracilis (Kinberg, 1867) (Annelida, Oligochaeta) were sampled in each of the study sites. The total number of earthworms collected per study site was as follows: 14 in MHT, 18 in MT, and 19 in LT, for histological analyses. An extra 4 earthworms per study site were collected for elemental concentration analyses.

There were no significant differences (H-test, $p > 0.05$) in the weight of earthworms between groups [weight (g): 1.69 ± 0.11 (MHT); 1.45 ± 0.097 (MT); 1.50 ± 0.16 (LT)].

The concentration of various elements – including As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Sn, V, and Zn – was assessed in earthworms from each study site to reflect both common road pollution contaminants and substances deemed harmful to health (Hjortenkrans et al., 2006; Johansson et al., 2009; Niede and Benbi, 2022; Jomova et al., 2025). Measurements were performed using inductively-coupled plasma mass spectrometry (ICP-MS) following QOP Hydrogeo Rev. 6.6, carried out in a certified and accredited laboratory (ActLabs, Activation Laboratories Ltd., Canada; ISO 17025 and ISO 9001:2015).

2.5. Earthworm histological slides preparation and analyses

For histological assessment, earthworms were fixed in 4% methanol-buffered formaldehyde and processed according to standard histological procedures. Transverse sections were obtained from the immediate post-clitellum region using a rotary microtome. This anatomical region was selected to ensure consistency among individuals and to minimize variation associated with the marked antero-posterior anatomical heterogeneity of earthworms.

Histological sections of 4 μm were obtained and mounted on glass slides, following the staining with Periodic Acid-Schiff–Alcian Blue (PAS-AB) for the identification and differentiation of mucous cell populations within the integument. After staining, slides were dehydrated, cleared, mounted with DPX mounting medium, and coverslipped.

Prepared slides were examined under a light microscope coupled to a digital camera. Only well-preserved transverse sections showing clear tissue organization and suitable orientation were selected for image acquisition and subsequent quantitative and semi-quantitative analyses. Histological evaluation focused on the integument and intestinal wall, namely the cuticle, epidermis, intestinal epithelium, peritrophic matrix, and epidermal mucous cells.

2.5.1. Histomorphometric analysis

Using a camera attached to an optic microscope and the Image Pro-Plus 5.0 software, photomicrographs were taken of the immediate post-clitellum region of each earthworm, with the selection of cuts separated by a minimum of 8 μm – to ensure the measured areas did not correspond to the exact same ones in the preceding cut, yet remained in that general region (Fig. 2).

A set of 4 photomicrographs per section (2 on the dorsal region and 2 on the ventral region of the earthworms) were taken with the 40X objective, totaling 8 photomicrographs for this purpose per earthworm. Data regarding epidermis thickness and cuticle thickness was collected from these photomicrographs. The epidermis thickness was measured in a straight, perpendicular line from the basal lamina towards the cuticle, whereas the cuticle was measured in a straight, perpendicular line from the apical surface of epidermal cells towards the exterior.

The intestinal epithelium thickness and peritrophic matrix thickness were measured in a set of 4 photomicrographs per section taken with the 40X objective over distinct areas of the intestine, totaling 8 photomicrographs for this purpose per earthworm. The intestinal epithelium thickness was measured in a straight, perpendicular line from the basal lamina towards the peritrophic matrix, whereas the peritrophic matrix was measured in a straight, perpendicular line from the apical surface of intestinal epithelial cells towards the intestinal lumen. Only areas where the delimitation of the basal lamina and peritrophic matrix were clear were selected for measurements. All morphometric data was finally registered as the average per earthworm, in μm. Examples of how morphometric data was measured can be found in Supplementary material, Fig. S1.

2.5.2. Histomorphologic analysis

Another set of 4 photomicrographs per section (2 on the dorsal region and 2 on the ventral region of the earthworms) were taken with the 20X objective, totaling 8 photomicrographs for this purpose per earthworm. The epidermis spatial proportion occupied by mucous cells (meta- and orthochromatic cells), and support cells over a 320 μm transept (which was the maximum area captured by the camera attached to the optic microscope) was analyzed semi-quantitatively through visual qualitative scales, per photomicrograph, per earthworm. The higher the assigned value on the scales, the greater the spatial proportion occupied by the studied cells. For each cell type, histomorphometric analyses were performed considering the scales "1" = 1–50%; "2" = 51–100%. Examples of photomicrographs assigned with extreme values can be found in Supplementary material, Fig. S2.

2.6. Earthworm elemental bioaccumulation factor (BAF)

The bioaccumulation factor (BAF) is an index used to gauge the degree to which a given element is concentrated in the tissue of an organism in relation to its concentration in the surrounding environment. This measurement includes uptake from all environmental sources, such as water, food, and sediment (Zhang et al., 2023).

The BAF is calculated as $BAF = C_{organism}/C_{environment}$, where $C_{organism}$ is the concentration of the element of interest in the organism, and $C_{environment}$ is the concentration of the element of interest in the surrounding medium. A BAF >1 indicates that the element accumulates in the studied organism at a higher concentration than its ambient environment, whereas a BAF < 1 indicates that there is no bioaccumulation (Zhang et al., 2023). A BAF > 1 in earthworms may represent a risk of secondary poisoning to predators, depending on whether the element is toxic (Yadav et al., 2023).

2.7. Statistical analysis

Statistical analysis was performed in the IBM SPSS Statistics® v. 31.0.1.0 (49) software. Statistical significance was determined at $p < 0.05$.

Data normality was analyzed via Q-Q plots. For soil and earthworm elemental concentrations data, along with morphometric data, differences of the means between groups MHT, MT, and LT were analyzed with One-way analysis of variance (ANOVA) tests [followed by post hoc Tukey's honest significance (HSD) tests, to assess which groups differed from each other] for normal data, and non-parametric independent samples Kruskal-Wallis tests (followed by pairwise comparisons, to assess which groups differed from each other) for non-normal data.

