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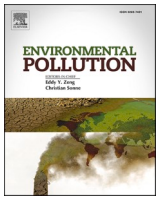

Pollution-related changes in nest microbiota: Implications for growth and fledging in three passerine birds☆

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ABSTRACT

Non-ferrous smelters emit toxic metals into the environment, posing a threat to wildlife health. Despite the acknowledged role of microbes in host health, the impact of such emissions on host-associated microbiota, especially in wild birds, remains largely unexplored. This study investigates the associations of metal pollution, fitness, and nest microbiota (serving as a proxy for early-life microbial environment) which may influence the nestling health and development. Our study focuses on three passerine birds, the great tit (*Parus major*), blue tit (*Cyanistes caeruleus*), and pied flycatcher (*Ficedula hypoleuca*), within control and metal-polluted sites around a Finnish copper-nickel smelter. The polluted sites had been contaminated with arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), and zinc (Zn). We performed bacterial 16S rRNA sequencing and metal analyses on 90 nests and monitored nestling body mass, fledging success, and various biotic and abiotic factors. Our findings revealed species-specific responses to metal exposure in terms of both fitness and nest microbiota. *P. major* and *C. caeruleus* showed sensitivity to pollution, with decreased nestling growth and fledging in the polluted zone. This was accompanied by a shift in the bacterial community composition, which was characterized by an increase in some pathogenic bacteria (in *P. major* and *C. caeruleus* nests) and by a decrease in plant-associated bacteria (within *C. caeruleus* nests). Conversely, *F. hypoleuca* and their nest microbiota showed limited responses to pollution, indicating greater tolerance to pollution-induced environmental changes. Although pollution did not correlate with nest alpha diversity or the most abundant bacterial taxa across all species, certain potential pathogens within the nests were enriched in polluted environments and negatively correlated with nestling fitness parameters. Our results suggest that metal pollution may alter the nest bacterial composition in some bird species, either directly or indirectly through environmental changes, promoting pathogenic bacteria and potentially impacting bird survival.

1. Introduction

Microbes, consisting of bacteria, archaea, fungi, and viruses, play an essential role for a wide range of developmental and physiological processes within animals. The importance of microbiota for these processes, such as early-life adaptive immune system functions (Honda and Littman, 2016), digestion and vitamin synthesis (Rowland et al., 2018), and metabolization of harmful substances (Claus et al., 2016) has recently been recognized in mammals. However, less is known about the health-related consequences and the factors shaping the microbiota within different bird species – especially concerning the nest microbiota (Evans et al., 2016; Grond et al., 2018; Zabłotni et al., 2020).

Bird nest forms an early-life microbial environment for altricial bird species, and plays a role in the colonization of nestling intestines, together with parental contact and food (Chen et al., 2020; Kimura et al., 1986; Mills et al., 1999; Teyssier et al., 2018a). The presence and load of

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these early-life environmental microbes, including those in the nest, may mediate bird development and condition (Ruuskanen, 2024). For example, bacterial load and various culturable strains in nest or plumage have been associated with the physiological condition (such as feather growth, body mass gain, and blood hemoglobin concentration) and breeding success in cavity-nesting passerines (Azcárate-García et al., 2019; González-Braojos et al., 2012; Saag et al., 2011; Zabłotni et al., 2020). However, it is unclear how the composition and diversity of these bacterial communities, including non-culturable bacteria, impact the condition of nestlings. Additionally, the impact of abiotic environmental factors such as metal pollution (Zhang et al., 2023; Zhou et al., 2023) and temperature (Sepulveda and Moeller, 2020; Zhu et al., 2019) on the bird-associated microbiota has been poorly studied, despite their potentially significant impact.

The characteristics of nest materials vary among bird species and habitats, likely influencing the early-life exposure of birds to microbes (Drobniak et al., 2021; Goossens et al., 2022; Grond et al., 2018; Ruuskanen, 2024). Consequently, the type of nest materials can affect the condition of nestlings. Feathers and aromatic plants in the nest may reduce harmful bacterial loads on eggs and nestlings by harboring antibiotic-producing bacteria and plant volatiles (Mennerat et al., 2009; Ruiz-Castellano et al., 2019, 2016). For example, a well-studied insectivorous passerine, the blue tit (*Cyanistes caeruleus*), utilizes these antibacterial materials (e.g. feathers, *Rhododendron tomentosum* leaves, *Juniperus communis* bark) to line its moss-based nest. Similarly, the great tit (*Parus major*) constructs its nest primarily using mosses, many of which are known for their antimicrobial properties (Rydgren et al., 2023; Veljic et al., 2008). However, unlike *C. caeruleus*, *P. major* typically lines its nest with animal hair (Alambiaga et al., 2020; Järvinen and Brommer, 2020), potentially influencing the microbiota within the nests. An opposing example of nest materials is provided by the pied flycatcher (*Ficedula hypoleuca*), a migratory species with nests mainly consisting of bark and leaves from pines and birches. Thus, the nest materials and their associated microbes could play a species-specific role in shaping the early-life microbiota and condition of birds, as implicated by previous comparisons of nest microbiota in *P. major* and *C. caeruleus* (Goodenough and Stallwood, 2010), as well as in various other bird species (Peralta-Sánchez et al., 2012).

One factor that may shape the nest microbiota of wild birds is anthropogenic pollution, such as toxic metals. While some metals, including Zn and Cu, are essential for life in small amounts, most can have toxic effects on microbes (Fashola et al., 2016), plants (Tiwari and Lata, 2018), and animals (De Francisco et al., 2003; Sun et al., 2022) even at low concentrations. Industrial activities, especially non-ferrous smelters, emit high levels of metal(loid)s (e.g., As, Cd, Cu, Ni, Zn, and Pb) into the environment, altering the soil microbiota and their functions (Chen et al., 2022; Fashola et al., 2016; Li et al., 2017). These persistent and bioaccumulative elements also occur within bird nest materials in polluted areas (Kiikkilä, 2003), potentially affecting the bird nest and gut microbiota via direct toxicity and indirectly via changes in the environment (Gall et al., 2015). There is likely a bidirectional relationship between the host-associated microbiota and metals, since gut microbes act as the first barrier against toxicity, while metal-related alterations in the composition and metabolic profile of the microbiota have been observed (Arun et al., 2021; Breton et al., 2013b, 2013a; Brila et al., 2021; Duan et al., 2020; Liu et al., 2014; Richardson et al., 2018). Recently, similar patterns have been suggested to occur in birds (Acheampong, 2022; Gillingham et al., 2021; Kou et al., 2019; Zhou et al., 2023).

In this study, we perform a non-invasive examination of associations between anthropogenic metal pollution, nest microbiota, nestling growth, and fledging success of three common, cavity-nesting bird species (*P. major*, *C. caeruleus*, and *F. hypoleuca*) in the wild, while also taking into consideration other potentially important variables such as the temperature and humidity inside the nest chamber, nest material pH, and brood size. We expected differences in the nest microbiota between metal-contaminated and control study areas, as well as among different bird species, likely due to factors such as the presence of inhibitory metals and variations in nest materials affecting bacterial communities. Moreover, we hypothesize that alterations in the nest microbiota, possibly induced by metal pollution, could be linked to fledging success and nestling growth. Several studies have considered the effects of metal pollution on the wildlife-associated microbiota (Brila et al., 2021; Giambò et al., 2021; Pinowski et al., 1994; Yan et al., 2020), but to our knowledge, this is the first investigation concerning associations between heavy metals, nest microbiota, and fitness in wild birds.

