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Metal-polluted environments across Europe show location dependent associations with gut microbiota and nestling performance in an insectivorous passerine

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ABSTRACT

While the direct toxicological effects of metal exposure on animals, including birds, are well documented, indirect mechanisms remain poorly understood. This applies to effects on microbiota, despite the emerging evidence of its crucial role in host's physiological functions. We investigated the metal exposure, growth, and fledging of great tits (*Parus major*) in six rural vs. industrial/urban area (IUA) comparisons across Europe to identify associations with bird gut microbiota. To capture a range of pollution profiles, IUAs included both city settings and industrial sites such as a copper-nickel smelter, a metallurgical plant, a pulp mill, and a lead mine. Fecal samples from 191 broods were analyzed for bacterial 16S rRNA and concentrations of 18 elements, of which nine common contaminants were selected for further analyses. Nestlings in IUAs showed higher metal exposure than those in rural sites, except near a pulp mill, with opposing results. An index describing impervious land cover was a weak predictor for most microbiota metrics. Instead, the rural vs. IUA comparison potentially caught the environmental characteristics better, showing effects on the fledgling number, body mass, microbial composition, and abundances of several taxa, though these patterns were location dependent and may reflect secondary effects of pollution, like changes in habitat quality and diet. Taxa with links to both metal levels and nestling performance were identified. Despite reduced emissions in Europe, wild birds remain exposed to metal pollution, particularly near industrial areas. Overall, our findings suggest that anthropogenic influence associates with wildlife microbiomes and health in a context-dependent manner.

1. Introduction

Environmental contamination by toxic metals and metalloids is a worldwide environmental health concern. Globally, up to 7% of cropland surface is estimated to exceed the human health and ecological thresholds for one or more toxic metals – directly risking up to 1.4 billion people living in these areas – while an even larger share (16%) exceeds agricultural thresholds, particularly in southern Eurasia (Hou et al., 2025). In the European Union, emissions of many harmful metals like lead, arsenic, and nickel (but not, for example, copper) have decreased

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drastically since 1990 (European Environment Agency, 2023). However, elemental levels in the soil still persist and move through food chains within ecosystems. Particularly high contamination levels tend to be found near extractive and manufacturing industries such as mines and smelters, but also in urban areas with major traffic-related emissions, and even agricultural areas with heavy use of fertilizers and pesticides (Briffa et al., 2020; Espín et al., 2020b).

Essential trace elements like iron, copper, selenium, and zinc are necessary for several physiological functions in small quantities but become toxic at higher concentrations. Moreover, elements including arsenic, lead, nickel, cadmium, and chromium can have adverse health effects on animals even in small amounts, although certain organisms also require some of these elements (Jomova et al., 2022). Excessive levels of these elements can cause oxidative stress, epigenetic changes (such as DNA methylation and histone acetylation), and DNA damage in cells, as well as various neurological diseases (Briffa et al., 2020; Jomova et al., 2025). These effects have also been studied in pollution-exposed wildlife. In birds, metal exposure can result in bioaccumulation and histopathological changes in kidneys, liver, reproductive organs, lungs, and brain, as well as decreased breeding success, weakened eggshell quality, and oxidative stress (Aljohani, 2023; Eeva and Lehikoinen, 1995; Janssens et al., 2003; Koivula et al., 2011). Since certain invertebrate taxa tend to accumulate specific metals over others (Gall et al., 2015), insectivorous birds may have varying levels of metal exposure depending on their dietary composition. Moreover, indirect effects of metal pollution on wild birds via habitat and dietary changes have been reported (Eeva et al., 1997, 2014).

A lesser-known potential indirect pathway for metal toxicity is the effect of metal exposure on host-associated microbiota, including bacteria, fungi, archaea, and viruses. In birds, although much less studied than in mammals, microbiota is recognized as crucial for the development and functions of the immune system, nutrient absorption and digestion, and as the first barrier against harmful substances in the gut (Grond et al., 2018). The normal gut microbiota in wild birds varies geographically and among species, but the shared core bacterial taxa usually include Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (Grond et al., 2018). It is unclear whether the intraspecific geographical variation is due to different methods between studies, habitat type, diet, or other environmental factors such as exposure to pollution. Thus, the composition and functions of the common microbiota are still unknown for most bird species. Nevertheless, subsequent health effects of microbiota modified by environmental factors may be especially pronounced in the sensitive early-life phase after hatching (Somers et al., 2023) when the colonization of the bird's intestines by bacteria begins.

Pollution can alter environmental microbiomes in soil, water, plants, and invertebrates (Gall et al., 2015), likely changing the profile of environmental microbial exposure in birds via, e.g., diet as well as nest materials, to which altricial birds are exposed immediately after hatching (Eeva et al., 2005; Leino et al., 2024). Furthermore, exposure to toxic metals such as arsenic, cadmium, lead, and mercury has been associated with gut microbial dysbiosis linked with immune system deficiencies as well as changes in lipid metabolism and gene expression (Kaur and Rawal, 2023). Examples of potential host-microbe-metal interactions include gut bacteria such as *Bacteroides*, *Desulfovibrionales*, and *Clostridium*, which may convert metals into either more or less toxic forms by methylation, while *Pseudomonas* and *Bacillus* may reduce the absorption of metals from the gut (Kaur and Rawal, 2023). This demonstrates the multidirectional impacts – it is not only metals affecting microbes and the host, but also microbes regulating the conversion and upcycling of metals to the host (Duan et al., 2020). Moreover, the host and its microbiota share a functional relationship which is also dependent on the environment. For example, bacterial genes involved in xenobiotic degradation and lipid metabolism are more abundant in urban birds, suggesting functional changes in microbiota due to diet or environmental conditions (Teyssier et al., 2018b, 2020). Detecting these complex interactions requires a large-scale ecological perspective.

However, comprehensive assessments on the effects of metal pollution on bird microbiomes are still scarce, and the consequences of metal mixtures, typical of polluted environments, are poorly understood. In addition to metal pollution, other environmental factors (e.g., pesticides or urbanization) can simultaneously contribute to shaping bird microbiota. For example, urbanization has been suggested to alter diet composition and potentially affect gut bacterial diversity, although the magnitude and direction of the effect vary between studies (Maraci et al., 2022; Murray et al., 2020; Scholier et al., 2023; Teyssier et al., 2020). Moreover, implications on bird breeding success, body condition, and overall health need to be thoroughly understood to inform conservation efforts. Therefore, empirical studies assessing the relationship between metal exposure in polluted areas and bird gut microbiota across different environmental settings are needed to determine potential effects on bird health.

We aim to fill this knowledge gap by examining the gut bacterial microbiota characteristics and variation among great tit (*Parus major*) populations from six rural vs. industrial/urban area (IUA) comparison setups across Europe. We assess the influences of urbanization and metal (loid) pollution on the nestling microbiota and links to nestling growth and fledging, by focusing on five main hypotheses: **1)** Metal levels in nestling feces are higher in IUAs than in their rural counterparts; **2 a)** Impervious surface index, **b)** Rural and IUAs, and **c)** Metals are associated with bacterial microbiota alterations, characterized by alpha diversity, beta diversity, and taxa abundances; **3)** Nestling performance, measured as relative body mass and fledgling number, is weaker in the IUAs than in their rural counterparts; **4)** Nestling performance is associated with bacterial microbiota characteristics; **5)** Those performance related microbiota characteristics are also (negatively) associated with anthropogenic disturbance (IUAs or metals). Moreover, various differences across the large geographical gradient are likely and are discussed along with the hypotheses, but are not the main focus of this paper.

2. Materials and methods

2.1. Study design and sites

We studied the great tit, an insectivorous passerine widely distributed across Eurasia. Great tits commonly serve as a model species in ecological research (Culina et al., 2021) due to their readiness to breed in nest boxes. To gather large-scale, representative data on the gut microbiota and breeding parameters of great tits, nest box sites were established in multiple European locations spanning north to south (Fig. 1): Harjavalta (Finland), Malmö (Sweden), Antwerp (Belgium), Prague (Czechia), Figueira da Foz (Portugal), and Murcia (Spain). For clarity, each location is referred to by the city name, although some nest box areas were located outside city borders.

Nest boxes were placed in both rural and IUA environments to assess the effects of metal pollution and urbanization. Rural areas had varying levels of forest cover, agricultural use, and settlement density. In contrast, IUAs included forests or parks near 1) city-like settings (traffic, settlements), or 2) industrial or mining activity. The study area pairs were chosen so that each IUA and the corresponding reference area had similar vegetation types for comparability. The urban, mining, and industrial areas were pooled under the functional (rather than homogenous) category of "IUAs", as the supposed stress factors such as pollutants, habitat change, changed food composition, disturbance, noise, and light are relatively similar in all cases. Further details on habitat types, pollution sources, known main contaminants, and previous research are provided (Table 1). Birds in these locations have been studied in various contexts, often related to the effects of urbanization or pollution. Despite their potential relevance to bird health and microbiota, other pollution types than metals (e.g., microplastics and other particulate matter, nitrogen oxides, and organic compounds common to urban and industrial areas) are not included in this study, since they

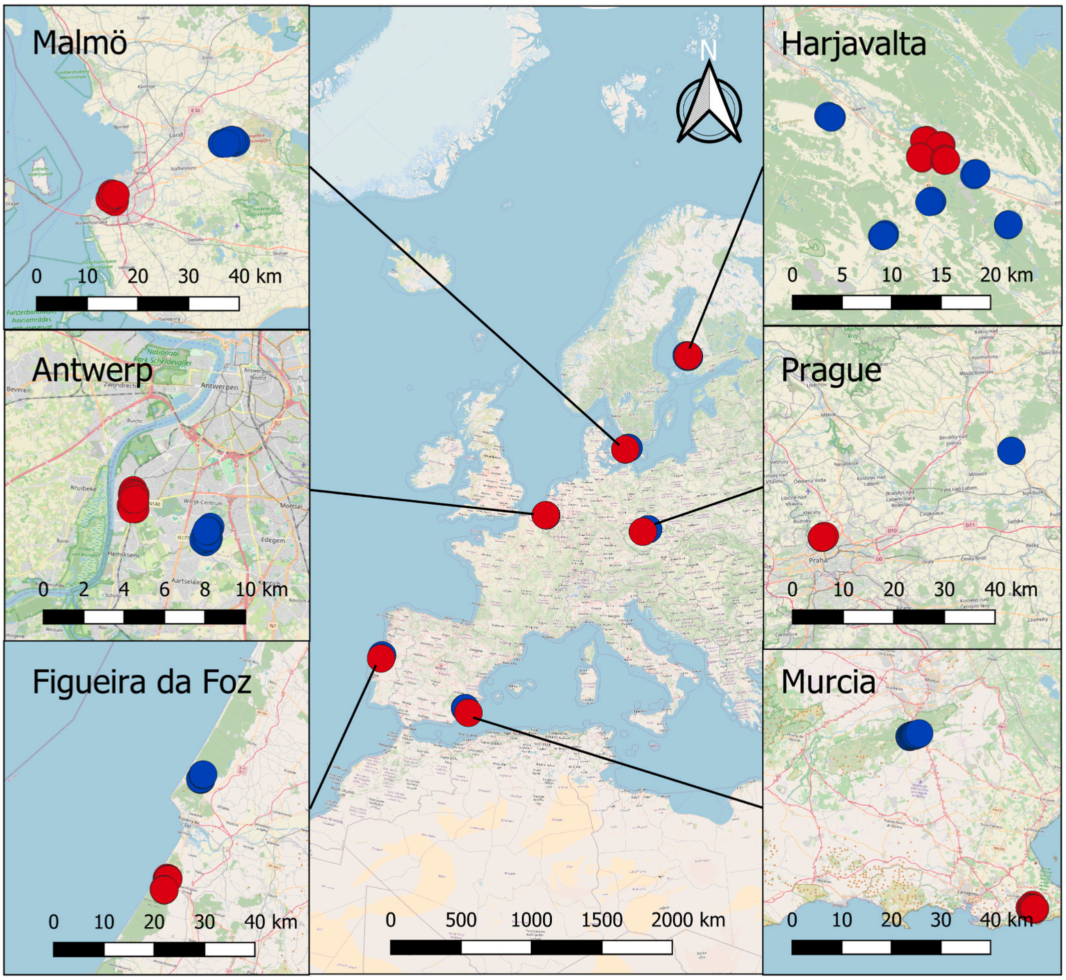


Fig. 1. Map of the six study locations across Europe: Harjavalta (Finland), Malmö (Sweden), Antwerp (Belgium), Prague (Czechia), Figueira da Foz (Portugal), and Murcia (Spain). Red dots indicate industrial/urban areas, while blue dots represent rural reference areas where nest box sites were established for sampling. Map data from Open Street Map (https://www.openstreetmap.org/copyright). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

require separate quantification methodologies.