For morphological data, non-parametric Kruskal-Wallis tests were used to assess and compare the distributions of values assigned in observations among the groups MHT, MT, and LT. The relative frequency of each category was calculated, per group, to better illustrate differences between their distributions per group.

Data associations between the concentration of elements in soil and road traffic, and elements in soil and in earthworms were determined via Pearson correlations, for normal data, and Spearman correlations, for non-normal data.

3. Results

3.1. Soil elemental analyses

In general, the soil from the MHT site presented the highest concentrations of the elements As, Ba, Bi, Cr, Pb, and Sb; the soil from the MT site presented the highest concentrations of the elements Co, Mn, Ni, Sc, Sr, and V, and the soil from the LT site presented the lowest concentrations of most selected elements. Differences in Cd, Cu, Hg, and Zn concentrations between study sites were not significant (Fig. 3). Further details such as variation and *p* values can be found in Supplementary Material Tables S1 and S2.

Results show that (i) the soil in MHT site presented elevated and the highest EF for Bi (26.30, significant), and (ii) the soil in MT site presented elevated EF for Bi (2.79, moderate), and elevated and the highest EF for Co (2.18, moderate) (Table 2).

3.2. Earthworm elemental analyses

In general, the earthworms from the MHT site presented the highest concentrations of the elements Cu, Pb, Sb, Sn, and Zn; earthworms from the MT site presented the highest concentrations of the elements As, Cd, Co, Cr, Hg, Mn, Ni, and V, and earthworms from the LT site presented the lowest concentrations of most selected elements. Differences in Mn concentrations in earthworms between study sites were not significant (Fig. 4). Further details such as variation and *p* values can be found in Supplementary Material Table S3.

3.3. Earthworm histological analyses

3.3.1. Histomorphometric analysis

Results show that (i) epidermis thickness was greater in earthworms

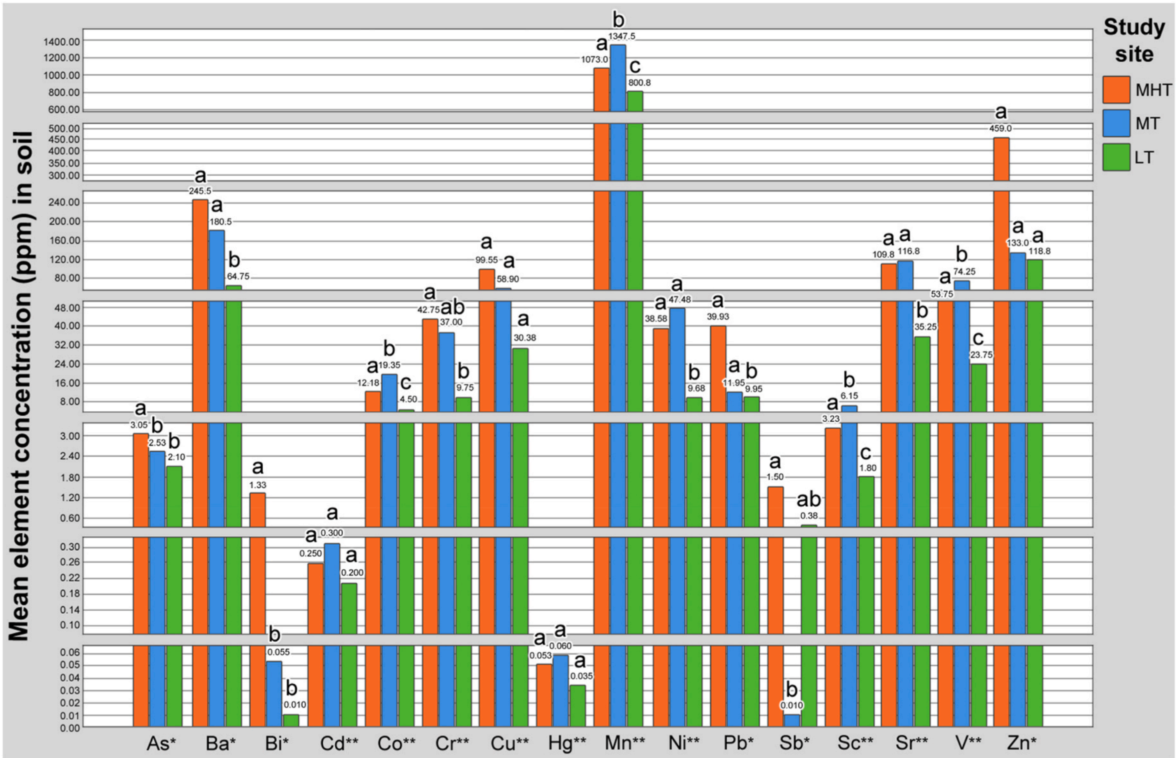

Fig. 3. Clustered bar charts with axis cuts of the mean concentrations of the studied elements in soil samples from "moderately high traffic" (MHT), "moderate traffic" (MT), and "low traffic" (LT) sites. **H*-test; ***F*-test; different letters (a, b, and c) indicate significant differences ($p < 0.05$).

Table 2
Enrichment factors (EF) of the studied elements in soil samples from "moderately high traffic" (MHT) and "moderate traffic" (MT) sites in relation to "low traffic" (LT) site, considering Ca as the reference element. Cells were colored different shades of gray to classify the EF as: significant (EF > 5, dark gray), moderate (EF 2–5, light gray), not significant (EF < 2, no shading) (scales adjusted to fit the low traffic levels of the study sites). Per element, between each site, values marked in bold indicate the highest EF, whereas underlined values indicate the lowest EF.