2. Materials and methods

2.1. Study design and sites

To investigate the associations between metal pollution and nest microbiota of birds, nest microbiota samples were collected from three wild bird species (*F. hypoleuca*, *P. major*, and *C. caeruleus*) breeding in nest boxes in metal-contaminated and relatively clean control areas. Our nine study sites were located within and in the surroundings of Harjavalta town (61°20′ N, 22°10′E) in southwestern Finland. Four of these sites were located within 2.5 km of a metal pollution source, a copper-nickel smelting industrial complex (Kiikkilä, 2003). Sites close to the smelter (hereafter: “polluted zone”), remain contaminated by several toxic metals such as As, Cd, Cu, Ni, and Zn, despite a significant reduction in emissions during the last decades (Eeva et al., 2018). Furthermore, five separate control sites (hereafter: “control zone”) were located at least 5 km away from the smelter. In these areas, metal levels in the soil approach the natural background levels (Ruiz et al., 2017). The study sites were chosen to represent similar *Pinus sylvestris*-dominated habitats with a mix of coniferous and deciduous trees, to avoid variation between the sites.

Each study site included 20–60 nest boxes that allowed us to track the breeding of the birds. Ca. 30 nests were chosen for each study species (*C. caeruleus* n = 29, *P. major* n = 31, *F. hypoleuca* n = 30), half of which located in the polluted zone. We inspected these nest boxes weekly during the breeding season to collect data on the timing of breeding, the number of nestlings, and their body mass and fledging success. Furthermore, we tracked the ambient growing conditions for microbes in the nests by placing temperature-humidity loggers (Hygrochron DS1923 iButton) inside the nest boxes. The loggers measured relative humidity and temperature in 3-h intervals during 8 days from hatching (i.e., until the sampling). When checking the nests, we carefully avoided any microbial contamination by wearing sterilized laboratory gloves and using sterilized equipment when there was a need to touch the nest e.g., to estimate the age of nestlings by measuring their wing chord length.

2.2. Sampling and nest metal analyses

Licenses for collecting, handling, and storing samples of the three protected bird species and their nests were acquired from the Centre for Economic Development, Transport and the Environment (licence number VARELY/3622/2017), and the Regional State Administrative Agency for Southern Finland (license number ESAVI/3021/04.July 10, 2017). These licenses fully complied with legal requirements and were respected throughout the process. The sampling was conducted by experienced bird ringers authorized by the Finnish Museum of Natural History.

The nest microbes were sampled at an average age (±SD) of 8 ± 1.0 days for the chicks, with efforts made to minimize variation (range 5–11 days due to logistical constraints). The chicks were individually ringed and weighed. Day 8 post-hatching is a convenient age for ringing the chicks to individualize them for later metrics (e.g., fledging), while providing a standardized time frame for them to interact with nest microbiota. Microbes from the nest materials were sampled by swiping a

sterile, phosphate-buffered saline (PBS) -moistened swab (Copan FLOQSwabs 30 mm) inside the nest cup for 30 s, i.e., sampling the nest surface materials in contact with the chicks. Next, the swab samples were stored at −20 °C on-site and later transferred to a −80 °C freezer for storage. After the chicks had fledged, the nests were collected for further analyses to determine the number of dead nestlings, nest material pH, and metal concentrations.

Metal levels (As, Cd, Cu, Ni, Zn) were measured by gathering <0.5 g of dried nest materials (e.g., fur, moss, bark, grass, depending on the nest composition) from each nest and by using ICP-OES Spectrometer Analyser (Thermo Scientific iCAP 6500 duo, CEBAS-CSIC Murcia University) according to Espín et al. (2016). Certified reference material (TORT-2 by National Research Council of Canada (NRC)) was used to validate the metal levels. Recoveries from the reference material were relatively high, ranging from 125% to 165%. For this reason, and to reduce dimensionality of the data, we produced principal components (PC) of these highly correlated metal concentrations instead of using absolute values. The two PCs (hereafter: PC_{Met1} and PC_{Met2}) with Promax rotation were formed together for all bird species using SAS Enterprise Guide (EG) 8.3 (SAS Institute Inc, 2013) and were used in the further analyses to describe the general metal levels within the nests. PC_{Met1} consisted mainly of As, Cu, Ni, and Cd (eigenvalue 4.29, explained 85.8% of the variation) and PC_{Met2} mainly of Zn (eigenvalue 0.51, 10.2% of the variation). To confirm the differential exposure of nestlings to metals in the polluted and control zones, PC_{Met1} and PC_{Met2} were tested using zone and species as explanatory variables in a linear model.

2.3. Molecular analysis

DNA extraction from the swab samples was conducted following Vesterinen et al. (2016). The locus-specific PCR was targeted to bacterial 16S rRNA gene hypervariable V4 region using primers 515FB (oligo: GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016; Walters et al., 2016) and 806RB (oligo: GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015; Caporaso et al., 2011). For the preparation of the next-generation sequencing (NGS) library, we followed the 2nd PCR protocol from Vesterinen et al. (2016). All the resulting reactions were then pooled and purified following a dual-SPRI (solid-phase reversible immobilization) magnetic bead protocol (Vesterinen et al., 2018). The sequencing run was carried out by Finnish Functional Genomics Centre (FFGC) (University of Turku and Åbo Akademi University, Finland), on Illumina NovaSeq6000 SP Flowcell 2 × 250 bp (Illumina Inc., San Diego, California, USA), including a PhiX control library. Our subsequent bioinformatics pipeline closely followed Kaunisto et al. (2020). Shortly, the raw reads were trimmed, merged, the PCR primers were removed using the software CUTADAPT 2.7 (Martin, 2011), reads were dereplicated, and then collapsed into sequence variants (ZOTUs) using 'unoise3' in USEARCH 11 (Edgar, 2010). The number of ZOTUs in each sample was assessed and all ZOTUs were assigned to taxa using USEARCH/V-SEARCH SINTAX algorithm using pre-built database (*16S RDP training set v16*) downloaded from https://drive5.com/usearch/manual/sintax_downloads.html; Edgar (2016). The DNA extractions, NGS library preparation, sequencing and bioinformatics were conducted by DNA-analysis company Bioname (Turku, Finland; www.bioname.fi). The final data was transformed to relative abundances (RA; see also Fig. S1) (McKnight et al., 2019) and this proportional data was used in all downstream analyses unless otherwise stated. Detailed molecular methods and bioinformatics are provided in the Supplements.

2.4. Statistical analysis

2.4.1. Study variables

The statistical analyses for fitness-related parameters, alpha diversity, and selected bacterial taxa were performed using generalized linear models (GLM) or linear models (LM) with the GLIMMIX procedure in SAS EG 8.3 (SAS Institute Inc, 2013). To investigate the associations between bird nest microbiota and potentially associated conditions, the analyses considered the following variables: bird species, zone (polluted or control), metal PCs, bacterial PCs, alpha diversity indices (Shannon index and observed richness, i.e. the number of ZOTUs, produced using *microbiome* 1.20.0 in R (Lahti and Shetty, 2022; R Core Team, 2023)), relative body mass of nestlings (RBM), probability of fledging, brood size and nestling age at the time of sampling, temperature and humidity within the nest chamber, and nest material pH. RBM indicates the relative (%) deviation of the mean body mass of a brood from the mass predicted by a long-term (1991–2022) growth curve derived from the same area. Furthermore, the bacterial PCs were derived from the log-transformed RAs of 21 orders (see Fig. S2 and Table S1 for the criteria and further details) with the Princomp procedure in SAS EG. Three most informative PCs were used in the further analyses: PC_{Bac1} (eigenvalue 6.02, explains 28.6% of the variance), PC_{Bac2} (eigenvalue 1.89, explains 9.0%), and PC_{Bac3} (eigenvalue 1.70, explains 8.1%). These variables were tested for variance inflation values (VIF) to avoid multicollinearity in each model. Statistical models consisting of the variables were reduced stepwise based on *p*-values, unless otherwise stated.