2.2. Sampling

The great tit nests were inspected weekly during the 2022 breeding season to collect data on egg-laying, hatching rate, brood size, and fledging rate. The nestlings were sampled at an average age of 9 days from hatching (SD ± 1.8, ranging from 5 to 16 days due to logistical constraints), with efforts to minimize variation in age. Despite the age range, >90% of broods were sampled just within ±2 days of the target age of 9 days, and there were no significant between-area age differences except in one of the locations (nestlings in the rural area of Prague were three days older than in the polluted area, but this was confirmed not to affect bacterial community composition). Moreover, the within-location hatching dates (Table 1) were similar in five locations but were significantly delayed in the rural Figueira da Foz as a result of two late broods. In theory, late hatching could have phenology-related effects on bird microbiota, but further testing on bacterial principal components and hatching date implicated no such effects here.

Fresh fecal samples were collected directly from defecating nestlings in sterile tubes and stored in −20 °C portable coolers until transported into a −80 °C final storage. Fecal sacks were pooled by brood already in the field to ensure an adequate amount of material for two analyses, to improve the reliability of metal exposure estimates (Eeva et al., 2020), and reduce the analysis costs. Each nestling was ringed and weighed individually at microbial sampling, and the wing length was measured. However, to match the pooled fecal data, we used brood means of these growth parameters in the analyses. Sterile gloves and equipment were used to avoid any microbial contamination whenever there was a need to touch the birds, e.g., to collect the fecal samples. Dead nestlings were recorded and removed from the nests as soon as they were found to avoid microbial contamination from the corpses.

Appropriate licenses for ringing, handling, and collecting fecal samples of great tit nestlings were acquired as follows: Regional State Administrative Agency for Southern Finland, License no. ESAVI/44280/2021 and Centre for Economic Development, Transport and the Environment, VARELY/6817/2021 (Finland); The Swedish board of agriculture, the ethical board of Malmö-Lund, License no. 5.8.18-14112/2019 (Sweden), Royal Belgian Institute of Natural Sciences (RBINS) (Belgium); License no. MZP/2019/630/1778 (Czechia); License no. 188/2022/CAPT (Portugal); Regional Environmental Authority of Murcia, AUF20190068 (Spain).

2.3. Molecular analysis

A well-established, relatively robust, and cost-effective 16S rRNA metabarcoding approach was chosen to address this broad spatial scale data with an ecological focus on the microbiota community shifts in polluted environments. Although the 16S approach is acknowledged to lack functional information (unlike, e.g., metagenomics or

Table 1
Study areas and their main habitat types and known pollution characteristics with references to previous pollution or bird-related research in nearby locations. "IUA" refers to industrial and urban study areas.

Location	Harjavalta (Finland)[a]	Malmö (Sweden)[b]	Antwerp (Belgium)[c]	Prague (Czechia)[d]	Figueira da Foz (Portugal)[e]	Murcia (Spain)[f]
Pollution source	Cu-Ni smelter	City, traffic	Non-ferrous metallurgical plant	City, minor industry	Pulp factory/industrial complex, agriculture fields near the rural sites	Ancient mining district (Cartagena-La Unión), lead mine
Habitat type	Coniferous, Deciduous, Mixed	Coniferous, Deciduous (beeches), Mixed	Deciduous	Deciduous	Coniferous	Coniferous (pine)
Known main pollutants	Cu, Ni, As, Pb, Zn	Nitrogen oxides, particulate matter	As, Cd, Cu, Pb, Zn	Zn, Pb, Cd, (Cr, Cu)	As, Cd, Cu, Hg, Ni, Pb, Se, Zn, carbon monoxide, nitrous oxides, particulate matter	Mainly Pb, also Cd, As, Zn, Cu, Sn, Hg
Study period (2022)	April-July	April-June	March-May	April-June	April-June	April-July
Mean hatching date (dd. m.) IUA/rural area (±SD in days)	25.5. (±3)/27.5. (±5)	18.5. (±5)/18.5. (±5)	24.4. (±5)/22.4. (±5)	4.5. (±4)/ 6.5. (±3)	7.5. (±13)/18.5. (±21)	18.5. (±9)/15.5. (±17)
Distance to pollution source (IUA/rural area, km)	0.6/11	0/10–25[g]	0.6/6	0/45[h]	1/20	0/38[i]
Number of metal samples (IUA/rural)	14/14	16/14	17/14	20/15	15/10	12/20
Number of microbiota samples after rarefaction (IUA/rural)	16/15	17/13	16/13	19/15	14/12	13/18

[a] (Espín et al., 2020; Kiikkilä, 2003).
[b] (Jensen et al., 2023; Salmón et al., 2018).
[c] (Grunst et al., 2018; Janssens et al., 2001).
[d] (Bauerová et al., 2017, 2020).
[e] (Costa et al., 2011, 2012, 2017).
[f] (Conesa and Schulin, 2010; Espín et al., 2014; Sánchez-Virosta et al., 2021).
[g] A non-point pollution source: the IUA was located 2 km from Malmö city centre (but still within the central zone), and the rural area 10 km and 25 km from the city centers of Lund and Malmö, respectively.
[h] A non-point pollution source: the IUA was located 6 km from Prague city center, remaining in close proximity to traffic and residential areas, whereas the control area was 45 km from the city center.
[i] Despite a busy highway beside the rural site, the reported numbers refer to distance from the mining area. The mining area is strongly impacted by mining and has a low population density.

metabolomics approaches), correlational studies using large, integrative data are vital to first detect ecological patterns and to create founding hypotheses for more targeted studies in the future. Microbial DNA was extracted from the fecal samples using Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (D6010, Zymo Research) by taking a standard mass of fecal material (150-180 mg) of each pooled sample. Both negative and positive extraction controls (Zymo Microbial Community DNA Standard, D6306) were added to validate the protocol and to assess potential contamination. The PCR protocol was carried out with technical duplicates, and the primers were targeted to the highly variable bacterial 16S rRNA gene region V3-V4 by using the primers from Herlemann et al. (2011) in the first PCR reaction: "Bakt_341F" (5′–CCTACG GGNGGCWGCAG–3′) and "Bakt_805R" (5′–GACTACHVGGGTA TCTAATCC–3′) and primers from Adapterama I (Glenn et al., 2019a) for the second PCR reaction: "iTru5" (5′-AATGATACGGCGACCACCGA-GATCTACAC [i5 index] ACACTCTTTCCCTAC-3′) and "iTru7" (5′-CAAGCAGAAGACGGCATACGAGAT [i7 index] GTGACTGGAG TTCAG-3′). The PCRs were based on the Adapterama II protocol by Glenn et al. (2019b), resulting in a quadruple-indexed (multiplexed) DNA library. Reagent mix '2x MyTaq™ HS Red Mix' (Bioline, UK) was used with the primers and the template in both PCR reactions, which included 35 (PCR-1) and 10 (PCR-2) cycles of denaturation, annealing, and extension. Details of the PCR protocols are provided in the Supplements. A purified and quality-checked DNA library was sequenced by the Finnish Functional Genomics Centre (FFGC) (University of Turku and Åbo Akademi University, Finland), using Illumina MiSeq v3, 2 × 300 bp (Illumina Inc. San Diego, California, USA).

The resulting sequence data were demultiplexed (mismatch rate of 1), trimmed, merged, and the primers were removed. The reads were then dereplicated and denoised into zero-radius operational taxonomic units (ZOTU), a type of sequence variant, using the 'unoise3' command (with settings minsize = 8 and unoise_alpha = 2) in USEARCH 11 (Edgar, 2010). ZOTUs are defined at 100% sequence identity, similarly to, e.g., amplicon sequence variants (ASVs), providing better chimera and Illumina artefact removal compared to traditional OTU clustering (Edgar and Flyvbjerg, 2015). The ZOTUs were assigned to taxa using the SINTAX algorithm in USEARCH/VSEARCH against the database *16S RDP training set v18 (21k seqs)* (Edgar, 2016). It should be noted that this database version includes taxa names that have been changed more recently; the updated nomenclature includes names such as Pseudomonadota (formerly Proteobacteria), Bacillota (for Firmicutes), and Actinomycetota (for Actinobacteria). To maintain the consistency and reproducibility of the data, names and taxonomy provided by the reported database are used throughout this article. The data was further processed using the R Statistical Software (v. 4.4.1; R Core Team, 2023), to, e.g., remove ZOTUs unique to only one of the duplicates, collapse the duplicates by averaging, remove non-bacterial reads and 226 probable contaminant ZOTUs (tested using the prevalence-based method (*decontam* v. 1.18.0) described by Davis et al. (2018)). To control the uneven sequencing depths while maintaining comparability across samples, the data was then rarefied and normalized to an even depth of 3171 reads (function 'rarefy_even_depth' in *phyloseq* v. 1.48.0; McMurdie and Holmes (2013)). The procedure excluded 10 low-read samples that did not have sufficient sequencing depths and 84 rare ZOTUs, resulting in 181 microbial samples, 3667 ZOTUs, and 573 951 reads (see Fig. S1 that demonstrates the sufficient capturing of taxa using the chosen threshold). The key results were confirmed to remain robust despite rarefaction, with an exception further discussed in the methods and discussion.

Further details of the molecular methods, bioinformatics, and subsequent processing are provided in the Supplements (section *1.1. Molecular methods*).

2.4. Metal analyses

After the DNA extraction, the fecal sample leftovers were dried for further elemental analysis. Elements measured were: aluminum (Al), arsenic (As), cadmium (Cd), calcium (Ca), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), magnesium (Mg), manganese (Mn), nickel (Ni), phosphorus (P), potassium (K), selenium (Se), sodium (Na), sulfur (S), and zinc (Zn). The samples were analyzed by the Servicio de Apoyo a las Ciencias Experimentales laboratory (SACE; University of Murcia) using inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, Model 7900), following the methods described by Martínez-López et al. (2019). The eventual number of broods with successful metal measurements was 181.

All elements measured were assessed for location-specific levels in bird feces and differences between rural and urban areas. However, a subset of 9 elements (As, Cd, Cr, Cu, Fe, Pb, Ni, Se, and Zn) was chosen for the microbiota and bird health-related analyses, to include only the most relevant and accuracy-confirmed metals, metalloids (As), and nonmetals (Se), hereafter referred to as 'metals' for simplicity. Full data of all the elemental levels, comparisons between rural and urban areas, and methodology can be found in the supplementary materials (Fig. S2, Tables S1 and S2).

Fecal metal concentrations indicate a combination of metal directly excreted via feces and that absorbed and again excreted via kidneys (uric acid) and liver (bile). So, they do not directly describe concentrations in internal organs but can be used as non-destructive proxy measures for internal exposure, especially for more toxic non-essential heavy metals that are less well physiologically regulated (Berglund et al., 2011). However, the ambient environment of microbes is the gut contents, and microbiota could be affected independently of the internal metal absorption of the gut.

2.5. Statistical analysis

2.5.1. Study variables

The primary classifying variables in the statistical models were 'location' and 'area'. 'Location' refers to the six European locations, while 'area' refers to the IUAs vs. rural reference areas within each location. Moreover, the degree of urbanization (specifically, impervious surfaces) around each nest box was estimated using the Copernicus online database (European Union's Copernicus Land Monitoring Service information, 2020).