Element	As	Ba	Bi	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	Sb	Sc	Sr	V	Zn
Site																
MHT	<u>0.29</u>	<u>0.75</u>	**26.30**	<u>0.25</u>	<u>0.54</u>	<u>0.87</u>	<u>0.65</u>	<u>0.30</u>	<u>0.27</u>	<u>0.79</u>	**0.80**	**0.79**	<u>0.36</u>	<u>0.62</u>	<u>0.45</u>	**0.77**
MT	**0.61**	**1.41**	<u>2.79</u>	**0.76**	**2.18**	**1.92**	**0.98**	**0.87**	**0.85**	**2.49**	<u>0.61</u>	<u>0.01</u>	**1.73**	**1.68**	**1.58**	<u>0.57</u>

from MHT and MT sites compared to LT site, (ii) cuticle thickness was greater in MHT site, followed by MT site, compared to LT site, (iii) intestinal epithelium thickness was lower in MHT site compared to MT and LT sites, and (iv) peritrophic matrix thickness was greater in MHT and MT sites compared to LT site (Fig. 5). Further details such as variation and *p* values can be found in Supplementary Material Table S4.

3.3.2. Histomorphologic analysis

Results show that (i) a greater epidermis spatial proportion was occupied by mucous cells in earthworms from MT site, followed by MHT site, compared to LT site, and (ii) a lower epidermis spatial proportion was occupied by support cells in earthworms from MT site, followed by MHT site, compared to LT site (Fig. 6). Further details such as *p* values can be found in Supplementary Material Table S5.

3.4. Earthworm elemental bioaccumulation factor (BAF)

The elements As, Cd, Hg, Sb, and Zn bioaccumulated in *A. gracilis* earthworms (BAF > 1) (Table 3). In particular, (i) earthworms from MHT site bioaccumulated As, Cd, and Hg, (ii) earthworms from MT site bioaccumulated As, Cd, Hg, Sb, and Zn, having presented the highest BAF for these elements between groups, and (iii) earthworms from LT site bioaccumulated Cd, Hg, and Zn, having presented the lowest BAF for the first two elements between groups.

3.4.1. Data associations

Road traffic was significantly positively correlated with the concentrations of the following elements in soil: (i) As [$rs(10) = 0.671$, $p = 0.017$], (ii) Ba [$rs(10) = 0.592$, $p = 0.042$], (iii) Bi [$rs(10) = 0.859$, $p < 0.001$], (iv) Cr [$rs(10) = 0.710$, $p = 0.010$], (v) Cu [$rs(10) = 0.591$, $p = 0.043$], (vi) Ni [$rs(10) = 0.591$, $p = 0.043$], (vii) Pb [$rs(10) = 0.770$, $p = 0.003$], and (viii) Sr [$rs(10) = 0.680$, $p = 0.015$].

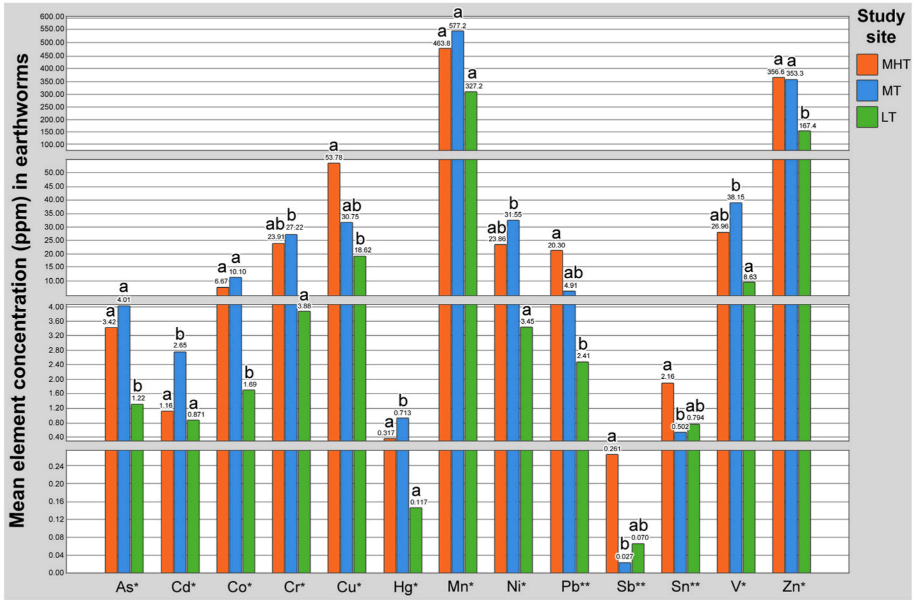

Fig. 4. Clustered bar charts with axis cuts of the mean concentrations of the studied elements in *Amynthas gracilis* (Kinberg, 1867) earthworms sourced from "moderately high traffic" (MHT), "moderate traffic" (MT), and "low traffic" (LT) sites. **F*-test; ***H*-test; different letters (a, b, and c) indicate significant differences ($p < 0.05$).