2.4.2. Beta diversity

Permutational multivariate analysis of variance (PERMANOVA) was performed for the bacterial community composition using "adonis2" (*vegan* 2.6–4; Oksanen et al., 2022) in R Statistical Software (4.2.2; R Core Team, 2023). The Bray-Curtis distances (999 permutations) were calculated based on the ZOTU RAs and used as the response variable for pollution zone, species, zone-species interaction, temperature, nestling RBM, humidity, age and brood size at sampling, and pH. Subsequently, the zone-species interaction was studied further with pairwise comparisons (*pairwiseAdonis* 0.4.1; Martinez Arbizu, 2017) using Benjamini-Hochberg adjusted *p*-values. The visualizations were created by using the function "ordinate" (*phyloseq* 1.42.0; McMurdie & Holmes, 2013) to perform principal coordinate analysis (PCoA) of the Bray-Curtis distances in R and by creating a biplot based on the bacterial order PCs in SAS EG.

Furthermore, to examine the patterns in bacterial composition in more detail, we used three bacterial PCs PC_{Bac1} (beta distribution, GLM), PC_{Bac2} and PC_{Bac3} (LM) to examine their associations with bird species, metal PCs, RBM, humidity, and temperature.

2.4.3. Differential abundance analysis

Differential abundance (DA) analysis was performed on five different tools in R to find a consensus of the DA taxa. Such multiestimator approach has been recommended, as different DA tools commonly produce differing results (Nearing et al., 2022). The tools used were ALDEx2 (1.30.0) (Fernandes et al., 2014, 2013; Gloor et al., 2016), LinDA (0.1.0) (Zhou et al., 2022), ANCOM-BC2 (2.0.2) (Kaul et al., 2017; Lin and Peddada, 2020; Peddada and Lin, 2023), DESeq2 (1.38.3) (Love et al., 2014), and Corncob (0.3.1) (Martin et al., 2020). The analysis was performed separately for each bird species on five taxonomic levels (phylum, class, order, family, and genus) by using 10% prevalence-filtered observed read counts as the input, and pollution zone (polluted versus control) as the grouping variable. Taxa that statistically significantly differed between the zones (Benjamini--Hochberg-adjusted *p*-value <0.05) according to the majority of the DA tools, i.e., at least three of the tools, were considered differentially abundant.

To investigate the significance of the identified DA taxa for bird fitness within each bird species, we calculated their effects on fledging and RBM and subsequently incorporated the significant taxa into more comprehensive statistical models, where the effects of several pathogens on fledging (GLM) and RBM (LM) were assessed.

2.4.4. Fitness parameters

Effects of various terms on the fledging probability (fledglings/

hatchlings) and nestling RBM were investigated for each bird species using binomial distribution for the fledging (GLM) and Gaussian for the RBM (LM). Such models, having these fitness-related terms as the response variables, included three main courses: 1. The effects of metal and bacterial PCs, pH, temperature, humidity, and brood size, while the effect of the zone on fitness was tested separately; 2. The effects of alpha diversity (i.e. Shannon index and observed richness); and 3. The effects of known avian pathogens and other relevant taxa including potentially

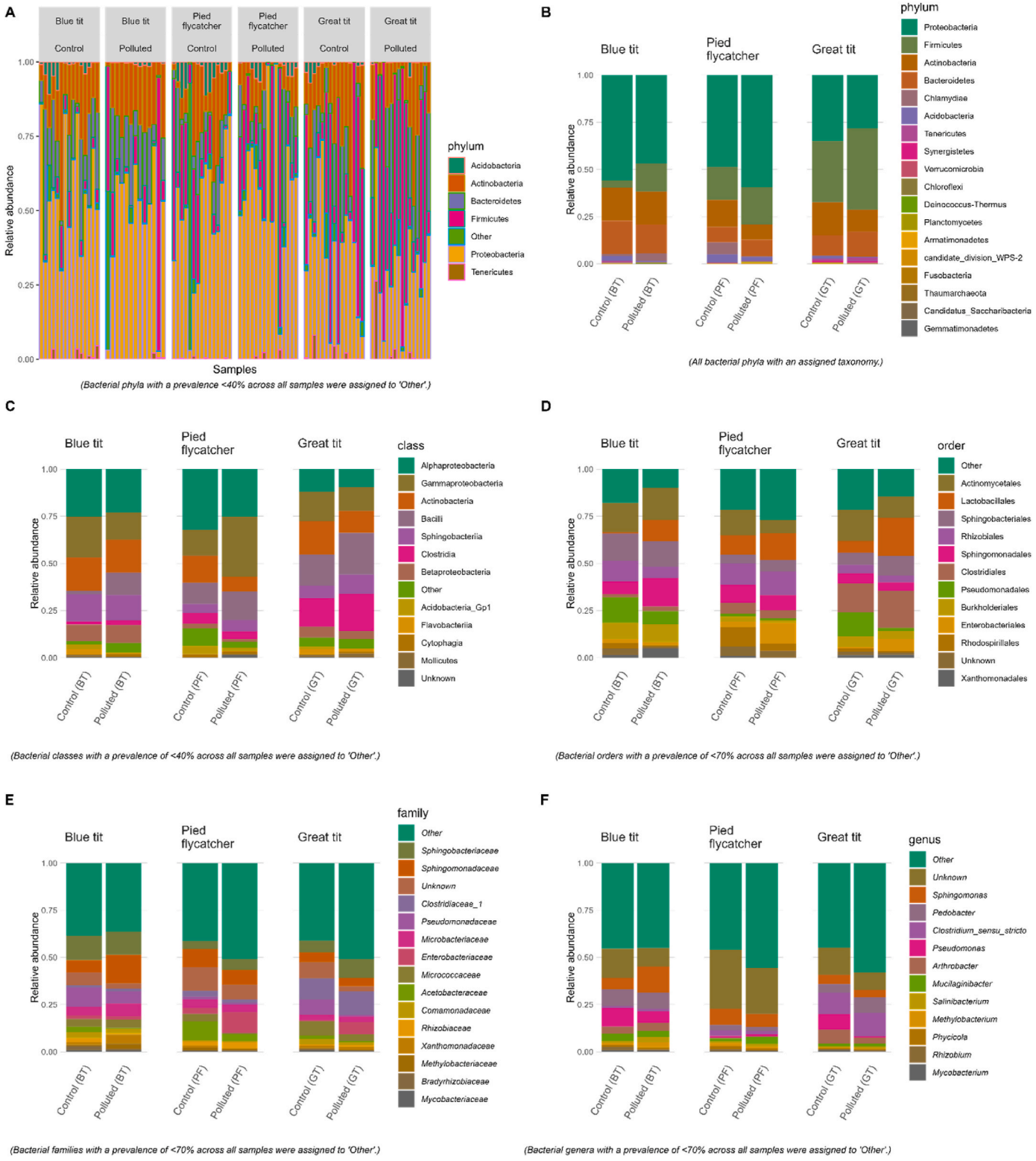

Fig. 1. Relative abundances (RA) of bacteria in the nests of blue tits, pied flycatchers, and great tits in the control zone and metal-contaminated polluted zone. **A)** Each nest sample displayed individually to show the variability of bacterial phyla between nests. Mean RAs are shown on the **B)** phylum-level **C)** class-level **D)** order-level **E)** family-level and **F)** genus-level with a specified prevalence threshold calculated from all samples across species. Taxa below the threshold (across all species) were aggregated into the class "other". (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pollution-associated bacteria based on literature and our DA analyses (Giacopello et al., 2016; Hubá, 2004; Ravichandran et al., 2021; Schmidt et al., 2022; Yang et al., 2019; Zhou et al., 2023).

Moreover, we explored the impacts of different biotic and abiotic factors on the following nest microbiota parameters potentially associated with bird fitness: alpha diversity, known pathogens and pollution-associated taxa, as well as the predominant taxa based on relative abundances. First, we tested the effects of the following factors on the alpha diversity indices: zone, nestling RBM, temperature, humidity, and brood size and age at sampling (GLM; beta distribution). Second, the RA of known avian pathogens and other relevant taxa were tested using metal PCs and bird species as explanatory variables (GLM and Tukey's post-hoc tests for species). Third, we examined a set of predominant taxa in relation to metal PCs and other conditions (LM for Proteobacteria and GLM for other taxa), and assessed their interspecific differences (Tukey's post-hoc tests for species).