Two primary measures were used to describe the nestling performance: relative body mass (RBM) and fledgling number. RBM represents the percentage deviation of a brood's mean body mass (at the age of sampling) from the value predicted by a long-term (1991–2022) growth curve derived from the great tit populations in Harjavalta. Additionally, relative wing length – defined as the percentage deviation from the average wing length at the sampling age – was used in a circular correlation plot to visualize relationships among nestling growth, microbes, and metal exposure. The number of fledglings describes a short-term aspect of survival, while higher nestling body mass and more advanced wing development may indicate better post-fledging survival (Jones et al., 2017; Rodríguez et al., 2016). The ratio of fledglings/hatchlings (i.e., fledging probability) was taken into consideration, but due to low to zero variation in multiple study groups, it could not be reliably used in further analyses. Two potentially predated broods were excluded from the fledgling number analyses.

2.5.2. Element principal components

We performed a principal component analysis (PCA) for the log10-transformed metal levels (As, Cd, Cr, Cu, Fe, Pb, Ni, Se, and Zn), since many were highly correlated. Two principal components, PC_{Met1} and PC_{Met2}, were formed using the Factor procedure (method = Prin) with Varimax rotation in SAS (SAS software 9.4; SAS Institute Inc, 2013). Before the PCA, mean element values by location and area were temporarily imputed for eight microbiota-sampled broods with missing metal information to avoid loss of statistical power in further analysis due to the combination of missing microbes, metal samples, or other variables. Imputation did not affect the allocation of metals between the PCs, and the main results were confirmed to remain robust regardless of imputation. The first component, $\mathbf{PC_{Met1}}$, showed strong positive correlations to **Cd, Zn, As, Pb, Cu, and Se** (eigenvalue 4.19, explains 46.6% of the total variation), whereas $\mathbf{PC_{Met2}}$ was strongly positively correlated to **Cr, Fe, and Ni** (eigenvalue 1.96, explains 21.8% of the total variation; Table 2 a and Fig. S3). Hence, PC_{Met1} represents the overall level of variably toxic non-ferrous metal pollution elements while PC_{Met2} represents relatively less toxic 'stainless steel' group of metals, yet toxic in excess and, e.g., some forms of Cr being highly toxic (Cervantes et al., 2001).

2.5.3. Common taxa and principal components

The fecal microbiota was analyzed primarily on three taxonomical levels to examine 1) broad patterns of microbiota (phyla), 2) an intermediate taxonomic level (orders), maintaining still a large read data coverage and sufficient read counts for statistical analyses across multiple study groups, and 3) the most precise taxonomical assignment level available for this data (genera).

A subset of common bacterial orders was determined by an abundance threshold of 0.01% (i.e., minimum of 58 reads per order in at least one sample) in addition to a prevalence threshold of 5% (i.e., order present in at least 10 samples) across all locations, to improve the interpretability of the analyses and results by limiting the number of rare orders with sparse reads data. The subset thus included 23 orders out of 115 and covered 95.5% of the total reads of the rarefied data. These common bacterial orders were used for creating three principal components with the Factor procedure (method = Prin) and Varimax rotation in SAS: $\mathbf{PC_{Bac1}}$, $\mathbf{PC_{Bac2}}$, and $\mathbf{PC_{Bac3}}$ had eigenvalues of 5.40, 3.21, and 2.48, explaining 23.5%, 14.0%, and 10.8% of the total variation, respectively (Table 2 b and Fig. S3).

2.5.4. Alpha and beta diversity

To assess the bacterial diversity within each great tit brood (i.e., alpha diversity), Shannon index values were calculated with the R package *microbiome* (v. 1.26.0; Lahti and Shetty, 2022) from the rarefied data using ZOTUs, and further statistically analyzed in SAS. The Shannon index results were confirmed to remain robust regardless of rarefaction.

Beta diversity, i.e., bacterial community composition among bird populations, was assessed as follows: The effects of area (i.e., IUA vs. rural), location (i.e., city), location-area interaction, degree of urbanization, and RBM on the microbial community composition (ZOTU level) were analyzed using permutational multivariate analysis of variance (PERMANOVA) in *vegan* (v. 2.6-8; Oksanen et al., 2024; function 'adonis2' with 9999 permutations), with Bray-Curtis distances calculated based on rarefied reads. To mitigate the data compositionality issues raised by Gloor et al. (2017), we applied consistent library size normalization (rarefaction to an even depth) prior to analysis and focused on between-group dissimilarities rather than correlations among taxa. Under these conditions and in similar studies (such as by Kropáčková et al., 2017), Bray–Curtis dissimilarity has been used and shown to perform robustly. A comparison of rarefied and non-rarefied data is further disclosed. The number of fledglings was excluded from PERMANOVA to preserve sample size, as 9 nests were either predated or lacked fledging information, which would have led to their exclusion from analyses of other variables as well. Afterward, to specify the location-area interactions, *pairwiseAdonis* (v. 0.4.1; Martinez Arbizu, 2017) was used for pairwise comparisons. To focus on ecologically relevant comparisons (IUA v.s. rural areas within each location), the

Table 2
Rotated principal component patterns for **A)** 9 metals and **B)** 23 common bacterial orders. The strongest loadings have been bolded, indicating the principal component to which each metal and order contributes most. Note that all metals and the majority of bacterial orders show positive correlations with their primary PC, but three bacterial orders show negative correlations: Lactobacillales with PC_{Bac2} and Chlamydiales and Legionellales with PC_{Bac3}. As an example, a trait that correlates positively with PC_{Bac2} indicates a negative correlation with Lactobacillales and a positive correlation with the other taxa within the said PC.

A)	PC_{Met1}	PC_{Met2}	B)	PC_{Bac1}	PC_{Bac2}	PC_{Bac3}
Cd	**0,905**	0,112	Caulobacterales	**0,786**	0,132	0,128
As	**0,758**	0,498	Sphingomonadales	**0,740**	0,138	0,192
Zn	**0,734**	−0,263	Rhizobiales	**0,714**	0,170	0,353
Pb	**0,697**	0,452	Xanthobacteriales	**0,698**	−0,326	−0,138
Cu	**0,631**	0,070	Sphingobacteriales	**0,649**	−0,146	−0,213
Se	**0,560**	0,342	Micrococcales	**0,640**	0,007	0,340
Cr	−0,003	**0,945**	Unknown from the class Spartobacteria	**0,576**	0,186	0,216
Fe	0,146	**0,871**	Pseudomonadales	**0,514**	0,360	−0,233
Ni	0,183	**0,813**	Burkholderiales	**0,503**	0,487	0,110
			Unknown from the class Acidobacteria Gp1	**0,461**	0,307	−0,144
			Mycoplasmatales	**0,459**	0,123	−0,230
			Unknown from the phylum Saccharibacteria	**0,420**	−0,111	0,200
			Synergistales	0,041	**0,918**	0,006
			Desulfovibrionales	0,041	**0,915**	−0,069
			Streptosporangiales	0,090	**0,734**	0,224
			Lactobacillales	−0,099	**−0,531**	0,417
			Erysipelotrichales	0,322	**0,384**	0,154
			Enterobacterales	−0,018	**0,253**	−0,013
			Mycobacterales	0,100	−0,007	**0,892**
			Streptomycetales	0,010	0,381	**0,758**
			Chlamydiales	0,101	0,451	**−0,575**
			Legionellales	−0,068	0,124	**−0,383**
			Clostridiales	0,034	0,042	**0,125**

results were sliced by location, and unadjusted *p*-values were used, as explained in the next chapter of Statistical modeling. Moreover, multivariate dispersion of Bray–Curtis distances (i.e., distances to group centroids) was evaluated using the betadisper function in *vegan*, with significance assessed by permutation tests (permutest with pairwise comparisons, 9999 permutations) for the Area × Location interaction. Tukey's HSD was also used to assess the adjusted *p*-values.

2.5.5. Statistical modeling

Linear models (LM) and generalized linear models (GLM) were used in the SAS GLIMMIX procedure for assessing: **1)** Levels of individual metals across six European locations and their rural and IUAs **2)** Metal PCs, urbanization, and nestling performance (RBM and fledgling number) across the locations and areas, **3)** Effects of metal PCs, urbanization, and nestling performance on the bacterial alpha diversity (Shannon index), **4)** Effects of location, area, urbanization, and nestling performance on three bacterial PCs, **5)** Differences in six main bacterial phyla between locations and areas, and **6)** Associations between significant bacterial phyla and orders, metal levels, and nestling performance.

The non-significant terms were sequentially excluded from the models based on the largest *p*-value, starting from the location-area interaction term. For the interaction term, only the ecologically relevant comparisons (i.e., rural versus urban areas within each location) were assessed in post-hoc tests. In these cases, unadjusted p-values were reported, as the focus was on the pre-determined rural-urban comparison within each individual study system instead of a random search among any area or location. The Variance Inflation Factor (VIF) values were inspected to eliminate multicollinearity, and residual distributions were controlled for (see details in supplements). Degrees of freedom may vary due to missing observations in some variables.

2.5.6. Differential abundance analysis

Differential abundance (DA) analysis of bacterial orders between IUAs and rural areas was performed separately for each location using five R packages: ALDEx2 (1.36.0) (Fernandes et al., 2014), LinDA (0.2.0) (Zhou et al., 2022), DESeq2 (1.44.0) (Love et al., 2014), ANCOM-BC2 (2.6.0) (Kaul et al., 2017; Lin et al., 2022; Lin and Peddada, 2020), and Corncob (0.4.1) (Martin et al., 2020), to account for method-specific variation as recommended by Nearing et al. (2022). Non-rarefied and filtered read data (threshold of 50 reads and presence in ≥10% of samples by location) and false discovery rate-corrected (FDR) *p*-values were used. Orders identified as differentially abundant by the majority of methods were selected for further analyses related to nestling performance. Clostridiales from Harjavalta were also retained based on biological relevance and near-significant results across multiple methods. Similar DA analyses were performed at the genus level for more detailed taxonomical information.

An additional DA analysis across locations was conducted using ANCOM-BC2 with mixed directional false discovery rate (mdFDR) control method (Holm–Bonferroni), suitable for multi-group pairwise comparisons (Lin and Peddada, 2024). Rare orders were excluded using a 0.01% total read abundance and 5% prevalence threshold.

The data used in this study is publicly accessible via the Mendeley Data repository at https://doi.org/10.17632/2grtt8j4sb.1 (Leino et al., 2025).

3. Results

3.1. Environmental characteristics and nestling performance

3.1.1. Elevated metal levels in IUAs, except in Figueira da Foz

Although metal profiles varied a lot among the locations, in five out of six IUA-rural comparisons, the individual metals showed higher values in IUAs. Figuera da Foz was an exception, as the levels were higher in the rural area (Table 3; see also the Supplementary Tables S1 and S2 for all 18 elements).

Principal components of metal levels (PC_{Met1} and PC_{Met2}) were statistically significantly dependent on the area, location, and their interaction (Table S3). More specifically, PC_{Met1} (including Cd, Zn, As, Pb, Cu, and Se) levels were higher in the IUAs of Antwerp, Harjavalta, Malmö, and Murcia, whereas PC_{Met2} (Cr, Fe, and Ni) levels were higher in the IUAs of Antwerp, Harjavalta, and Prague. Corresponding to individually tested metals, Figueira da Foz showed higher levels of PC_{Met1} and PC_{Met2} in rural area than IUA.

3.1.2. Location-specific effects of IUAs and imperviousness on nestling performance

The urbanization index, describing land surface imperviousness,

Table 3
Geometric means and 95% confidence limits for concentrations of 9 elements (ppm = mg/kg, dry weight), measured from the great tit (*Parus major*) nestling feces across six European locations in Belgium, Portugal, Finland, Sweden, Spain, and Czechia. Elemental levels between rural and urban/industrial areas (IUA) were compared using linear and generalized linear (Fe) models. Statistically significant results (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; . $p < 0.1$) where element levels were higher in the urban areas are highlighted in yellow, while those higher in rural areas are highlighted in grey. Values are shown with three significant digits. The elements were analyzed using constant-added (1), log10-transformed concentrations that followed a normal distribution, then back-transformed using the exponential function.