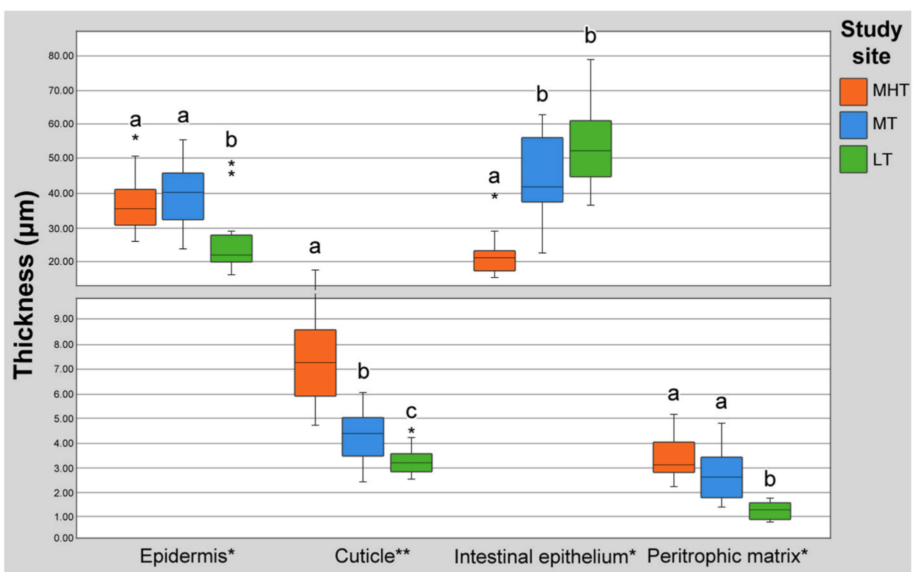

Fig. 5. Box plot charts with an axis cut of the epidermis, cuticle, intestinal epithelium, and peritrophic matrix thickness of the *Amynthas gracilis* (Kinberg, 1867) earthworms sourced from "moderately high traffic" (MHT), "moderate traffic" (MT), and "low traffic" (LT) sites. **H*-test; ***F*-test; different letters (a, b, and c) indicate significant differences between sites ($p < 0.05$). The line in the middle of each box plot represents the median of the respective group; an asterisk within the graphs indicates a group outlier.

Concentrations in soil were significantly positively correlated with the concentrations of the following elements in earthworms: (i) As [$rs(10) = 0.624$, $p = 0.030$], (ii) Co [$r(10) = 0.890$, $p < 0.001$], (iii) Cr [$r(10) = 0.858$, $p < 0.001$], (iv) Cu [$r(10) = 0.954$, $p < 0.001$], (v) Hg [$r(10) = 0.679$, $p = 0.015$], (vi) Ni [$r(10) = 0.818$, $p = 0.001$], (vii) V [$r(10) = 0.811$, $p = 0.001$], (viii) Pb [$rs(10) = 0.718$, $p = 0.009$], and (ix) Sb [$rs(10) = 0.938$, $p < 0.001$].

4. Discussion

Earthworms have been extensively used as bioindicators in a plethora of contexts, from soil pollution assessment to agricultural management (Fründ et al., 2011; Parelho et al., 2014, 2017; Wang et al., 2025; Feng et al., 2026). Some studies have also used earthworms as bioindicators of road pollution, particularly in high traffic roads and highways (Mariño et al., 1992; Bamgbose et al., 2000; Maher, 2013; Oketola and Olaoye, 2015; Chatelain et al., 2024). However, to our best knowledge, this is the first study using *A. gracilis* earthworms as bioindicators of moderate traffic road pollution. The followed methodology revealed, even in roads below the limit of what is considered "heavy traffic" (i.e., roads with an AADT of 50.000 and above), significant histological alterations and elemental bioaccumulations in earthworms chronically exposed to soil contaminated with moderate levels of road pollution-derived elements.