In all the models, residual-type (R-side) random component was included in all the models to account for model overdispersion. The suitable distribution was determined for each model either by the Shapiro-Wilk test for residual normality (if $p > 0.05$, Gaussian distribution was used) or by comparing the Pearson Chi-Square degrees of freedom (df) for non-Gaussian residual distributions. All distribution assumptions were used with the default link functions in SAS.

The data used in this study is publicly accessible via the Mendeley Data service at https://doi.org/10.17632/vs2g9cdfx4.1 (Leino et al., 2024).

3. Results

3.1. Core microbiota

In total, 18 bacteria phyla (Fig. 1) were found from the nests of the three bird species, six of which (Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Acidobacteria, and Tenericutes) were prevalent in at least 40% of all samples. By species, the majority of the nest microbiota in *C. caeruleus* (mean RA: 51.7%, SD = 20.0) and *F. hypoleuca* (54.0%, SD = 22.6) belonged to Proteobacteria, while Proteobacteria RA was statistically significantly lower in *P. major* (31.4%, SD = 16.8; Tukey's post-hoc test, $t_{df} = 3.85_{85}$, $p = 0.0007$ (*C. caeruleus–P. major*) and $t_{df} = 4.40_{85}$, $p_{adj} > 0.0001$ (*F. hypoleuca–P. major*), see Table S2). In contrast, Firmicutes, the most abundant phylum in *P. major* (mean 37.9%, SD = 30.5), was less abundant in the other species ($t_{df} = -4.79_{85}$, $p_{adj} < 0.0001$ (*C. caeruleus–P. major*) and $t_{df} = -2.56_{85}$, $p_{adj} = 0.032$ (*F. hypoleuca–P. major*)). The number of genera present in at least half of the samples were 60, 38, and 38 in *C. caeruleus*, *P. major*, and *F. hypoleuca* nests, respectively. On average, the most abundant genera within *C. caeruleus* nests were *Sphingomonas* (mean RA: 9.6%, SD = 6.9), *Pedobacter* (8.7%, SD = 9.5), and *Pseudomonas* (8.1%, SD = 14.1). The *P. major* nests were characterized by *Clostridium sensu stricto* (11.8%, SD = 20.5), *Catellicoccus* (7.3%, SD = 18.6), and *Pedobacter* (6.5%, SD = 8.8), and the *F. hypoleuca* nests by *Diplorickettsia* (13.9%, SD = 24.9), *Enterococcus* (8.2%, SD = 18.2), and *Sphingomonas* (7.8%, SD = 9.2).

3.2. Metal exposure within different zones

To confirm the differential metal burden within the nests between the polluted and control zones (Fig. 2), PC_{Met1} and PC_{Met2} were tested using zone and species as explanatory variables. Statistically significant elevations in metal levels (both PC_{Met1} and PC_{Met2}) were identified in polluted zone compared to control zone (LM; PC_{Met1}: $F_{df} = 552.90_{1,\ 86}$, $p < 0.0001$; PC_{Met2}: $F_{df} = 63.15_{1,\ 86}$, $p < 0.0001$). Bird species showed differences regarding only PC_{Met2} (i.e. Zn), with the *F. hypoleuca* nests carrying lower levels of PC_{Met2} metals compared to both tit species (Tukey's post-hoc test, *C. caeruleus* $t_{df} = 4.07_{86}$, $p = 0.0003$ and *P. major*

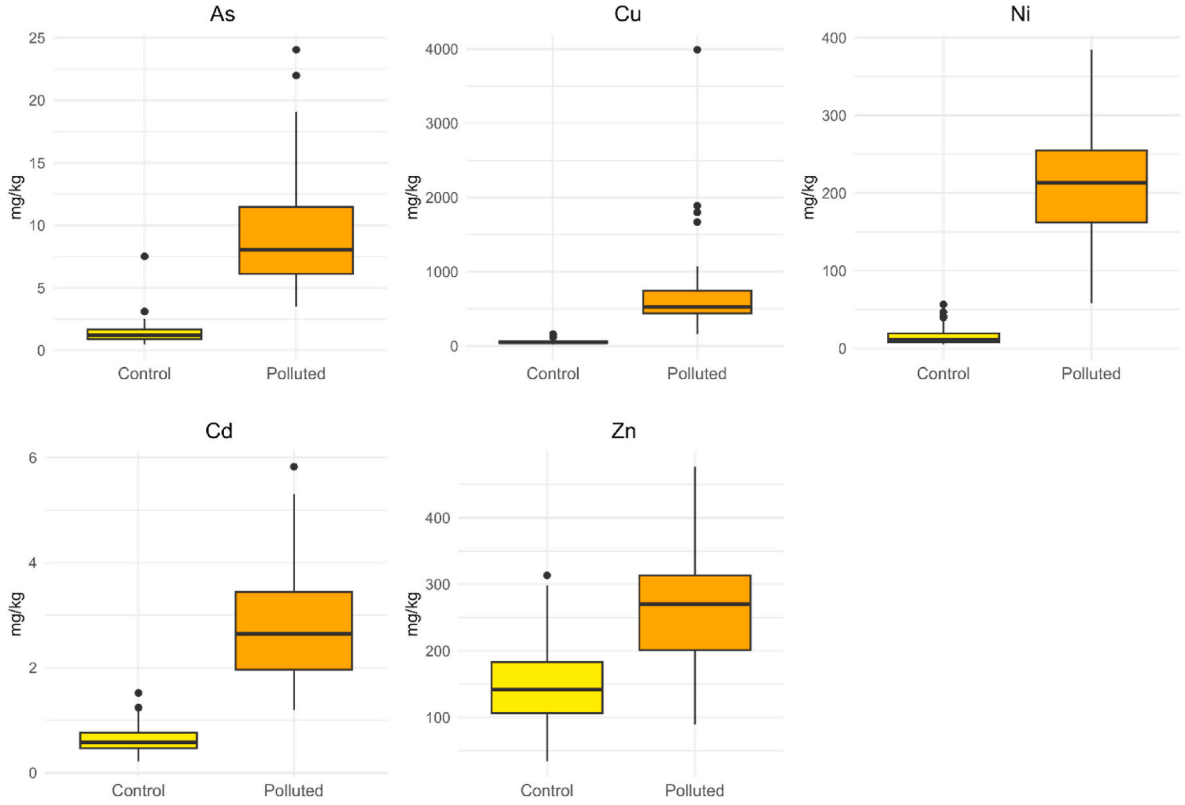

Fig. 2. Mean concentrations (mg/kg) of As, Cu, Ni, Cd, and Zn as measured from the nest materials of great tits, blue tits, and pied flycatchers (n = 90) within the polluted zone (<2.5 km from the pollution source) and control zone (>5 km from the pollution source). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

$t_{df} = -4.85_{86}$, $p < 0.0001$).

3.3. Beta diversity

The microbial composition among bird nests demonstrated statistically significant differences concerning the zone, bird species, temperature, nestling RBM, and the interaction of zone and species (PERMANOVA in R, Table S3). In contrast, the nest chamber humidity, age at sampling, brood size, and nest pH did not show any significance and were consequently removed from the model (Table S3). The microbial composition differed between the zones in both tit species but not in *F. hypoleuca* (pairwise comparisons with Benjamini-Hochberg-adjusted *p*-values for *P. major* $F = 2.03$, $R^2 = 0.065$, $p_{adj} = 0.005$ (n = 31); *C. caeruleus* $F = 1.59$, $R^2 = 0.062$, $p_{adj} = 0.010$ (n = 26); and *F. hypoleuca* $F = 1.38$, $R^2 = 0.047$, $p_{adj} = 0.103$ (n = 30). Moreover, the composition within the *F. hypoleuca* nests differed from all other groups (Fig. 3, see Table S3 for the full results).