	Antwerp				Figueira da Foz				Harjavalta			
Element	Rural	IUA	F_{df}		Rural	IUA	F_{df}		Rural	IUA	F_{df}	
Arsenic (As)	0.736 (0.514-1.06)	10.3 (7.45-14.3)	123 $_{1,29}$	***	1.40 (0.730-2.68)	0.582 (0.342-0.990)	4.67 $_{1,23}$	*	1.05 (0.768-1.44)	6.43 (4.70-8.80)	70.6 $_{1,26}$	***
Cadmium (Cd)	1.76 (1.32-2.35)	7.27 (5.59-9.45)	55.0 $_{1,29}$	***	1.90 (1.12-3.22)	0.350 (0.227-0.540)	26.1 $_{1,23}$	***	1.29 (0.939-1.78)	1.94 (1.41-2.68)	3.42 $_{1,26}$	.
Chromium (Cr)	2.19 (1.48-3.23)	3.88 (2.73-5.53)	4.96 $_{1,29}$	*	0.619 (0.398-0.964)	0.432 (0.301-0.619)	1.71 $_{1,23}$		2.84 (1.80-4.50)	6.08 (3.84-9.62)	5.79 $_{1,26}$	*
Copper (Cu)	28.3 (23.6-33.8)	54.6 (46.4-64.1)	31.3 $_{1,29}$	***	71.1 (49.3-103)	35.4 (26.2-47.7)	9.34 $_{1,23}$	**	70.5 (56.2-88.6)	121 (96.4-152)	11.9 $_{1,26}$	**
Iron (Fe)[1]	962 (645-1430)	2110 (1470-3040)	8.96 $_{1,29}$	**	785 (489-1260)	276 (188-407)	12.5 $_{1,23}$	**	2560 (1670-3930)	3750 (2440-5750)	1.67 $_{1,26}$	
Lead (Pb)	4.96 (3.52-6.98)	61,6 (45.2-84)	125 $_{1,29}$	***	1.72 (1.06-2.80)	0.782 (0.525-1.16)	6.70 $_{1,23}$	*	2.90 (2.32-3.64)	5.97 (4.76-7.48)	21.6 $_{1,26}$	***
Nickel (Ni)	1.14 (0.916-1.42)	3.21 (2.63-3.92)	50.3 $_{1,29}$	***	0.656 (0.415-1.04)	0.350 (0.241-0.509)	4.81 $_{1,23}$	*	3.73 (2.80-4.97)	26.9 (20.2-35.8)	101 $_{1,26}$	***
Selenium (Se)	0.436 (0.347-0.548)	6.63 (5.39-8.15)	327 $_{1,29}$	***	0.518 (0.372-0.719)	0.287 (0.220-0.376)	8.21 $_{1,23}$	**	0.686 (0.552-0.851)	1.50 (1.21-1.86)	27.5 $_{1,26}$	***
Zinc (Zn)	209 (181-242)	260 (227-297)	5.00 $_{1,29}$	*	389 (281-537)	254 (195-330)	4.46 $_{1,23}$	*	254 (213-304)	195 (164-233)	4.79 $_{1,26}$	*
	Malmö				**Murcia**				**Prague**			
Element	Rural	IUA	F_{df}		Rural	IUA	F_{df}		Rural	IUA	F_{df}	
Arsenic (As)	0.466 (0.297-0.730)	1.08 (0.708-1.64)	7.79 $_{1,28}$	**	0.955 (0.709-1.29)	10.1 (6.98-14.6)	103 $_{1,31}$	***	0.919 (0.679-1.24)	1.76 (1.35-2.28)	11.0 $_{1,33}$	**
Cadmium (Cd)	0.364 (0.245-0.541)	0.837 (0.578-1.21)	9.91 $_{1,28}$	**	0.702 (0.537-0.918)	4.09 (2.93-5.71)	70.9 $_{1,31}$	***	0.593 (0.478-0.735)	0.560 (0.464-0.674)	0.170 $_{1,33}$	
Chromium (Cr)	1.70 (0.872-3.31)	4.14 (2.22-7.72)	4.00 $_{1,28}$	.	2.53 (1.88-3.40)	3.40 (2.36-4.91)	1.63 $_{1,31}$		2.58 (1.73-3.85)	4.76 (3.37-6.73)	5.54 $_{1,33}$	*
Copper (Cu)	26.7 (20.9-34.1)	48.6 (38.6-61.2)	13.3 $_{1,28}$	**	75.9 (64.4-89.5)	82.8 (67.5-102)	0.450 $_{1,31}$	.	40.3 (34-47.8)	38.4 (33.1-44.5)	0.190 $_{1,33}$	
Iron (Fe)[1]	1860 (1180-2920)	2520 (1650-3850)	1.03 $_{1,28}$		1510 (1110-2070)	6280 (4270-9220)	34.4 $_{1,31}$	***	1530 (1030-2270)	2170 (1540-3050)	1.86 $_{1,33}$	
Lead (Pb)	2.24 (1.48-3.39)	4.12 (2.79-6.06)	4.81 $_{1,28}$	*	1.70 (1.20-2.43)	200 (129-310)	298 $_{1,31}$	***	2.04 (1.44-2.87)	3.35 (2.49-4.51)	4.93 $_{1,33}$	*
Nickel (Ni)	1.02 (0.617-1.67)	2.36 (1.48-3.76)	6.37 $_{1,28}$	*	1.80 (1.39-2.35)	2.40 (1.73-3.32)	1.91 $_{1,31}$		1.33 (0.983-1.80)	2.25 (1.73-2.92)	7.19 $_{1,33}$	*
Selenium (Se)	0.508 (0.367-0.703)	0.951 (0.702-1.29)	8.32 $_{1,28}$	**	0.738 (0.631-0.862)	0.701 (0.578-0.851)	0.180 $_{1,31}$		0.298 (0.202-0.440)	0.397 (0.284-0.557)	1.28 $_{1,33}$	
Zinc (Zn)	167 (135-207)	221 (181-269)	3.83 $_{1,28}$	.	284 (233-347)	673 (525-862)	30.5 $_{1,31}$	***	216 (190-245)	218 (195-243)	0.0100 $_{1,33}$	

[1] Original (i.e., non-transformed) iron values were used with a negative binomial distribution assumption and the default SAS link function.

expectedly showed that Antwerp, Harjavalta, Malmö, and Prague had a higher degree of urbanization in IUAs than the "rural" areas (Table S3). Instead, the urbanization values did not differ between rural areas and IUAs in Figueira da Foz (pulp mill) and Murcia (lead mine).

Both nestling performance-related parameters (fledgling number and RBM) showed location dependent differences between areas. The fledgling number was significantly lower in the IUAs only in Harjavalta and Malmö (Fig. S4), while nestlings had a significantly decreased RBM only in the IUA of Figueira da Foz. However, neither of the parameters showed associations with the degree of urbanization, nor with each other (Table S3).

3.2. Microbiota characteristics

3.2.1. Major taxa across locations

Overall, the bird fecal microbiota was dominated by phyla Proteobacteria, Firmicutes, and Actinobacteria (mean ± SD relative abundances 47.2% ± 22.2, 31.7% ± 23.8, and 7.3% ± 10.5, respectively) (Fig. 2). Differences from this general abundance ranking were found in Murcia, where Firmicutes (53.5%, SD = 21.9) were more abundant than Proteobacteria (35.4%, SD = 23.0); Harjavalta, where Chlamydiae (9.85%, SD = 13.0) was the third most abundant phylum instead of Actinobacteria; and Figueira da Foz, where the elsewhere rare Synergistetes covered 9.08% (SD = 13.77) of the microbiota (Table S4; we also refer to Table S5 for more information about the occurrence of bacterial phyla across locations). The top five dominant bacterial genera were *Diplorickettsia* (18.2%, SD = 20.0), *Enterococcus* (9.07%, SD = 15.2), *Sarcina* (8.7%, SD = 14.2), *Sphingomonas* (6.9%, SD = 8.2), and *Escherichia* (3.4%, SD = 8.7%). However, the ranking order varied substantially by location – for example, *Enterococcus* was the dominant genus in Murcia (31.8%, SD = 22.8) while being rare in some other locations such as Figueira da Foz (1.87%, SD = 1.71).

3.2.2. Abundances of major phyla vary by area and location

The six most abundant gut bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria, Chlamydiae, Tenericutes, and Synergistetes) were tested for differences between areas and locations. Location was a statistically significant factor for the abundance of all six phyla (Table S6). The study area was a significant factor for some bacteria: Firmicutes were elevated in the IUAs, whereas Actinobacteria and Chlamydiae were higher in the rural areas. Moreover, Chlamydiae also showed a significant area-location interaction in GLM: only Figueira da Foz ($F_{df} = 5.12_{1,169}$, $p = 0.025$), Harjavalta ($F_{df} = 6.30_{1,169}$, $p = 0.013$), and Murcia ($F_{df} = 4.19_{1,169}$, $p = 0.042$) had a significantly larger abundance of Chlamydiae in the rural areas. Additionally, Proteobacteria showed significantly elevated abundances in the Malmö IUA ($F_{df} = 9.35_{1,169}$, $p = 0.003$; LM). Further details and Tukey's post-hoc comparisons for locations (i.e., abundance differences between locations) are shown in Table S6.

3.2.3. Differential abundance analysis of orders and genera across areas and locations

Differences were found between rural and IUAs in some common bacterial orders, but not in all locations. Bacteria significantly elevated in the IUAs were (log2 fold-changes ±SE): **1)** Clostridiales (Harjavalta: 2.14 ± 0.55, $p = 0.0004$, $n_{rur/IUA} = 16/16$; and Prague: 1.69 ± 0.49, $p = 0.006$, $n_{rur/IUA} = 15/20$, with contribution from the genus *Sarcina*), **2)** Sphingomonadales (Malmö: 1.95 ± 0.51, $p = 0.004$, $n_{rur/IUA} = 15/17$ – contribution from the genera *Altererythrobacter* and *Sphingomonas*), and **3)** Enterobacterales (Murcia: 1.72 ± 0.41, $p = 0.001$, $n_{rur/IUA} = 20/14$). Moreover, **4)** Pseudomonadales (contribution from *Acinetobacter*) were elevated in the Prague rural areas (−4.39 ± 1.08, $p = 0.0008$, $n_{rur/IUA} = 15/20$) (Fig. 3). The results were validated using multiple DA estimators; however, only DESeq2 values with Benjamini-Hochberg adjusted *p*-values are presented here. The genus-level analyses showed overall 9 differentially abundant genera (*Rhodoplanes, Rhizobium, Clostridium*