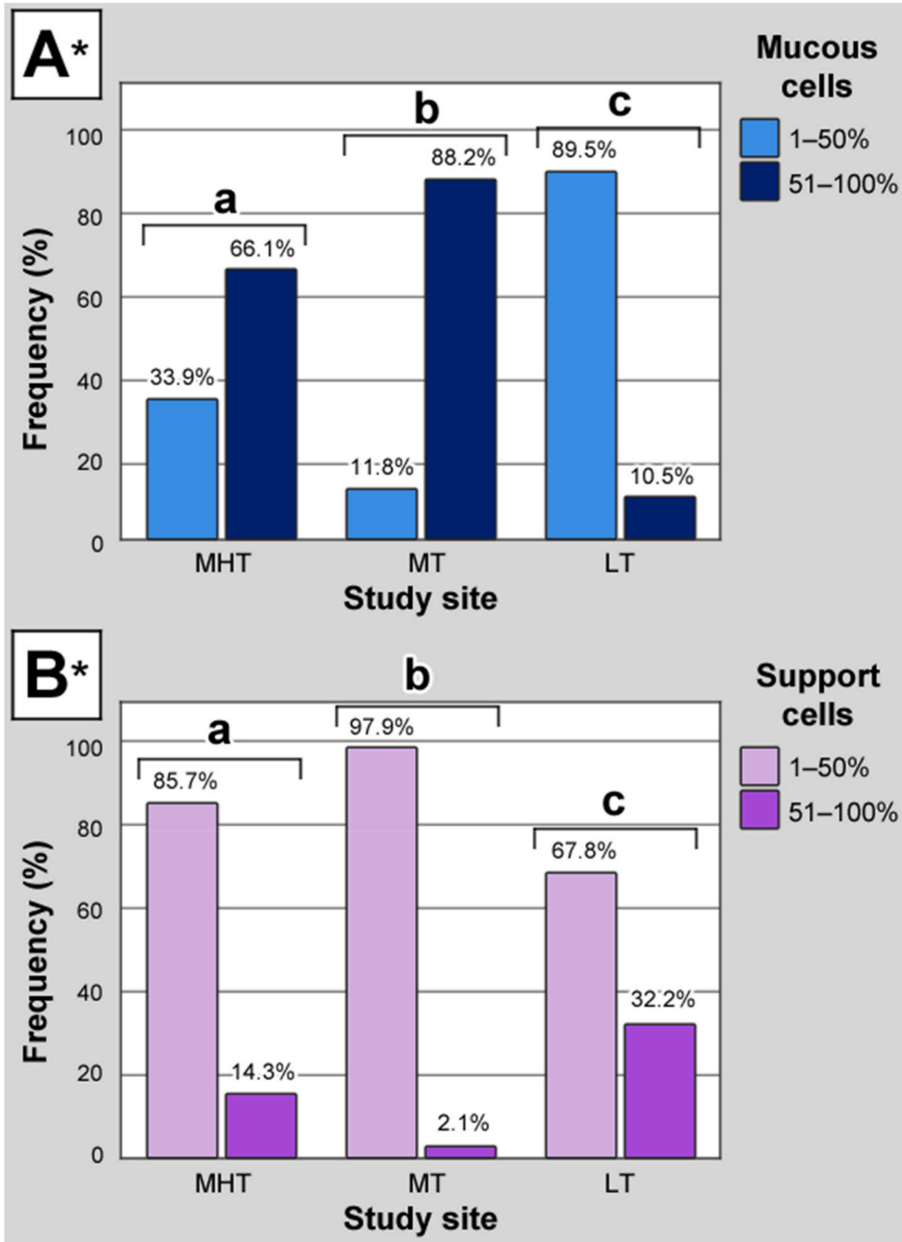

Fig. 6. Clustered bar charts of the epidermis spatial proportion occupied by (A) mucous cells and (B) support cells of the *Amynthas gracilis* (Kinberg, 1867) earthworms sourced from "moderately high traffic" (MHT), "moderate traffic" (MT), and "low traffic" (LT) sites. **H*-test; different letters (a, b, and c) indicate significant differences between sites ($p < 0.05$); relative frequencies per category per group are shown.

Volcanic soils tend to be naturally enriched in elements such as Al, As, Br, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, K, Li, Mg, Hg, Na, Ni, Pb, S, Se, Sn, Sr, Ti, Tl, V, and Z (Memoli et al., 2017; Baillie et al., 2018; Ma et al., 2019). However, the significantly greater concentrations of the elements As, Ba, Bi, Co, Cr, Mn, Ni, Pb, Sc, Sr, and V in the MHT and MT sites, including several significant strong positive correlations between soil elements and amount of traffic, showcase the impact of moderate levels of road pollution on the nearby soil. It is noteworthy that the 3 data points for road traffic correspond to a single value per site representing the AADT; for correlation purposes, it might have been better to have this data divided into more values (e.g., average monthly daily traffic for the year) for statistical robustness. However, significant differences were found between all 3 road types for most metals in both soils and earthworms, with few exceptions. Considering this, correlations are merely illustrative. The elevated EFs further show anthropogenic contribution to soil Bi and Co contamination. The Picos volcanic complex is the most densely populated and used for agricultural/industrial purposes, making it difficult to find truly pristine areas for the calculation of EFs. Although it is common practice to use pristine soils for the calculation of EFs in environmental impact assessments, using pristine soils from other geological complexes as a reference would not be correct due to differences in the geochemical composition of the soils between complexes. For this reason, the LT site was used as a reference because it only differs in traffic intensity. In any case, if it were possible to use a pristine soil as a reference, the EFs obtained would potentially be even more pronounced at the MHT and MT sites; however, it is important to emphasize that traffic is very low at LT, which allowed the impact assessment of road traffic on the input of the elements under study in the soils. The elements Bi and Co are less frequently investigated than many other PTEs, and their environmental fate and biological effects remain comparatively poorly understood. The element Bi is considered generally safe in its elemental form for humans.

Table 3
Elemental bioaccumulation factors (BAF) of the studied elements in *Amynthas gracilis* (Kinberg, 1867) earthworms sourced from "moderately high traffic" (HT), "moderate traffic" (MT), and "low traffic" (LT) sites. Cells were colored gray when BAF > 1 to indicate elements that bioaccumulated in earthworms. Per element, between each site, values marked in bold indicate the highest BAF, whereas underlined values indicate the lowest BAF.

Element Site	As	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	Sb	V	Zn
MHT	1,12	4,66	**0,55**	0,56	0,54	6,03	**0,43**	0,62	**0,51**	0,17	0,50	0,78
MT	**1,59**	**8,85**	0,52	**0,74**	0,52	**11,89**	**0,43**	**0,66**	0,41	**2,71**	**0,51**	**2,66**
LT	0,58	4,36	0,38	0,40	**0,61**	3,34	0,41	0,36	0,24	0,19	0,36	1,41