The bacterial principal components (PC_{Bac1}, PC_{Bac2}, and PC_{Bac3}) represented highly correlating bacterial orders within the nests. PC_{Bac1}, consisting mainly of soil and plant-associated bacteria, included Burkholderiales, Sphingobacteriales, Sphingomonadales, Caulobacterales, Actinomycetales, Rhizobiales, Cytophagales, Rhodospirillales, and Solirubrobacterales. In contrast, PC_{Bac2} was characterized by several common taxa of gut bacteria: Erysipelotrichales, Clostridiales, Bacteroidales, and Enterobacteriales. PC_{Bac3} included taxa with variable origins and ecological roles: Flavobacteriales, Mycoplasmatales, Pseudomonadales, Bacillales, and Xanthomonadales. See Fig. S2 and Table S1 for further details.

These bacterial PCs were mostly not explained by the nest metal levels when investigating the effects of various conditions on the bacteria, with the exception of PC_{Bac2}, which showed a species-specific association to the metal level (Table 1). Nevertheless, all bacterial PCs exhibited among-species differences and were associated with various environmental conditions, depending on the specific component. For example, bacteria represented by PC_{Bac1} showed a positive correlation with nestling RBM and a negative correlation with ambient temperature, while PC_{Bac3}-associated bacteria increased with rising humidity within the nest boxes.

3.4. Differential abundance analysis

Differential abundance analysis revealed that the abundance of Enterobacteriales (LinDA, Log2FC = 4.80, SE = 1.19, $p = 0.009$) and *Catellicoccus* (ANCOM-BC2, Log2FC = 3.00, SE = 0.92, $p = 0.031$) showed increased RA in the polluted zone within *P. major* nests (Fig. 4). Within the *C. caeruleus* nests in the polluted zone, *Intrasporangiaceae* (LinDA, Log2FC = 2.58, SE = 0.66, $p = 0.022$) were increased and *Acetobacteraceae* (LinDA, Log2FC = −1.85, SE = 0.50, $p = 0.022$), Rhodospirillales (LinDA, Log2FC = −1.96, SE = 0.54, $p = 0.033$), and *Roseiarcaceae* (LinDA, Log2FC = −3.08, SE = 0.70, $p = 0.014$) decreased compared to the control nests. In contrast, no significantly different bacterial taxa within *F. hypoleuca* nests between the zones were identified by a minimum of three DA tools. The total number of different DA taxa found by ALDEx2, ANCOM-BC2, LinDA, DESeq2, or Corncob were 31, 26, and 17 for *C. caeruleus*, *P. major*, and *F. hypoleuca*, respectively. All results of the DA analysis for each tool can be found in Table S4.

3.5. Fitness parameters in relation to metals and microbes

The pollution zone, in addition to various other biotic and abiotic factors, showed no associations with the nest alpha diversity (Fig. S3). Only the nestling RBM and temperature correlated with the alpha diversity (Table S5): higher RBM corresponded to higher alpha diversity (Shannon index) in the nests of *F. hypoleuca* (GLM, $F_{df} = 11.26_{1,\ 28}$, $p = 0.002$) and *P. major* ($F_{df} = 4.49_{1,\ 28}$, $p = 0.043$), while the ambient temperature was negatively linked with alpha diversity in *P. major* nests (Shannon index $F_{df} = 8.29_{1,\ 28}$, $p = 0.008$; observed richness $F_{df} = 8.42_{1,\ 29}$, $p = 0.007$). However, no significant impact of alpha diversity on fledging probability was detected across species.

Nest metals, microbiota, and other potential covariates were assessed for each bird species to investigate their effects on fledging probability or nestling RBM (hereafter: 'fitness parameters') (Table 2). The effect of zone on fitness parameters was assessed separately: the polluted zone was associated with lower fitness in *C. caeruleus* (fledging $F_{df} = 13.25_{1,\ 27}$, $p = 0.001$; RBM $F_{df} = 9.43_{1,\ 25}$, $p = 0.005$) and *P. major* (fledging $F_{df} = 8.48_{1,\ 29}$, $p = 0.007$; RBM $F_{df} = 18.64_{1,\ 29}$, $p = 0.0002$), but not in *F. hypoleuca* (fledging $F = 0.00_{1,\ 28}$, $p = 0.985$; RBM $F = 0.15_{1,\ 28}$, $p = 0.703$), resembling the effect of measured metal levels on the bird fitness.

The bacterial genus *Catellicoccus* that was elevated within the

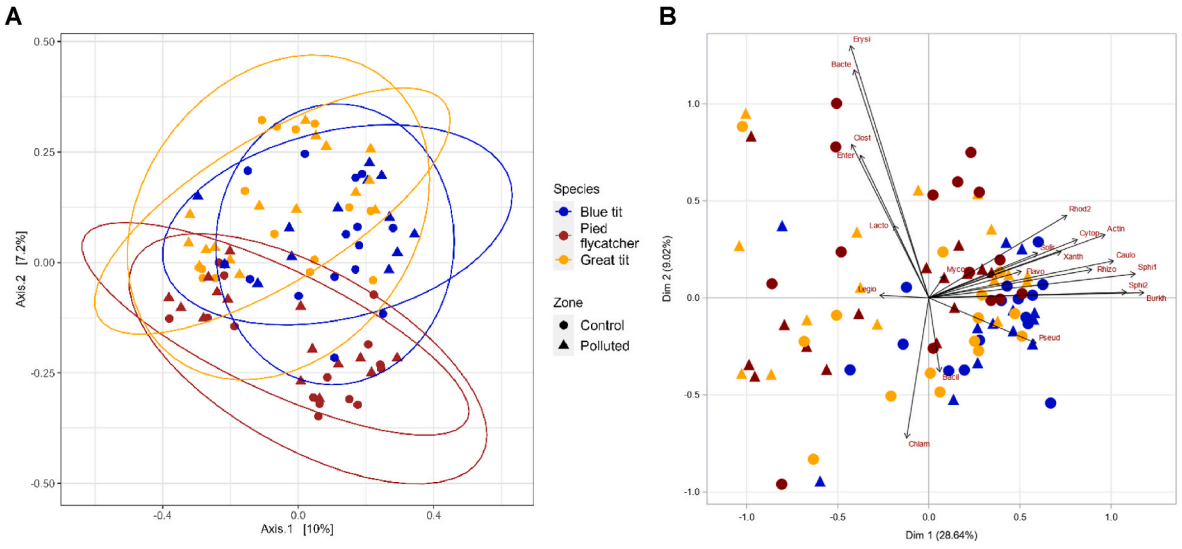

Fig. 3. Analysis of microbial community composition within the nests of three bird species (N = 89) in metal-polluted and control zones. **A)** Dissimilarity metrics of the overall microbiota among samples (PCoA with Bray-Curtis distances), based on relative ZOTU abundances. The ellipses represent 95% confidence levels. **B)** A biplot illustrating the samples and the distribution of 21 bacterial orders across bacterial principal components (Dim 1 and 2) with associated percentages of explained variance. The length and orientation of the vectors describe the impact of each order on individual PCs and their correlation with one another.

Table 1
Associations between nest bacterial orders and various conditions, with principal components PC_{Bac1}, PC_{Bac2}, and PC_{Bac3} serving as the response variables. The fixed factors included the bird species and the most informative metal principal component, PC_{Met1}, owing to their significance in the study. Otherwise, the model for each PC was reduced according to the *p*-values. Statistically significant results are bolded. The total sample size with all species included was n = 89 (28, 30, and 31 for blue tits (*Cyanistes caeruleus*), pied flycatchers (*Ficedula hypoleuca*), and great tits (*Parus major*), respectively, but may vary between models due to some missing values).