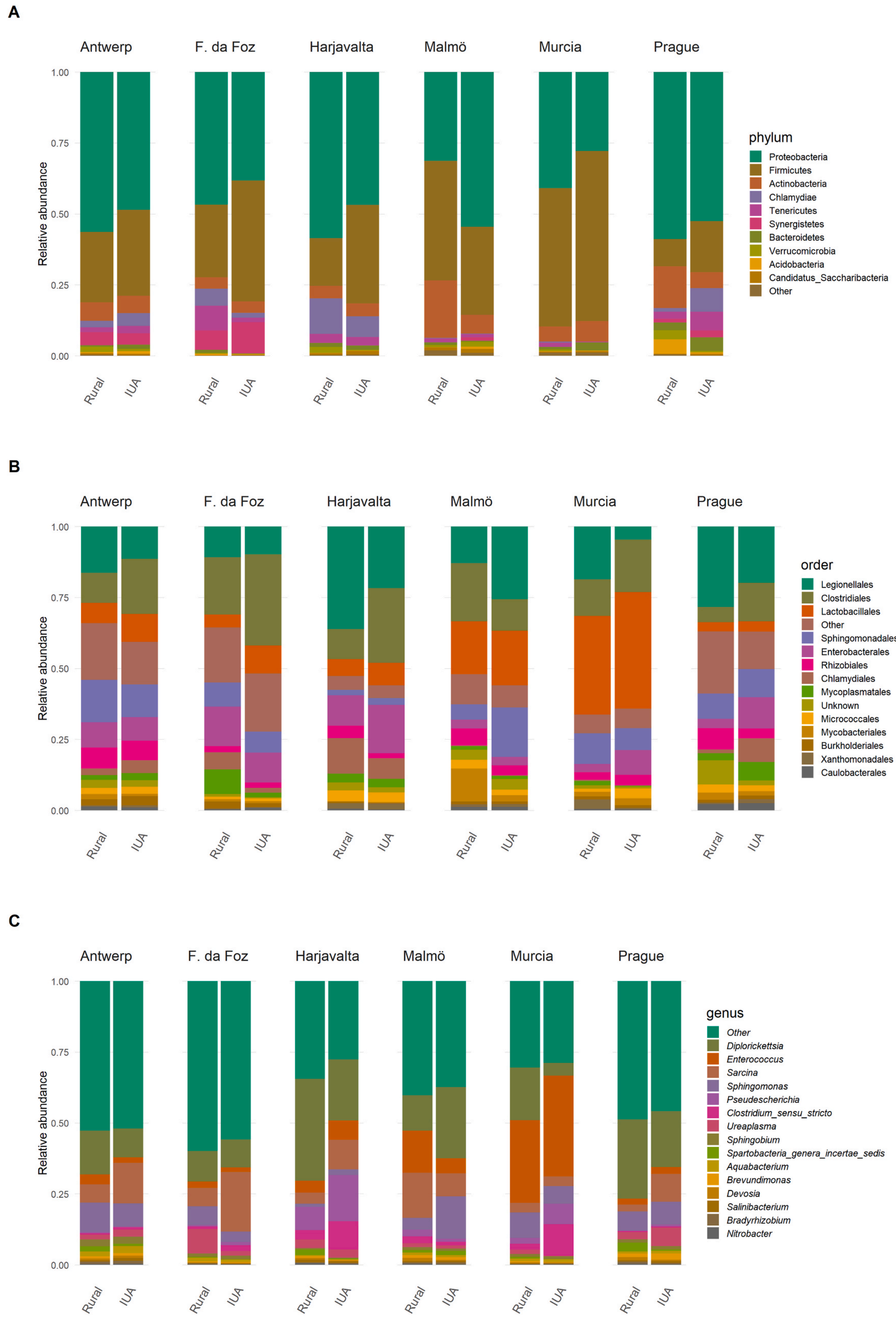


Fig. 2. Relative read abundances of **A)** bacterial phyla, **B)** orders, and **C)** genera, measured from the feces of great tits (*Parus major*) nestlings from rural and industrial/urban areas (IUA) in six European locations. Rare phyla, orders, and genera (prevalence of <40%, <70%, and <70% across all samples, respectively) were aggregated to class 'Other' to limit the number of shown taxa, for clarity. Note: the *Escherichia* genus was likely misidentified as *Pseudescherichia* (shown in the figure) from the same family of *Enterobacteriaceae.*

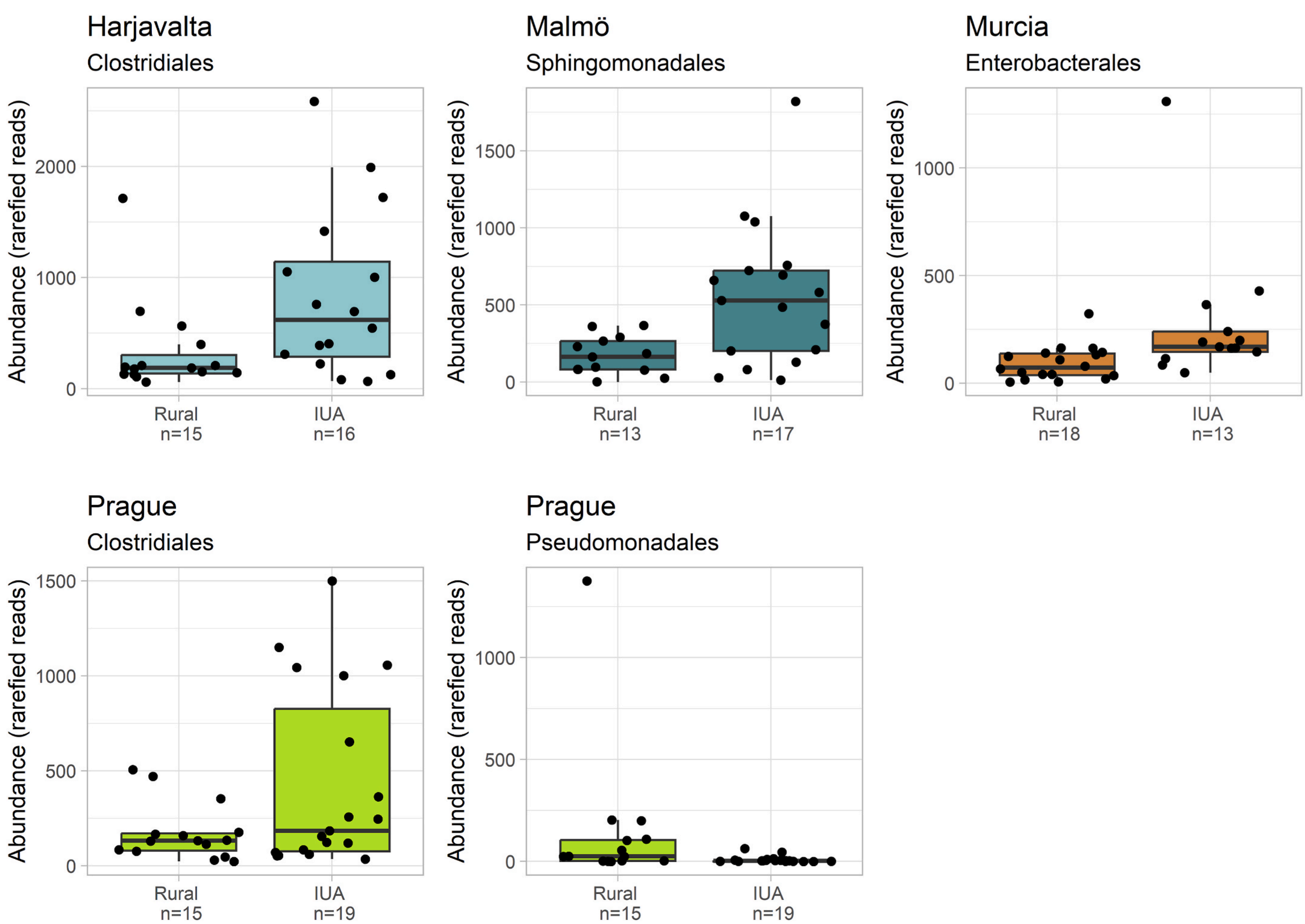


Fig. 3. Read abundances of bacterial orders identified as differentially abundant (DA) between rural and industrial/urban areas (IUA) in four study locations. DA analyses were performed on non-rarefied data (with rare taxa excluded) using five statistical methods: ALDEx2, LinDA, DESeq2, ANCOM-BC2, and corncob. For consistency in visualization, the figure displays data based on rarefied read counts.

sensu stricto, Ruminococcus, and *Tomitella*, in addition to the previously mentioned), detected from Malmö, Murcia, or Prague. For more detailed genus-level results, we refer to the Supplements (Fig. S9) and a separate supplementary file for the full DA data. Additionally, the following bacterial orders showed significant abundance differences among some of the six locations: Chlamydiales, Enterobacterales, Mycobacteriales, Lactobacillales, Desulfovibrionales, Burkholderiales, Sphingomonadales, and Streptomycetales (Fig. 4).

3.2.4. *Relationships between bacterial PCs, sites, and nestling performance*

The abundance of 23 gut bacterial orders, represented by PC_{Bac1}, PC_{Bac2}, and PC_{Bac3}, primarily depended on the location (Table 4; see also Table 2 for the contribution of orders to each PC). The PC_{Bac2} bacteria were also significantly positively associated with RBM, although the effect size was small (standardized $\beta = 0.13$). Moreover, PC_{Bac3} showed a significant area-location interaction, as these bacteria were elevated in only Malmö's rural area compared to the IUA ($F_{df} = 5.41_{1,\ 169}$, $p = 0.021$; LM). No associations were detected between the bacterial PCs and urbanization index or fledgling numbers.

Tukey's pairwise comparisons for locations showed that Antwerp and Prague had significantly higher PC_{Bac1} rates compared to Figueira da Foz and Murcia. Secondly, PC_{Bac2} showed higher rates in the "middle-latitude" locations – Antwerp and Prague, as well as Figueira da Foz – compared to Harjavalta, Malmö, and Murcia (Table 4; Fig. S5).

3.3. *Alpha and beta diversity*

The effects of metal levels, urbanization, and nestling performance on gut microbial alpha diversity (Shannon index, ZOTU level) were assessed using GLMs with a beta distribution. For all locations combined, the fledgling number was positively linked with alpha diversity ($F_{df} = 14.52_{1,\ 171}$, $p = 0.0002$). However, such an effect did not appear when analyzed separately by location. Moreover, the diversity was negatively associated with PC_{Met1} ($F_{df} = 5.27_{1,\ 26}$, $p = 0.030$) and positively with RBM ($F_{df} = 4.32_{1,\ 26}$, $p = 0.048$) in Antwerp, and positively associated with PC_{Met2} in Malmö ($F_{df} = 4.25_{1,\ 28}$, $p = 0.049$). No significant associations were found between the urbanization index and alpha diversity. For more information, see the supplementary materials for alpha diversity boxplots across areas and locations (Fig. S6) and Venn diagrams for the unique and overlapping ZOTUs between the urban and rural areas (Fig. S7).

The composition of nestling microbiota (assessed using PERMANOVA on the ZOTU level) was significantly affected by area, depending on the location (interaction term $F_{df} = 2.00_{5,\ 168}$, $p = 0.0001$). Both terms were also individually significant (area $F_{df} = 2.18_{1,\ 168}$, $p = 0.004$; location $F_{df} = 10.24_{1,\ 168}$, $p = 0.0001$). Furthermore, pairwise comparisons, sliced by location, showed that Antwerp was the only location where the composition did not significantly differ between the rural and IUAs. Additionally, the urbanization index significantly affected community composition ($F_{df} = 1.68_{1,\ 168}$, $p = 0.032$; however, the use of non-rarefied data with additional low-read samples led to a non-