Nevertheless, high environmental Bi concentrations have been associated with ecosystem repercussions such as alterations in soil microbiome composition (abundance and diversity of bacterial populations), negative effects on plant development, and bioaccumulation in *Eisenia andrei* (Bouché, 1972) earthworms (up to 21 ppm), including negative impacts on earthworm survival (at a lethal concentration 50, LC50, of 416 ppm) and reproduction (at an LC50 for juveniles of 123 ppm) (Omouri et al., 2017, 2018; Kolesnikov et al., 2022; Zacchini, 2024). Despite the very high EF for this element in MHT soil and high EF in MT soil, its concentrations did not surpass 1.5 ppm. Because Bi concentration and bioaccumulation in earthworms was not assessed, it cannot be said with certainty whether it contributed to histomorphological alterations, but its low concentrations in soil make this unlikely. On the other hand, Co is considered an essential element as part of the cobalamin or B_{12} vitamin, though it can be toxic in its free forms. Ecosystem repercussions of high Co concentrations include alterations of soil chemistry, the disruption of microbial nutrient cycling and nitrogen fixation, oxidative stress in plants, and trophic-level bioaccumulation (Srivastava et al., 2022; Khan et al., 2023). For earthworms like *Eisenia fetida* (Savigny in Cuvier, 1826) and *Lumbricus rubellus* (Hoffmeister, 1843), high Co levels have been reported to weaken ecosystem engineering capacity and are implicated in growth and reproductive problems (Coutris et al., 2011; Vittori Antisari et al., 2016). In this study, Co only showed an EF > 1 in MT soil; concentrations did not surpass 20 ppm in soil and 10.5 ppm in earthworms, and there was no significant bioaccumulation in earthworm tissue (BAF < 1). Nonetheless, considering that chronic exposure to low levels of PTES can cause physiological stress buildup and histopathological changes over time, this element may have contributed to alterations in earthworm histomorphology, although to a lesser extent (Coutris et al., 2011; Tang et al., 2017). For humans, high environmental Co levels have been linked to thyroid disruption and consequent development of thyroid disorders (with emphasis on hypothyroidism), given the capacity of this element to interfere with iodine uptake and thyroid hormone synthesis (Yu, 2017; Chai et al., 2025). The review of Coelho et al. (2025) highlights that "cobalt, though essential in small amounts, can inhibit iodine uptake and damage thyroid cells when present in excess". Further evidence indicates that chronic or high-level exposure to Co may lead to other adverse health effects like respiratory and cardiovascular disorders, neurological impairments, skin allergies, and potential carcinogenic outcomes (Leyssens et al., 2017; Motlagh et al., 2026). With all findings considered, it was unexpected that the MT site with comparatively less traffic would have exhibited similar elemental concentrations to the MHT site (9092 AADT vs. 44.300 AADT), in some cases significantly greater, as seen with the elements Co, Mn, Sc, and V. The similarities observed can plausibly be explained by the broader historical and current industrial background of the MT site in comparison to the MHT site. In addition to road traffic, various pollution sources have most likely contributed to this situation, including nearby mining operations, industrial parks, and ongoing civil construction activities that frequently involve heavy machinery passing through the area. The observed accumulation of pollutants has thus developed over time as a result of the combination between these factors. These findings underscore the importance of detailing study site industrial backgrounds in ecotoxicological studies.

It is known that soil physicochemical characteristics influence element mobility and bioavailability (Rocco et al., 2018). The soil in the MHT site presented the highest pH, electrical conductivity, sand, and clay-silt content, and lowest gravel content. In turn, the soil in the MT site presented the highest organic matter and gravel content, and the lowest pH and sand content. In comparison, the soil in the LT site presented the lowest electrical conductivity, organic matter, and clay-silt content. An alkaline or high soil pH (usually above 7.0) is associated with a reduction of mobility and bioavailability of cationic PTEs (such as Cd, Pb, and Zn) but an increase in that of anionic PTEs (e.g., As, Mo, and W), whereas an acidic or low pH (typically below 5.5) tends to significantly increase the mobility and bioavailability of most PTEs (Reijonen et al., 2016; de Figueiredo et al., 2025). The pH varied between approximately 6.75 and 6.85, meaning this parameter most likely did not influence elemental bioavailability between sites as much as other parameters. On the other hand, high electrical conductivity is associated with increased PTE bioavailability (particularly for Cd, Cu, Ni, and Zn), with ions like calcium and chlorides competing for binding sites on soil particles and pushing PTEs out of the soil matrix and into the soil pore water, and vice-versa (Rocco et al., 2018). Although with a large standard error, mean electrical conductivity was much greater in the MHT site (approximately 94 μS/cm) than in the MT and LT sites (approximately 38 μS/cm and 35 μS/cm, respectively), making this parameter a likely contributor to elemental bioavailability differences between sites. Soil organic matter content, including humic substances, also influences elemental bioavailability: while a high organic content binds and immobilizes PTES, a low organic content results in reduced PTE absorption and absence of a natural buffer against rapid chemical or pH changes, consequently leading to a greater bioavailability of PTEs (Ondrasek and Rengel, 2012). Organic matter content varied between approximately 1.7% and 2.7%, which makes it unlikely to have contributed to elemental bioavailability differences between sites. Finally, a predominantly coarse granulometry – when soil is mostly composed of gravel and sand – is translated into fewer reactive surfaces, with PTEs usually being bound in primary, less-weathered minerals, therefore posing a much lower risk of biological uptake; by contrast, a predominantly fine granulometry – when soil is mostly composed of clay-silt minerals – means soil possesses high surface reactivity, tightly binding higher concentrations and more labile (easily released) forms of PTEs (Li et al., 2021; Vasiluk et al., 2023). Soil from the MHT site possessed a higher sand and clay-silt content than the MT and LT sites, which may have translated into a greater elemental bioavailability; on the other hand, the soil from MT site possessed the highest gravel, lowest sand, and nearly lowest clay-silt content, potentially translated into the opposite. The distinct physicochemical characteristics exhibited by each of the study sites help explain elemental bioavailability to worms. Considering that clay-heavy and dry sandy soils are not favorable to earthworm development and spreading, study soil characteristics drive changes to optimize earthworm survival, digestion, and nutrient turnover. Moreover, it has been demonstrated that stressful or suboptimal soil environments can induce histological alterations in earthworms indicative of physiological strain (FiBL, 2022; Kooch et al., 2024; Zi et al., 2025). Although earthworms do reflect the bioavailable fraction of contaminants, it would have been pertinent to measure soil elemental fractions to further support the BAFs and histomorphological changes found in this study. Future studies should consider measuring different elemental species/fractions to support inferences related to bioavailability and consequent biological effects.