Model[a]	PC_{Bac1}[b]		PC_{Bac2}[c]		PC_{Bac3}[c]	
	F_{df}	*p*	F_{df}	*p*	F_{df}	*p*
Species (fixed)	$10.08_{2, 81}$	**0.0001**	$3.35_{2, 83}$	**0.040**	$17.59_{2, 84}$	**<0.0001**
PC_{Met1} (fixed)	$0.11_{1, 81}$	0.742	$0.01_{1, 83}$	0.919	$3.12_{1, 84}$	0.081
PC_{Met2}	$0.04_{1, 77}$	0.850	$1.73_{1, 79}$	0.192	$0.00_{1, 77}$	0.993
Temperature	$11.99_{1, 81}$ (Est. −0.03, SE 0.01)	**0.0009**	$0.08_{1, 78}$	0.784	$0.17_{1, 78}$	0.683
Humidity	$0.68_{1, 80}$	0.412	$0.00_{1, 77}$	0.975	$5.79_{1, 84}$ (Est. 0.03, SE 0.01)	**0.018**
Rel. body mass	$10.07_{1, 81}$ (Est. 0.01, SE 0.002)	**0.002**	$1.30_{1, 80}$	0.257	$0.28_{1, 79}$	0.597
Species* PC_{Met1}	$0.71_{2, 78}$	0.494	$3.97_{2, 83}$	**0.023**	$1.00_{2, 82}$	0.372
Comparison	**Tukey's post-hoc tests for species**					
	t_{df}	**Adj. *p***	t_{df}	**Adj. *p***	t_{df}	**Adj. *p***
C. caeruleus* – *F. hypoleuca	3.20_{81} (Est. 0.22, SE 0.07) *C. caeruleus* ↑	**0.006**	-2.60_{83} (Est. −0.89, SE 0.34) *F. hypoleuca* ↑	**0.029**	4.74_{84} (Est. 1.31, SE 0.28) *C. caeruleus* ↑	**<0.0001**
C. caeruleus* – *P. major	4.21_{81} (Est. 0.26, SE 0.06) *C. caeruleus* ↑	**0.0002**	-1.44_{83}	0.327	-0.75_{84}	0.735
F. hypoleuca* – *P. major	0.60_{81}	0.822	1.21_{83}	0.453	-5.48_{83} (Est. −1.52, 0.28) *P. major* ↑	**<0.0001**

[a] Brood size was not included in the final models, as it showed no statistical significance.
[b] Beta distribution with the Logit link function.
[c] Normal distribution with the Identity link function.

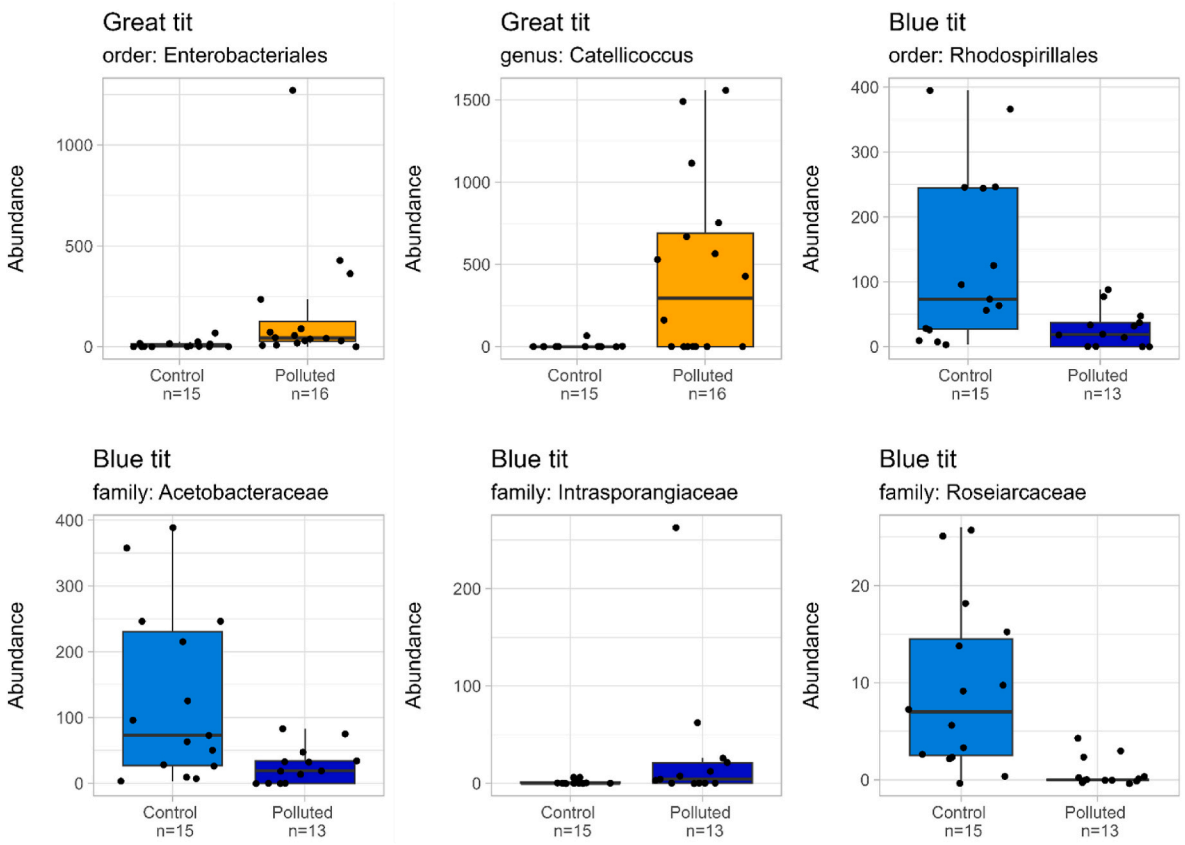

Fig. 4. Observed abundances (also used as the input for differential abundance (DA) analyses) of taxa that were differentially abundant in the bird nests among polluted and control zones according to three or more DA tools. Namely, the tools used were ALDEx2, LinDA, ANCOM-BC2, DESeq2, and Corncob, and the Benjamini-Hochberg adjusted p-value threshold was <0.05. Note that the order Rhodospirillales included only few bacterial members other than the family of *Acetobacteraceae*, making these taxa redundant.

P. major nests in the polluted zone, was additionally associated with decreased nestling RBM in both tit species but not in *F. hypoleuca* (Table S6). Other potential pathogens that were significantly negatively linked with the fitness parameters in one or more bird species were Enterobacteriales, *Enterococcaceae, Mycoplasmataceae, Escherichia/Shigella, Mycobacterium* (positive association), and *Clostridium sensu*

Table 2
Effects of nest material metal levels (PC_{Met1}, PC_{Met2}) and bacterial composition (PC_{Bac1}, PC_{Bac2}), together with other variables (relative nestling body mass, nest pH, temperature, humidity, and brood size at the time of sampling), on the probability of fledging and the relative body mass of nestlings. The primary focus was on the most informative metal and bacterial principal components, PC_{Met1} and PC_{Bac1}, which were set as fixed terms. Subsequently, for each species, the model was systematically reduced based on *p*-values. Statistically significant results are bolded. Sample sizes were n = 28, 30, and 31 for blue tits, pied flycatchers, and great tits, respectively, but may vary between models due to some missing values.