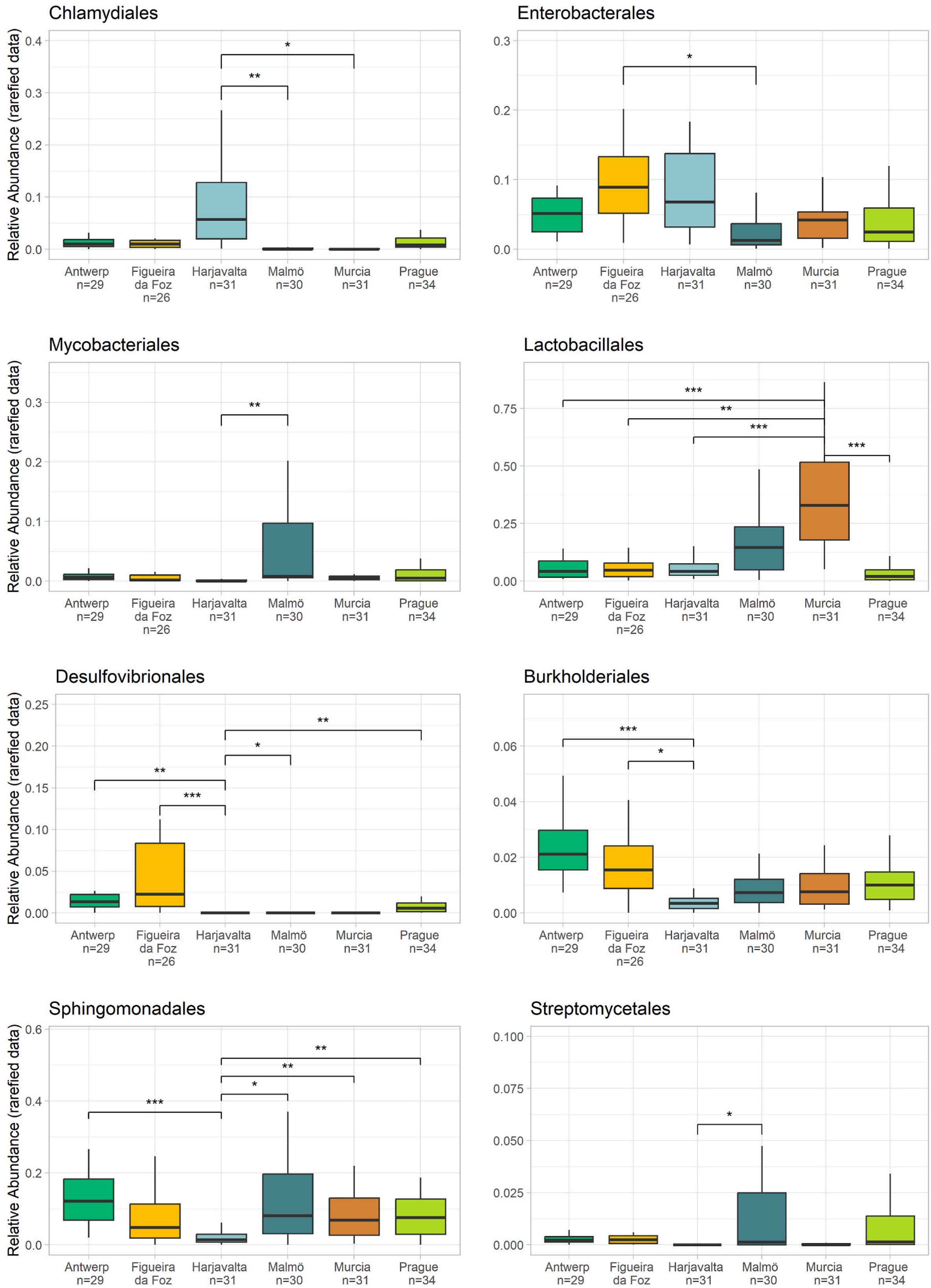


Fig. 4. Differentially abundant bacterial orders between six European locations, according to the ANCOM-BC2 differential abundance estimator. Statistically significant comparisons are shown (Holm-Bonferroni corrected *p*-value: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). Non-rarefied data with rare taxa excluded was used as input for the ANCOM-BC2 analysis; however, relative abundances of rarefied data are shown for clarity in the figure.

Table 4
Association of location, area (rural vs. industrial/urban area, IUA), imperviousness (UrbInd), fledgling number (FN), and nestlings' relative body mass (RBM) with three bacterial principal components (PC). Bolded variables were included in the final model, while others were excluded sequentially based on the highest p-value. Statistically significant Location term was further inspected with Tukey's post-hoc comparisons (adjusted p-values are considered: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ○ $p < 0.1$). For PC_{Bac3}, the significant Location*Area interaction was further examined by testing each rural-IUA comparison individually. The residuals of each model were normally distributed.

	PC_{Bac1}	PC_{Bac2}	PC_{Bac3} [a]
	F_{df} (Est. ±SE)	F_{df} (Est. ±SE)	F_{df}
Area (Rural-IUA)	$3.06_{1,\ 174}$ (0.25, ±0.14) ○	$2.52_{1,\ 173}$	**$0.01_{1,\ 169}$**
Location	**$5.03_{5,\ 175}$*** **	**$57.36_{5,\ 174}$*** **	**$18.09_{5,\ 169}$*** **
Location*Area	$0.31_{5,\ 158}$	$1.16_{5,\ 168}$	**$3.08_{5,\ 169}$* **
UrbInd	$0.33_{1,\ 163}$	$0.83_{1,\ 167}$	$0.12_{1,\ 167}$
FN	$1.12_{1,\ 165}$	$0.00_{1,\ 158}$	$0.02_{1,\ 158}$
RBM	$0.84_{1,\ 164}$	**$6.23_{1,\ 174}$ (0.007, ±0.003) ***	$0.44_{1,\ 168}$
Tukey's post-hoc comparisons for Location			
	t_{df} (Est. ±SE)	t_{df} (Est. ±SE)	-
Antwerp-F. da Foz	4.09_{175} (1.05, ±0.26) ***	-0.80_{174}	-
Antwerp-Harjavalta	2.59_{175}	8.95_{174} (1.44, ±0.16) ***	-
Antwerp-Malmö	1.84_{175}	10.99_{174} (1.63, ±0.15) ***	-
Antwerp-Murcia	3.04_{175} (0.75, ±0.25) *	10.28_{174} (1.71, ±0.17) ***	-
Antwerp-Prague	0.46_{175}	2.00_{174}	-
F. da Foz-Harjavalta	-1.63_{175}	9.59_{174} (1.56, ±0.16) ***	-
F. da Foz-Malmö	-2.33_{175}	11.51_{174} (1.75, ±0.15) ***	-
F. da Foz-Murcia	-1.20_{175}	10.91_{174} (1.83, ±0.17) ***	-
F. da Foz-Prague	-3.79_{175} (−0.94, ±0.25) **	2.77_{174} (0.42, ±0.15) ○	-
Harjavalta-Malmö	-0.74_{175}	1.22_{174}	-
Harjavalta-Murcia	0.46_{175}	1.85_{174}	-
Harjavalta-Prague	-2.23_{175}	-7.94_{174} (−1.14, ±0.14) ***	-
Malmö-Murcia	1.20_{175}	0.49_{174}	-
Malmö-Prague	-1.45_{175}	-9.23_{174} (−1.33, ±0.14) ***	-
Murcia-Prague	-2.69_{175} (−0.63, ±0.24) ○	-9.60_{174} (−1.41, ±0.15) ***	-

[a] The significant Location*Area interaction pairwise comparisons are presented in the article's main text.

significant result) whereas RBM did not (see the supplements: Table S7 for the full results and Fig. S8 for a visualization of the used Bray-Curtis distances).

Overall microbiota dispersion from the study group centroids differed among some groups (permutation test based on betadisper; $p < 0.001$). However, considering only the biologically relevant within-location comparisons, only urban Prague indicated a tendency toward higher bacterial variation compared to its rural counterpart (unadjusted $p = 0.02$), although this effect was not significant after multiple-testing correction (adjusted $p = 0.56$). Consequently, the PERMANOVA results for this comparison should be interpreted with slight caution.

3.4. Microbes in relation to metals and nestling performance

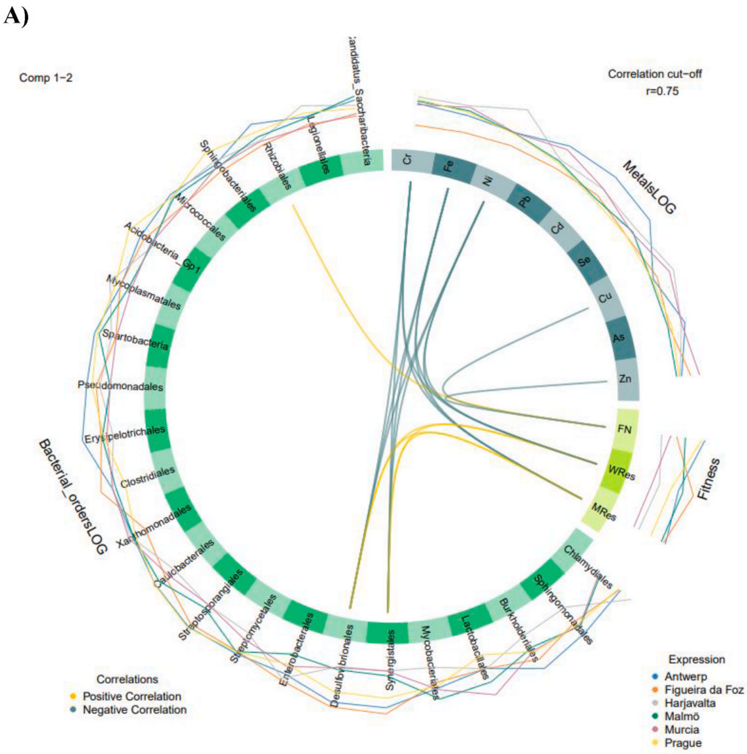
Six major phyla (covering a large proportion of the total microbiota) and four common orders (differing between rural and urban areas) were tested for associations with metal levels (PC_{Met1} and PC_{Met2}; GLM), and with RBM and fledgling number (LM), as these taxa were presumably relevant in the context of metal exposure or nestling performance. The bacteria were divided into two groups to avoid multicollinearity: the first group included Actinobacteria, Chlamydiae, Tenericutes, Synergistetes, Clostridiales, Sphingomonadales, and Enterobacterales, and the second Proteobacteria, Firmicutes, and Pseudomonadales. Actinobacteria showed a significant negative association with PC_{Met1} ($F_{df} = 5.25_{1,\ 179}$, $p = 0.023$) but not with PC_{Met2}, and a positive association with the fledgling number ($F_{df} = 7.15_{1,\ 170}$, p = 0.008) but not with RBM. Synergistetes was only associated negatively with PC_{Met2} and positively with RBM ($F_{df} = 11.54_{1,\ 178}$, $p = 0.0008$). Sphingomonadales showed positive associations with both fledging ($F_{df} = 10.71_{1,\ 170}$, $p = 0.001$) and RBM ($F_{df} = 7.58_{1,\ 178}$, $p = 0.007$), but only approached a significant negative association with PC_{Met1} ($p < 0.1$). Finally, Firmicutes showed a contrasting pattern by showing a positive association with PC_{Met1} ($F_{df} = 7.51_{1,\ 179}$, $p = 0.007$) and a negative association with fledging number ($F_{df} = 9.5_{1,\ 171}$, $p = 0.002$), but only closing in a significant negative association with RBM ($p = 0.054$). The rest of the inspected taxa (i.e., Chlamydiae, Tenericutes, Clostridiales, Enterobacterales, Proteobacteria, and Pseudomonadales) showed no statistically significant associations with metals or nestling performance.

Individual metals (Cr, Fe, Ni, Cu, Zn) tended to have negative correlations with some individual bacterial orders (Desulfovibrionales, Synergistales, Rhizobiales) and nestling performance (RBM, relative wing length, fledgling number), while, in contrast, these bacteria showed positive correlations with the performance. Pearson's correlations and a circular correlation plot are provided: the correlation plot was constructed using R package *mixOmics* v. 6.29.1 (Singh et al., 2019) following the González et al. (2012) approach (Fig. 5).