In consistency with available literature, earthworms clearly reflected the elemental concentrations in road traffic-polluted soil, showing elevated concentrations in MHT and MT in comparison to the low contents in LT (Mariño et al., 1992; Bamgbose et al., 2000; Oketola and Olaoye, 2015). Interestingly, the concentration of certain elements seemed to be heightened and significantly different in earthworms between groups, with emphasis on As, Cd, and Hg, even if their concentration in soil had not differed between groups nor had shown high EF (namely Cd and Hg). In specific forms or aggregates, these PTEs have been documented to demonstrate significant bioavailability even at low concentrations, raising potential concerns. Examples include inorganic arsenic species like As(III), which are more toxic and mobile than As(V); cadmium that is loosely attached to soil particle surfaces or linked with carbonates, exhibiting high mobility and bioavailability to plants; and methylmercury (MeHg), similar to ethylmercury (EtHg), which exhibit greater bioavailability than inorganic or mineral mercury compounds (Różański et al., 2016; Al-Makishah et al., 2020; Chen et al., 2025). Other elements more concentrated in earthworms from MHT and or MT sites included Co, Cr, Cu, Ni, Pb, Sb, V, and Zn. Consistently, earthworm BAFs were elevated especially for the elements As, Cd, and Hg, followed by Sb, and Zn. Mirroring the findings of this study for soil, these results, as well as several significant strong positive correlations between elements in soil and in earthworms, showcase the impact of low levels of road pollution on nearby earthworms and how these bioindicators clearly reflect the elemental concentrations of the soil they inhabit. Results are in accordance with the concentrations and BAFs observed in earthworms for elements like As, Cd, and Hg in the study of Šestinová et al. (2019) conducted in Košice, Slovakia. However, these findings contrast with those of Shen et al. (2026), which indicated that earthworms in soils from Punjab, Pakistan, accumulated metals at low to moderate levels, showing a weak correlation with soil concentrations. It is important to note that the earthworm species used as a bioindicator was different between studies, with Šestinová et al. using *Dendrobaena veneta* (Rosa, 1886), Shen et al. not specifying the species, and ours using *A. gracilis*. Research by Huang et al. (2021) suggests that distinct species uptake elements from the soil differently, highlighting the importance of using the same earthworm species (or at least closely related) for reliable result comparisons. Together, our results refer to the fact that, even if certain elemental concentrations do not significantly differ between soils, those elements may be differently (more or less) bioavailable to worms, with factors playing a part in this ranging from soil physicochemical characteristics to, likely, elemental forms present in soil (which were not distinguished in this study) (Bradham et al., 2018; Natasha et al., 2020; Petruzzelli et al., 2023; Galić et al., 2025). In this sense, it is possible that elements in MT site could have been even more bioavailable to earthworms – and therefore were yet more concentrated in them – if the soil was composed of a greater fraction of clay-silt.

The integument serves a crucial role in protecting earthworms from physical, mechanical, and chemical stress. Together, the non-cellular cuticle and epidermis specialized in producing mucus limit their environmental exposure to hazardous substances, prevent desiccation, deter pathogens, minimize friction, and shield them from the harsh soil environment. In spite of such protection, the integument is semi-permeable primarily because gas exchange occurs at the integumental level – a process called cutaneous respiration, where environmental oxygen dissolves in mucus and diffuses into capillaries and blood vessels, while carbon dioxide is released to the environment –, which inevitably allows the passage of sometimes undesirable particles into earthworms (Świątek and Bednarska, 2019). Damages to the integumentary system can lead to serious injury and even compromise earthworm survival (Aouaichia et al., 2024). The simultaneous significant thickening of the cuticle and epidermis observed in earthworms from MHT and MT sites seem to reflect a physiological attempt to increase the physical barrier between the earthworms and the surrounding soil, making it difficult for PTEs in the harsher soil environment to traverse the integument and make their way inside them. It has been reported that the exposure of earthworms to elements like Cd, Cu, and Ni directly disrupts the cuticle and erodes the epidermis, with other histopathological alterations and malformations including body wall blistering, abnormal clitellum swelling, along with circular and longitudinal muscular layers disintegration (Hirano and Tamae, 2010; Lourenço et al., 2011; Homa et al., 2016; Ijomah et al., 2020; Duo et al., 2021). Many of these effects can be attributed to oxidative stress, with extensive evidence suggesting that the accumulation of PTEs tends to overwhelm antioxidant enzymes, accelerating lipid peroxidation via the creation of reactive oxygen species (ROS) and leading to DNA damage – processes which go beyond the integument of earthworms (Ercal et al., 2001; He et al., 2021; Mansoor et al., 2023).

The role of the integument on earthworm external protection is mirrored by the intestinal epithelium peritrophic matrix on internal protection. Being functionally analogous to the intestinal mucosal layer of invertebrates, the peritrophic matrix both mechanically and chemically shields the delicate digestive epithelial cells from food and any other ingested particle abrasion. Inevitably, because the intestinal epithelium exhibits an absorptive function – of nutrients and minerals needed for survival, tissue maintenance and reproductive needs –, it is semi-permeable, meaning undesirable particles will still make their way into the earthworm to variable extent (Tellam et al., 1999; Hirano and Tamae, 2010). Earthworm intestinal lining damage has serious implications, ranging from immediately impaired nutrient absorption and osmotic balance, inhibited growth and weight loss to lethality (Yang et al., 2020; Aouaichia et al., 2024). In this study, the peritrophic matrix was significantly thickened while the intestinal epithelium was significantly thinned in earthworms from the MHT and MT sites (the latter especially in MHT worms), which also reflects the physicochemical harshness of the ingested soil and the according physiological response to cope with environmental circumstances. On one hand, a thick peritrophic matrix makes for a larger barrier between the ingested soil within the intestinal lumen and earthworm tissues; on the other, a thin intestinal epithelium may reflect situations of direct injury to the intestinal lining by the particles constantly being absorbed through it, resulting in a higher cellular turnover – cells have less time to develop the normal columnar height of healthy, unstressed intestinal epithelium cells due to constant damage, going through apoptosis (cell death) and other tissue renewal processes earlier. A high cellular turnover is an evolutionary mechanism shared by organisms of vast taxonomic origin where cell division and removal are accelerated to eliminate unrepairable cells, prevent mutations, and ensure that tissues are rapidly repaired – a mechanism that plausibly explains the results found in terms of the thin epithelium of MHT earthworms (Wodarz, 2007; Fulda et al., 2010). In accordance, several studies refer to the intestinal epithelium disruption of earthworms exposed to PTEs, including other effects such as digestion and metabolic alterations, gut microbiome disturbance, and oxidative stress (Hirano and Tamae, 2010; Šrut et al., 2019; Yang et al., 2020).