Probability of fledging[b]						
Model[a]	Blue tit		Pied flycatcher		Great tit	
	F_{df}	*p*	F_{df}	*p*	F_{df}	*p*
PC_{Met1} (fixed)	$15.37_{1, 23}$ (Est. −1.07, SE 0.27)	**0.0007**	$1.11_{1, 26}$	0.302	$0.04_{1, 26}$	0.846
PC_{Bac1} (fixed)	$0.14_{1, 23}$	0.709	$0.00_{1, 26}$	0.967	$0.03_{1, 26}$	0.869
PC_{Met2}	$2.18_{1, 22}$	0.154	$4.43_{1, 26}$ (Est. 0.96, SE 0.46)	**0.045**	$0.49_{1, 24}$	0.491
PC_{Bac2}	$0.96_{1, 17}$	0.340	$0.21_{1, 22}$	0.649	$0.19_{1, 22}$	0.671
Rel. body mass	$1.23_{1, 19}$	0.281	$3.38_{1, 24}$	0.079	$5.94_{1, 26}$ (Est. 0.03, SE 0.01)	**0.022**
pH	$8.69_{1, 23}$ (Est −2.90, SE 0.98)	**0.007**	$1.35_{1, 23}$	0.258	$0.46_{1, 25}$	0.506
Temperature	$4.81_{1, 23}$ (Est. 0.21, SE 0.10)	**0.039**	$0.00_{1, 20}$	0.976	$0.03_{1, 21}$	0.876
Humidity	$0.18_{1, 16}$	0.681	$0.12_{1, 21}$	0.729	$0.24_{1, 23}$	0.632
Brood size[c]	$1.86_{1, 18}$	0.189	$4.12_{1, 25}$	0.053	$14.85_{1, 26}$ (Est. 0.33, SE 0.08)	**0.0007**
Relative body mass of nestling[d]						
Model[a]	**Blue tit**		**Pied flycatcher**		**Great tit**	
	F_{df}	*p*	F_{df}	*p*	F_{df}	*p*
PC_{Met1} (fixed)	$9.69_{1, 23}$ (Est. −8.90, SE 2.86)	**0.005**	$0.89_{1, 26}$	0.354	$4.86_{1, 27}$ (Est. −6.07, SE 2.75)	**0.036**
PC_{Bac1} (fixed)	$0.00_{1, 23}$	0.997	$2.62_{1, 26}$	0.118	$9.63_{1, 27}$ (Est. 2.74, SE 0.88)	**0.005**
PC_{Met2}	$0.61_{1, 19}$	0.444	$0.47_{1, 23}$	0.498	$10.77_{1, 27}$ (Est. −10.99, SE 3.35)	**0.003**
PC_{Bac2}	$0.89_{1, 20}$	0.356	$7.33_{1, 26}$ (Est. 2.61, SE 0.96)	**0.012**	$0.85_{1, 23}$	0.366
pH	$0.03_{1, 1}$	0.862	$2.60_{1, 24}$	0.120	$0.73_{1, 22}$	0.401
Temperature	$0.86_{1, 21}$	0.365	$0.14_{1, 21}$	0.716	$0.88_{1, 25}$	0.358
Humidity	$0.00_{1, 17}$	0.965	$1.60_{1, 25}$	0.217	$1.68_{1, 24}$	0.208
Brood size[c]	$0.47_{1, 22}$	0.500	$0.12_{1, 22}$	0.733	$2.50_{1, 26}$	0.126

[a] Bacterial PC_{Bac3} excluded from the models as it showed no statistical significance.
[b] Binomial distribution.
[c] Brood size at the time of sampling.
[d] Normal distribution.

stricto (see Table S6 for the full results). Of these pathogens, the genus *Escherichia/Shigella* showed higher RA with increasing metal levels (GLM; PC_{Met1}: $F_{df} = 8.95_{1, 85}$, $p = 0.004$). Other terms including PC_{Met2}, brood size, ambient temperature, and humidity were not associated with the abundance of the investigated pathogens (Table S6).

The RA of the most abundant nest bacterial phyla and orders (Proteobacteria, Actinobacteria, Rhizobiales, Actinomycetales, Sphingobacteriales) correlated positively with the RBM of the nestlings, with the exception of Firmicutes, which showed a negative correlation and Bacteroidetes and Lactobacillales, which showed no associations with the RBM (Table S2). Moreover, the RA of these taxa commonly depended on the bird species and the ambient temperature within the nest box. In contrast, no associations were found between these abundant taxa and metal levels (PC_{Met1} and PC_{Met2}), humidity, brood size, age at the time of sampling, or the PC_{Met1}–species interaction (Table S2).

4. Discussion

4.1. Core microbiota

The characteristics of the bird nest microbiota were influenced by several factors, one of which was the bird species. Analysis of the most abundant bacteria showed that Proteobacteria were predominantly found in *C. caeruleus* and *F. hypoleuca* nests, while Firmicutes dominated within *P. major* nests. Additionally, Actinobacteria and Bacteroidetes were among the most abundant phyla across all nests, albeit in varying proportions. Moreover, the most abundant nest bacteria on the order-level consisted of Actinomycetales, Lactobacillales, Sphingobacteriales, and Rhizobiales, for all three species considered. Data on the functions of these taxa within bird nests are currently lacking. However, Actinomycetales and Lactobacillales are recognized for their production of antibacterial and antifungal metabolites (Dalié et al., 2010; Selim et al., 2021). Additionally, *Sphingobacterium* was found to be positively correlated with As and Co in soil in a previous study by Zhang et al. (2022). Our results resemble earlier findings on the microbiota of the tit species and birds in general (Bodawatta et al., 2020; Drobniak et al., 2021; Goossens et al., 2022; Grond et al., 2018; Kohl, 2012), but the nest microbiota specifically has been little studied (Devaynes et al., 2018; Goodenough et al., 2017; Goodenough and Stallwood, 2010; Zabłotni et al., 2020). Furthermore, our results for the first time comprehensively characterize the nest bacterial composition (including non-culturable bacteria) of *F. hypoleuca* nests.

In addition to the differential RA of the nest-dominating taxa, species-specific differences were observed in the overall bacterial community composition, as well as in the association of bird fitness to metal pollution and pathogens. This highlights the importance of caution when generalizing microbiota results to other, even closely related species.

4.2. Metal pollution and nest microbiota

While the nest alpha diversity (i.e., Shannon index and observed richness within individual nests) was similar between polluted and control areas (in contrast to *Passer montanus* gut microbiota in another study by Zhang et al., 2023), we found species-specific differences in the bacterial composition among nests (i.e., beta diversity). Specifically, the bacterial composition differed between metal-polluted and control zones for *C. caeruleus* and *P. major*, but no significant differences were observed in *F. hypoleuca*. Previous reports of such differences have mostly focused on the bird gut microbiota rather than nests (Zhang et al., 2023), and considered environmental factors beyond direct metal pollution, such as urbanization (Maraci et al., 2022; Stephens et al., 2021; Teyssier et al., 2018b, 2018a). In our study areas, pollution and/or urbanization impact the availability and quality of diet and nest materials (Eeva et al., 2005; Kiikkilä, 2003). This can potentially lead to changes in the composition of the nest microbial community,

particularly among the parids. Unlike *F. hypoleuca*, both parid species may have to resort to alternative nest materials (such as grass, roots, and twigs) within the polluted zone due to the scarcity of their usual mosses (demonstrated in Figs. S4 and S5). The lack of microbial differences observed in *F. hypoleuca* nests potentially reflects the lesser variations in the availability of their typical nest materials, such as bark and leaves, between the zones. As expected, the nest community composition was overall more similar between the closely related parid species compared to *F. hypoleuca*, likely because the nest materials and nestling diet resemble each other more.

Pollution was associated with several individual bacterial taxa within the nests. *Escherichia/Shigella* positively correlated with the metal levels (PC_{Met1}), and the differential abundance analysis revealed higher abundance of Enterobacteriales and *Catellicoccus* (in *P. major* nests) and *Intrasporangiaceae* (in *C. caeruleus* nests) within the polluted zone. In support of our results, increased Enterobacteriales and *Catellicoccus* have previously been detected within *P. major* guts in urban areas (Maraci et al., 2022). Accordingly, neither of the mentioned taxa here corresponded to actual metal levels within the nests, which emphasizes the likely importance of indirect effects of pollution via habitat alteration. Moreover, members of *Intrasporangiaceae* have previously been identified from the cloaca and skin of woodlarks (van Veelen et al., 2017), as well as in soil and wastewater samples from metal-polluted mining areas (Huang et al., 2021; Liu et al., 2012). They may exhibit metal detoxification functions (Yang et al., 2009), which could be connected to their larger abundance in the polluted nest materials.