4. Discussion

4.1. Pollution across the European rural and IUAs

We hypothesized that fecal metal concentrations would be elevated in IUAs, as commonly observed, and that such metal exposure would be associated with alterations of the nestling gut microbiota (Adewumi and Ogundele, 2024; Eeva et al., 2009; Zhang et al., 2023). We further expected associations between pollution-related microbiota and impaired nestling growth and fledging. These hypotheses were tested using multiple complementary approaches. Consistent with our predictions, metal concentrations (tested using LM for Fe and GLM for other metals) were increased in the majority of IUAs. Microbiota composition differed between IUAs and rural areas in five out of six locations and was associated with the urbanization index (PERMANOVA), although this continuous index explained little of the other microbiota metrics, such as alpha diversity. The between-area differences were characterized by phylum-level shifts (LM & GLM) and by several individual taxa identified in the DA analyses. Moreover, some specified metal-associated taxa also correlated with nestling performance metrics (LM & GLM), which may be consistent with the hypothesis of microbiota-mediated health



B)

Link	Pearson correlation coefficient *r (n)*
Cr - MRes	−0.07 (181)
Cr - WRes	−0.14 (181) ○
Cr - Synergistales	−0.20 (173) **
Cr - Desulfovibrionales	−0.19 (173) *
Cu - FN	−0.30 (173) ***
Fe - MRes	−0.11 (181)
Fe - WRes	−0.16 (181) *
Fe - Synergistales	−0.31 (173) ***
Fe - Desulfovibrionales	−0.31 (173) ***
Ni - MRes	−0.25 (181) ***
Ni - WRes	−0.26 (181) ***
Ni - Synergistales	−0.34 (173) ***
Ni - Desulfovibrionales	−0.33 (173) ***
Zn - FN	−0.07 (173)
FN - Rhizobiales	0.32 (173) ***
MRes - Synergistales	0.42 (181) ***
MRes - Desulfovibrionales	0.40 (181) ***
WRes - Synergistales	0.45 (181) ***
WRes - Desulfovibrionales	0.43 (181) ***

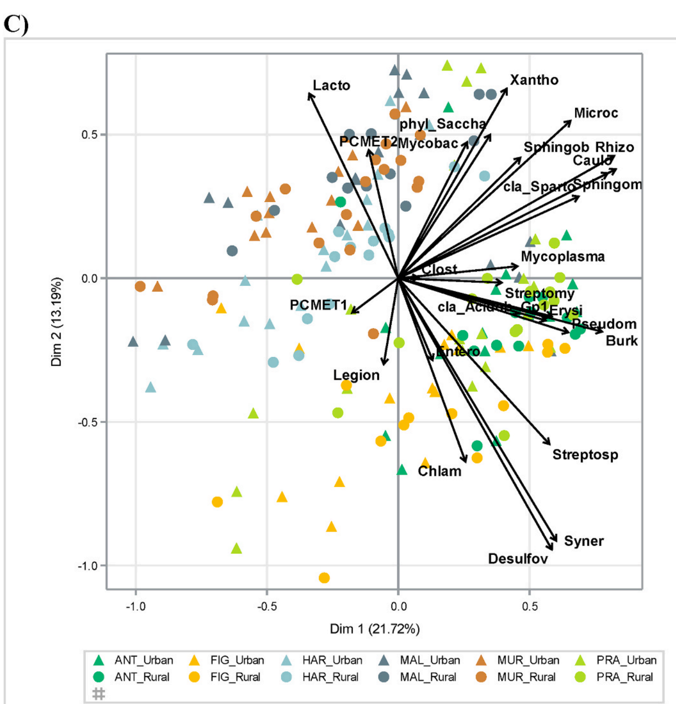


Fig. 5. **A)** Circular correlation plot showing positive correlations with yellow and negative with blue links among three variable blocks: log10-transformed read counts of 23 bacterial orders (n = 181) and levels for 9 metals (n = 173), as well as nestling performance traits ("Fitness": fledgling number FN, relative wing length WRes, and relative body mass MRes; n = 174–181). Intra-block correlations and low correlations with r < |0.75| are excluded. Correlations are based on latent component scores, not raw values. Outer lines indicate the expression of the variables in each of the six European locations. E.g., the peaking grey line represents high Ni levels in Harjavalta. The figure is based on DIABLO modeling. B) Significance of the links shown in the circular correlation plot tested with separate Pearson's correlations on raw data (metals and bacteria log-transformed). $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; ○ $p < 0.1$). C) Biplot of Varimax-rotated metal principal components (PC_{MET1}: Cd, Zn, As, Pb, Cu, Se; PC_{MET2}: Cr, Fe, Ni) with 23 common bacterial orders at the industrial/urban and rural areas of six European locations: Antwerp (ANT), Figueira da Foz (FIG), Harjavalta (HAR), Malmö (MAL), Murcia (MUR), and Prague (PRA). The datapoint distribution primarily describes bacterial communities, as the metal PCs have little effect on the distribution; removing them changes Dim 1 and 2 explanatory rates to 23.48% and 13.96%. Created using SGPLOT procedure in SAS. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

associations with metal exposure (Zhang et al., 2023). Such mechanistic interpretations, however, remain speculative, as they could not be directly tested within this observational study design; reverse causation and shared environmental drivers may also underlie these patterns and are further discussed.

The metal exposure profile (As, Cd, Cr, Cu, Fe, Pb, Ni, Se, Zn) measured from the nestling feces considerably varied among the European locations (Fig. S2). As predicted in our first hypothesis, exposure levels were, overall, higher in the IUAs than in rural areas, except for in Figueira da Foz, as discussed later. The emission sources varied from metallurgical industry to agriculture and urban traffic/energy supply, which are known to contaminate air, soil, and water with metals that further bioaccumulate in food webs. Besides varying emission profiles, local food webs likely modify the metal exposure of birds (Gall et al., 2015).

Malmö and Prague, both representing urban rather than industrial environments, showed similar element profiles, with no excessive peaks in any of these elements compared to other locations. However, both locations exhibited moderately elevated levels of most elements in their urban areas – particularly copper in Malmö and arsenic in Prague. In addition to natural sources, copper contamination in urban areas can result from, e.g., traffic (brake wear), while arsenic may originate from fossil fuel combustion, biomass burning, or pesticide use (Adewumi and Ogundele, 2024; Denier van der Gon et al., 2007; European Environment Agency, 2023).

Levels of As were peaking in the industrial areas of Antwerp, Harjavalta, and Murcia, which was expected based on previous research on birds on these sites (see Table 1). The peaking of Cd was in common for the urban Antwerp and Murcia, as well as high Pb, especially in Murcia, where mining has been practiced since ancient Roman Empire times until the activity was finally ceased in 1992 (Conesa and Schulin, 2010). In addition, Murcia's distinctive pollution characteristics were the high Cu levels in both rural and industrial areas and the highest mean levels of the common side-product contaminants of mining, Fe and Zn, among all the IUAs.

Of all locations, Harjavalta had distinctively high Cu and Ni levels in the IUA due to the Cu smelter activity since 1945 and Ni smelter activity since 1960 (Kiikkilä, 2003). The observed mean level of Ni was over 8-fold higher compared to the second-ranking area, the industrial Antwerp. Of all the urban locations, Antwerp showed the highest levels of Se – a contaminant produced mainly by metal industry in Belgium (European Environment Agency, 2023).

Figueira da Foz showed an interesting but somewhat expected contrast to other locations. The surroundings of a paper mill represented the industrial study areas; however, the great tit nestlings had been more exposed to metals in the rural areas. Depending on the sample type studied, higher levels of Cd, As, and occasionally other metals have previously been measured in the rural areas, potentially resulting from the use of pesticides or fertilizers in the nearby agricultural fields (Costa et al., 2012, 2013, 2017). Metal levels near the paper mill have generally been low, but tend to show elevated mercury, which was not measured in this study due to methodological constraints. Overall, the measured metal levels in great tit feces were low in both Portuguese areas, in accordance with the previous research.

4.2. Gut microbiota of the great tit across Europe

4.2.1. Abundance and functions

The observed major bacterial phyla (Proteobacteria, Firmicutes, and Actinobacteria, respectively) largely reflected patterns found in earlier studies on great tits (Kropáčková et al., 2017; Rainio et al., 2025). However, the ranking of the three phyla seems to vary from study to study (see e.g., Maraci et al., 2022). Our results show that the proportions of major phyla vary depending on location, as, for example, the Murcian bird microbiota was dominated by Firmicutes rather than Proteobacteria. One of the major drivers of the location effect in this study is likely the diet, which can vary substantially among the European locations. However, we did not have comparable diet data available on these specific locations. It is noteworthy that the phyla composition may also shift with aging (e.g., increasing Firmicutes) (Teyssier et al., 2018a) and depend on the section of the gastrointestinal tract, creating variability among studies – in birds such as passerines that lack a developed caeca, the fecal microbiota likely largely represents that of the lower gastrointestinal tract (Schmiedová et al., 2024).

Proteobacteria are a large and variable group of gram-negative bacteria tolerant to various oxygen conditions, with rather speculative functions within the bird intestines (Grond et al., 2018). In mammalian intestines, although present in much smaller proportions compared to avians (suggesting some unknown but potentially noteworthy functions in birds) (Grond et al., 2018), they likely contribute to the digestion of various organic molecules and have been hypothesized to pioneer the colonization of the intestines by consuming oxygen and creating a suitable habitat for strict anaerobes (Nalage et al., 2024; Shin et al., 2015; Těšický et al., 2024). Various potential pathogens are included: for example, *Escherichia* is part of the normal gut flora, but some strains found in birds can cause disease. *Escherichia* was the 2nd most abundant genus in Harjavalta nestlings, and the 4th, considering all locations. Moreover, genera such as *Brucella, Rickettsia, Neisseria, Shigella, Yersinia, Pseudomonas, Acinetobacter,* and *Enterobacter* (but also, e.g., *Helicobacter* and *Campylobacter,* which belong to Campylobacterota), were present in low abundances in the studied nestlings and have been described as pathogens in humans and animals (Nalage et al., 2024; Poosakkannu et al., 2025; Rizzatti et al., 2017).

The second largest group in our data, the predominantly gram-positive Firmicutes, is known for producing short-chain fatty acids (SCFAs) by digesting fibers and fermenting carbohydrates in the animal gut. SCFAs are well-known microbial metabolites that have several benefits for the host physiology, including the use for cellular energy, aiding nutrient absorption and defense against oxidative stress, reducing inflammation, and promoting the intestinal barrier against harmful substances and pathogens (Grond et al., 2018; Maiuolo et al., 2024). On the other hand, Firmicutes also include several potential pathogen groups often found in birds, such as Mycoplasmatales and Clostridiales (the latter of which also includes health-promoting species), which were detected in this study.

Some bacterial orders were distinguished as particularly different in abundance between the European locations, with known functions varying from pathogens to antibiotic producers, normal gut flora, and environmental microbiota. Such were Enterobacterales, Desulfovibrionales, Burkholderiales, and Sphingomonadales (phylum: Proteobacteria); Mycobacteriales and Streptomycetales (Actinobacteria); Chlamydiales (Chlamydiae); and Lactobacillales (Firmicutes). To mention a few examples, Burkholderiales (typical for both environmental and intestinal habitats) and Sphingomonadales (usually found in environmental habitats) had relatively low abundances in Harjavalta, while the pathogen-associated Chlamydiales (Ravichandran et al., 2021) were surprisingly largely abundant. Chlamydiales has previously been associated with metal(loid)-contaminated soil (Abbaszade et al., 2023), but we found no such association in bird feces, despite Harjavalta being among the most contaminated locations. Contrastingly, Malmö and Murcia had especially low Chlamydiales and high Lactobacillales levels. However, we did not detect significant associations between the pathogenic Chlamydiae and nestling performance, potentially due to the lack of separate analyses for locations.

Interestingly, the study area of Figueira da Foz, influenced by agriculture and the pulp mill, showed the highest levels of Desulfovibrionales in the bird microbiota. These sulfate-reducing bacteria are associated with pulp production, producing foul-smelling hydrogen sulfide (H_2S) (Maukonen et al., 2006). Additionally, plants can use sulfate-reducing bacteria to enhance sulfur uptake as sulfate (SO_4^{2-}), an essential nutrient often supplied through chemical fertilizers (Narayan et al., 2023). The abundance of Desulfovibrionales suggests influence from local sulfur sources, reaching the gut microbiota of birds in nearby areas. However, similar or lower sulfur levels in bird feces compared to Murcia, where Desulfovibrionales was not elevated, highlight the impact of factors other than general environmental sulfur availability.

4.2.2. Two urbanization approaches and metal exposure as predictors of microbiota variations

The effects of urbanization on wild bird gut microbiota are better studied than those of metal pollution, yet separating these impacts remains challenging since urbanization, as well as industrial land use, often overlap with pollution. We assessed the associations between microbiota and anthropogenic disturbance using two complementary approaches: a continuous urbanization index based on impervious surfaces, and categorical comparisons between urban/industrial and rural areas. While several microbiota alterations were observed between areas, only beta diversity (community composition) showed a significant association with the continuous urbanization index, indicating relatively weak support for our hypothesis 2 a), especially since the used rarefaction protocol affected the significance. The lack of significance regarding the urbanization index may be influenced by high variation within and between IUAs. For example, in Murcia and Figueira da Foz, imperviousness levels did not differ significantly between rural areas and IUAs. Such heterogeneity was also noted by Maraci et al. (2022). The insensitivity of the urbanization index to explain microbial abundance could also mean that it does not capture all the essential characteristics of the relatively complex phenomena of industrialization and urbanization, and can therefore act merely as one complementary approach to study microbiota variations.