Within the epidermis, meta- and orthochromatic cells produce different types of secretions, therefore reacting differently to histological stains. Metachromatic cells (stained pink in PAS-AB stain) produce an acidic, slightly sulfated, carboxylated mucus of low viscosity, primarily destined for heavy lubrication, ensuring adequate burrowing and moisture retention; conversely, orthochromatic cells (stained blue in PAS-AB stain) produce a neutral, dense mucus that grants structural protection and binding (Licata et al., 2002; Çinar et al., 2014). Earthworms from the MHT and MT sites exhibited a significantly higher spatial proportion of epidermis occupied by these mucous cells in response to the harsher soil environment compared to the LT site, potentially as a way to retain some PTEs and prevent their dermal absorption. It is important to note that neither of these glandular cells has a direct role in chelating metals or detoxification. These functions are more closely associated with metallothionein expression – cysteine-rich proteins that sequester and bind metals in almost all living organisms for metal regulation and detoxification. Alterations to both metallothionein expression and the innate immune system of earthworms, which includes chloragocytes (or chloragogen cells, akin to the vertebrate liver) and coelomocytes (immune-competent cells), have been used as biomarkers for earthworm exposure to PTEs (Gastaldi et al., 2007; Itziou, 2025).

This study addresses some knowledge gaps identified by Feng et al. (2026) by assessing the relationship between soil and earthworm elemental profiles, as well as histological effect biomarkers, directly in the context and complexity of a real environment. This is particularly relevant given the extensive evidence from laboratory studies and the scarcity of data from real-world ecological studies. Measurements of *A. gracilis* earthworm epidermis, cuticle, intestinal epithelium and peritrophic matrix thickness, as performed in this study, proved to be quick and easy ways to ascertain environmental pressures from the accumulation of elements in the surrounding soil and in these bioindicators. A plethora of other sub-lethal biomarkers could also be implemented depending on the objectives, spanning from the observation of behavioral changes (avoidance, coiling) to the assessment of elemental accumulations per tissue type (muscle layers, reproductive tissue, coelomocytes – free-floating immune system cells in the coelom –, chloragogenous tissues – composed of star-shaped cells surrounding the intestine and dorsal blood vessel, with metabolic and excretory functions analogous to the vertebrate liver –, etc.; the use of these biomarkers allows the distinction of dermal and intestinal uptake), oxidative stress, cellular and histological damage (lysosomal membrane stability), expression of metallothioneins and genotoxicity (comet assay) (Gastaldi et al., 2007; Itziou, 2025). The lack of data on the impact and biomonitoring of areas subject to moderate traffic road pollution makes the development of further studies within the scope crucial. Given results showed that even moderate traffic road pollution leads to elemental accumulation in soil and visible histological alterations in earthworms due to the bioaccumulation of PTEs like As, Cd, Hg, with potential agricultural and human health impacts (in first instance for those who reside near roads), emphasis is put on the need to monitor soil health near roads, to which earthworms can serve as sensitive, time- and cost-efficient bioindicators. Besides the intensity of traffic pollution, potential contributors to the observed effects are polycyclic aromatic hydrocarbons (PAH), microplastics, and elevated vibration and noise, which are typical of the road traffic exposome and have been demonstrated to alter earthworm behavior and physiological state (Nikolaeva et al., 2019; Velilla et al., 2021; Zhang et al., 2022). If possible, the nature of the pollution (e.g., tire wear vs. exhaust emissions, runoff vs. atmospheric deposition) should also be considered. Together, these findings underline the importance of a holistic approach for road traffic-related health risk assessments.

5. Conclusion

Moderate traffic road pollution was associated with a higher concentration of elements in soil – including PTEs like As, Cd, and Hg – and its enrichment in specific elements – particularly Bi and Co –, with such accumulation generally being greater and closely tied between the MHT site (road with 44.300 AADT) and MT site (road with 9092 AADT) in comparison to the LT site (road with <400 AADT). These findings also point to the importance of noting the industrial backgrounds of road surroundings to discern whether element accumulations are due to the history of their industrial context or current levels of road traffic, or a mix of both.

The initial hypothesis was supported in that clear histological alterations were found in *A. gracilis* earthworms in response to chronic exposure to slightly elevated soil elemental concentrations from moderate traffic road pollution, translated into a significant enlargement of epidermis, cuticle, and peritrophic matrix thickness, a significant reduction in intestinal epithelium thickness, and a significantly higher proportion of epidermis occupied by mucous cells. The potential of using *A. gracilis* earthworms as sensitive, easy-to-study bioindicators to moderate levels of road pollution is highlighted, with great potential in the biomonitoring of areas surrounding moderate traffic roads, such as agricultural fields and residences. However, employing a holistic approach considering other sources of earthworm physiological status disruption – such as microplastics, vibration levels, and nature of the pollution – is advisable in road traffic-related health risk assessments.

CRediT authorship contribution statement

Nádia M.P. Coelho: Data curation, Formal analysis, Investigation, Writing – original draft. **Ricardo Camarinho:** Methodology, Writing – review & editing. **Patrícia Garcia:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Armindo S. Rodrigues:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of generative AI and AI-associated technologies in the writing process

The authors declare that there were no generative AI and AI-associated technologies in the writing process.

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Declaration of competing interests

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2026.125521.

Data availability

The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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