More evident differences in microbiota were revealed at the area level (rural vs. IUA): the area was a significant factor in determining community composition, the abundance of major phyla (Proteobacteria, Firmicutes, Actinobacteria, Chlamydiae), bacterial orders belonging to PC_{Bac3} (including especially Mycobacterales, Streptomycetales, and Chlamydiales), and several orders and genera identified through the differential abundance analysis (e.g., Clostridiales, Sphingomonadales, Enterobacterales, and Pseudomonadales). Maraci et al. (2022) similarly observed changes in microbiota composition in juvenile great tits when comparing urban and rural environments and identified an increase of some related taxa in urban areas, such as *Enterobacteriaceae* and *Sphingomonadaceae*, although the different taxonomic levels make comparisons challenging. This is demonstrated by our results, where some genera belonging to the same order show opposing abundance patterns between study areas (Fig. S9). Overall, our hypothesis 2 b) of the IUA-related microbiota alterations seems to get support, although the specific nature of these alterations was highly dependent on location.

The third approach to examine anthropogenic disturbance, fecal metal levels, also seemed to reveal location-specific impacts on microbiota and, as such, partial support for hypothesis 2 c): PC_{Met1} metals (Cd, Zn, As, Pb, Cu, and Se) showed a negative association with the microbial alpha diversity in Antwerp, and the metals in PC_{Met2} (Cr, Fe, and Ni) a positive association in Malmö. Moreover, higher metal levels were generally associated with lower Actinobacteria abundance (and potentially lower Sphingomonadales, which approached significance), while, surprisingly, Firmicutes were positively associated with metals. When looking at the connections between microbes and Cr, Fe, and Ni individually (Fig. 5), an increase in these metals was linked with lower fecal abundances of Synergistetes/Synergistales and Desulfovibrionales. This may also reflect potential underlying drivers such as reduced habitat and diet quality, as the nestling body condition was positively linked with these bacteria.

Importantly, the associations found may reflect secondary effects of urbanization or pollution, as the consequent environmental alterations can affect insect availability/quality (Eeva et al., 2005) and even the great tit's foraging behavior (Grunst et al., 2019), likely leading to shifts in the bird gut microbiota. Evidence of habitat change-related shifts in great tit diet between rural and IUAs has been found, e.g., in Harjavalta, where parental provisioning of chicks with caterpillars and the biomass of ground-living arthropods near the metal smelter were decreased (Eeva et al., 1997). In a three-year study by Sinkovics et al. (2021), a rural-urban area comparison of great tit food provisioning showed that urban parents could not fully compensate for the decrease of high-quality caterpillar prey by providing the chicks with other arthropods or non-arthropods (such as spiders) during poor years. Diet is a key factor for microbiota composition and has been connected to microbiota alterations also in great tits (Bodawatta et al., 2021). Therefore, differences in nestling gut bacterial communities between rural and IUAs may be a secondary effect of pollution/urbanization, mediated by the supposedly lower caterpillar availability and compensation with other invertebrates in the IUAs. Microbiota alterations could, therefore, act as one potential biomarker for anthropogenic changes in habitat quality.

4.3. Fledging and growth

4.3.1. Nestling performance differs between rural and IUAs only in some locations

The number of fledglings varied by location and area: mid-latitude locations, Antwerp and Prague, showed higher overall offspring number, while the northernmost urban areas in Harjavalta had the lowest (note that the fledgling number largely depends on the initial clutch size; Fig. S4). In the IUAs of Harjavalta and Prague, fledgling numbers were significantly lower (corresponding with, e.g., de Satgé et al., 2019), and RBM was lower in the IUA of Figueira da Foz, but most locations showed no rural–IUA differences in fledgling number or RBM. Thus, our hypothesis no. 3 predicting weaker nestling performance in IUAs held true in only two to three locations out of six. In Harjavalta, population density-dependent decline in clutch size has likely intensified in the polluted environment, despite the dramatic reduction in metal emissions over recent decades (Eeva and Lehikoinen, 2013; Kiikkilä, 2003). In Figueira da Foz, the paper mill has not previously shown negative effects on breeding success or nestling body mass – likely due to good food availability and low pollution – but we found decreased RBM near the mill (Costa et al., 2011).

4.3.2. Associations among nestling performance, microbes, and anthropogenic disturbance

The associations between bird performance parameters and bacterial alpha diversity (Shannon index) were sporadic and location-specific, showing no clear patterns and weak support for hypothesis no. 4 regarding this specific microbiota metric. Moreover, the gut bacterial community composition (PERMANOVA for beta diversity) was unrelated to body mass – a result that seems to have varying support from other studies (Teyssier et al., 2018a; Worsley et al., 2021). Also, only one of the bacterial PCs showed a significant link to RBM, and the effect size was small, suggesting questionable biological relevance.

Individual bacterial taxa appeared to have more evident associations with fledgling numbers and relative body mass than diversity, better aligning with our predictions. Moreover, some bacterial taxa that were associated with nestling performance were also associated with metals or IUAs (hypothesis no. 5), indicating either a direct or indirect relationship among these bacteria, nestling health, and anthropogenic disturbance. Firmicutes were linked to fewer fledged chicks and showed a near-significant association with lower body mass, contrasting with earlier findings in birds and mammals, where Firmicutes have been linked to weight gain and immune function (Grond et al., 2018; Maiuolo et al., 2024). As also observed in a study on sparrows by Zhang et al. (2023), Firmicutes were more abundant in our IUAs and associated with higher metal levels. Based on other literature, their capability to form highly resistant endospores under various stress factors, including heavy metal exposure, potentially allows them to outcompete other phyla (Fajardo et al., 2019; Koopman et al., 2022). Some opportunistic pathogens belonging to Firmicutes, such as two Clostridiales genera (*Sarcina* and *Clostridium sensu stricto*), were elevated in some IUAs and have been associated with disease in mammals and poultry (Makovska et al., 2023; Yang et al., 2019). Moreover, *Ruminococcus,* another Clostridiales genus, was less abundant in an urban area. They are often considered generally beneficial for gut health through SCFA production, but the genus also includes potentially pathogenic species (La Reau and Suen, 2018).

At the order level, both Synergistales and Desulfovibrionales were linked to higher RBM and longer relative wing length, while showing negative correlations with Cr, Fe, and Ni. Overall, taxa contributing to PC_{Bac2} – particularly Synergistales, Desulfovibrionales, Streptosporangiales, and Lactobacillales (the latter with a negative contribution) – were associated with heavier nestlings, possibly reflecting local differences in food availability. Desulfovibrionales have accordingly also been associated with obesity and metabolic disorders in humans and rodents, but better condition and survival in birds, whereas certain probiotic Lactobacillales may counteract these effects (Qin et al., 2012; Wang et al., 2015; but see also, Marková et al., 2024).

Among the bacterial orders that differed between rural areas and IUAs (Clostridiales, Sphingomonadales, Enterobacterales, and Pseudomonadales), only Sphingomonadales – more abundant in urban Murcia – showed a positive link to fledging and RBM. Though typically soil- and plant-associated, Sphingomonadales have been detected in birds and their ability to degrade metallic compounds has been discussed (Asaf et al., 2020; Giorgio et al., 2018; Hird et al., 2015). A bacterial PC including Sphingomonadales (from the great tit nest materials) was also found to correlate positively with nestling RBM in our earlier study on Harjavalta birds (Leino et al., 2024).

4.3.3. Limitations and future directions

This study focused on gut microbiota characteristics in relation to pollution gradients, urbanization, and nestling growth and fledging across Europe, using a study design of control and polluted areas of similar habitat types to ensure comparability of the areas. However, several potentially influential environmental factors, such as pollution types other than metal(loid)s, diet composition, latitude, climate, vegetation (although the impervious surface degree likely also reflects vegetation abundance), and yearly variation, were beyond our scope. Notably, a recent study on adult great tits across Europe found no effects of latitude, rainfall, or temperature on microbiota, nor differences between deciduous and mixed forests during summer (Liukkonen et al., 2024). Nonetheless, the environment is widely recognized as a key factor in shaping gut microbiota, particularly during the sensitive early-life stages in birds (Somers et al., 2023).

We used pooled fecal samples to measure brood level microbiome and metal exposure. Pooled samples are preferred in enabling reliable metal exposure estimates (see Methods) but can potentially also affect our microbiome measures such as the alpha diversity. In general, however, the gut microbiomes of nestlings tend to be more similar within than between broods (Benskin et al., 2015; Teyssier et al., 2018a), because siblings share the same parents and environment, and similar early-life diet and microbial inoculation. Potentially affected diversity values would not, however, bias our comparison between polluted and control sites because the sampling was similar everywhere. Measuring individual microbiomes would still produce more information about pollution-related effects on within-brood variability.

Bacterial taxa linked to bird health may be influenced by metal exposure – a notable ecotoxicological concern, especially given the complex metal mixtures typical of urban and industrial pollution. However, multifaceted interactions, including reverse causation, are likely, and these causalities cannot be confirmed with an observational study design. As discussed, metal exposure may be the primary factor affecting bird physiology either directly or through dietary changes, with microbiota changes being secondary. Furthermore, bacteria can modify their metabolic functions involved in heavy metal resistance, as well as affect metal toxicity and the host intake of metals, reflecting the active role of microbes in host-pollution dynamics (Duan et al., 2020; Zhang et al., 2023).

Microbiota characteristics seem to vary with the type and source of pollution (e.g., urbanization or mining), making generalizations difficult and emphasizing the importance of studies in versatile real-life conditions (see, e.g., Schmitt et al. (2025), where pigeons in captivity showed little microbiota changes under zinc/lead administrations). While this study is correlational, it is nevertheless, to our knowledge, the first cross-European approach giving initial results that would deserve to be confirmed by an experimental approach. Our high-throughput 16S rRNA metabarcoding approach provides a baseline for the large-scale ecological situation of microbiota characteristics across various pollution sources and reference areas, offering directions for future studies that could address functional aspects of bacteriomes under pollution.

5. Conclusions

Great tit nestlings in metal-polluted urban or industrial environments showed partially different gut microbiota profiles than those in the rural reference areas. However, one of the key findings was the heterogeneity of these alterations among locations across Europe. For example, the bacterial composition was altered in five out of six locations. The relative abundances of several major taxa were altered, and the body mass and fledgling number decreased in urban areas, but this did not apply to all study locations equally, emphasizing the context-dependent impacts of urbanization or pollution on wildlife in natural habitats. This study further provides methodologically comparable data on metal levels to which birds are exposed at varying pollution source types across Europe, as well as information on the related gut microbiota alterations and implications for nestling survival. As wild birds in industrial and urban areas are often exposed to complex mixtures of metals, alongside numerous other environmental stressors, future experimental research in natural habitats and analyses of bacterial functions are needed to further disentangle the consequences of pollution-altered microbiota on wildlife.

CRediT authorship contribution statement

Lyydia I. Leino: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rute A. Costa:** Writing – review & editing, Investigation. **Marcel Eens:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Caroline Isaksson:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Pere Puigbò:** Writing – review & editing, Methodology. **Miia J. Rainio:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Pablo Sánchez-Virosta:** Writing – review & editing, Resources, Investigation, Data curation. **Eero Vesterinen:** Writing – review & editing, Supervision, Methodology. **Michal Vinkler:** Writing – review & editing, Resources, Investigation, Data curation. **Antonio Zamora-López:** Writing – review & editing, Resources, Investigation, Data curation. **Jose Manuel Zamora Marin:** Writing – review & editing, Resources, Investigation, Data curation. **Ann-Kathrin Ziegler:** Writing – review & editing, Investigation. **Tapio Eeva:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used GPT-4-5 solely to improve the language and readability of the article. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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.

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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.2026.124012.

Data availability

Research data available at: https://doi.org/10.17632/2grtt8j4sb.1 Data: Living in polluted and urban habitats across Europe is related to altered gut microbiota and nestling performance in a common passerine bird (Parus major) (Original data) (Mendeley Data)

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