In opposition, Rhodospirillales (which includes the family *Acetobacteraceae*) and *Roseiarcaceae* in *C. caeruleus* nests showed higher abundance within the control zone compared to polluted zone. These findings may reflect the characteristics of our control sites with denser and healthier ground floor vegetation layer, including typical *C. caeruleus* nest materials, moss and juniper, where members of these bacterial taxa have previously been observed (Kulichevskaya et al., 2014; Navarro-Noya et al., 2012). In general, aromatic plants such as *J. communis* and *R. tomentosum* that are favoured by *C. caeruleus* may act against harmful bacteria, fungi, and insects within the nests (Korpinen et al., 2021; Petit et al., 2002; Semerdjieva et al., 2021). Thus, the effects of metal pollution on the availability of important nest materials should be considered in future microbiological research.

4.3. Fitness-related consequences of metals and microbes

Metals detected in the nest materials (represented by PC_{Met1} that consisted of Cu, Ni, As, and Cd) predicted lower fledging probability in *C. caeruleus* and decreased nestling RBM (relative body mass) in both tit species. Additionally, the RBM and fledging success of both parids were lower in the polluted zone. This indicates harmful effects of pollution on the bird condition via direct and/or indirect mechanisms. Our findings are consistent with prior research on the same location, noting that the decreased success of the parids may be attributed to barren and food-limited environment of the polluted zone (Kiikkilä, 2003). Sparse forest canopy in the polluted area could further accelerate the progression of phenology in spring, leading to earlier egg-laying and a reduced quality of diet (Eeva et al., 2009). Potentially as response to these sub-optimal conditions, the nest microbiota of both tit species showed several signs of alteration, supporting earlier findings about the importance of habitat type for the microbiota of these species (Drobniak et al., 2021; Goossens et al., 2022).

In contrast, we observed few associations between pollution, fitness, and nest microbiota in *F. hypoleuca*, following the promising trend of recovery of this insectivorous species after drastically reduced emissions from the local Cu–Ni smelter since 1990's (Eeva et al., 2020; Eeva and Lehikoinen, 2015; Espín et al., 2016). *F. hypoleuca*, a migratory bird that arrives at the breeding sites later than parids in spring, partly evade the variable weather of the early season. Moreover, their flexible diet mitigates harm from altered habitat and diet quality in polluted areas, given that levels of calcium metabolism-disrupting metals remain at non-toxic levels (Eeva et al., 2005; Espín et al., 2024). Indeed, these birds appear to cope better in moderately polluted environments compared to e.g., *P. major* (Espín et al., 2016), although there can be yearly variation.

Nest alpha diversity (i.e. Shannon index and richness) was not associated with the fledging success in any of the study species, but higher Shannon diversity did predict greater RBM of *P. major* and *F. hypoleuca*. The results partially align with previous findings on the gut microbiota of *P. major* and *Passer domesticus* nestlings (Davidson et al., 2021; Teyssier et al., 2018a, 2018b), but diverge from those of *Acrocephalus sechellensis* (Worsley et al., 2021). Combined with the observation of a reduced *P. major* nestling mass under pollution, the results suggest potential indirect consequences of pollution to the nestling growth and nest alpha diversity. We do not know the causal relationship of these findings, and earlier results of such effects vary, highlighting the importance of further experimental research.

Nestling RBM was one of the factors associated with the nest community composition, implying a connection between the nestling growth and the early-life microbial environment. On a more detailed perspective, bacteria belonging to PC_{Bac1} increased with the *P. major* RBM. This PC represented the orders of Burkholderiales, Sphingobacteriales, Sphingomonadales, Caulobacterales, Actinomycetales, Rhizobiales, Cytophagales, Rhodospirillales, and Solirubrobacterales. These primarily soil and plant-associated bacteria are uncommon in birds, yet Sphingomonadales that have been found in birds, are also recognized for their ability to degrade metallic compounds (Asaf et al., 2020; Giorgio et al., 2018; Hird et al., 2015). The result perhaps non-causally reflects the more vegetation-rich areas that also provide a better quality of diet for the birds, increasing the nestling weight. However, PC_{Bac1} was not affected by metals. Moreover, PC_{Bac2} was associated to increased growth of *F. hypoleuca* nestlings. PC_{Bac2} mostly included bacteria often found in the guts of birds and other vertebrates (Erysipelotrichales, Clostridiales, Bacteroidales, and Enterobacteriales). Additionally, in a separate model, larger RBM also predicted higher abundance of some of the dominating taxa (Proteobacteria, Actinobacteria, Rhizobiales, Actinomycetales, and Sphingobacteriales), many of which likely originate from the surroundings, rather than bird guts (Haichar et al., 2008; Masson-Boivin et al., 2009; Ngamcharungchit et al., 2023). The causal relationship remains unknown, but the results emphasize the role of the environment and nest microbiota as potential determinants of nestling growth.

A common bacterial order Enterobacteriales that includes several pathogenic strains exhibited elevated levels in the polluted zone within *P. major* nests, and negatively correlated with fledging success in *F. hypoleuca*. Members of Enterobacteriales, such as toxin-producing *Escherichia coli* are commonly found in passerine birds, and are considered potential sources of hazardous human-associated zoonoses (Giacopello et al., 2016; Hubá, 2004). In our study, *Escherichia/Shigella* exhibited a dual association, showing both an increase alongside nest metal levels and a correlation with nestling growth (i.e., decreased RBM in *C. caeruleus*). This is noteworthy, since heavy metal exposure may disrupt immune functions, potentially increasing the susceptibility to opportunistic pathogens such as *Escherichia* (Zheng et al., 2023). Furthermore, *Catellicoccus,* a genus that can be found in bird guts (Green et al., 2012; Sottas et al., 2021), showed higher abundance in the polluted zone in *P. major* nests and correlated with decreased nestling RBM in both tit species. In contrast to earlier suggestions (Benskin et al., 2010), our results imply that *Catellicoccus* might act as an opportunistic pathogen, benefitting from polluted environments.

Other pathogenic taxa with negative fitness effects included *Enterococcaceae, Mycoplasmataceae,* and *Clostridium sensu stricto* (Hubá, 2004; Yang et al., 2019), but these taxa showed no significant associations with metal pollution. *Pseudomonas* and *Massilia* were included in the analysis since they had been suggested to benefit wildlife under metal exposure (Fakhar et al., 2022; Zhou et al., 2023), but we found no associations with metal pollution or bird fitness here. Surprisingly, *Mycobacterium* showed a positive association with fledging in *P. major*,

despite its reputation as a pathogen (Hubá, 2004; Schmidt et al., 2022). This may lack causation, and it is plausible that some unidentified environmental condition positively influenced both.

4.4. Other nest microbiota-associated factors

Regarding biotic factors, we found no association of nest microbiota with the brood size nor nestling age at the time of sampling, which was expected since we tried to avoid variability in the sampling age. Abiotic factors such as nest chamber humidity and nest pH showed limited effects on the nest microbiota, despite of being considered important abiotic factors for bacterial growth in other context (Griffiths et al., 2011).

In contrast, a majority of nest microbiota parameters appear to exhibit a negative association with temperature: warmer conditions predicted a decrease in alpha diversity (both Shannon index and observed richness), PC_{Bac1} (consisting mainly of soil and plant-associated microbes), and the RA of some of the most common nest bacterial taxa (Actinobacteria, Actinomycetales, Bacteroidetes, and Sphingobacteriales). Additionally, temperature influenced the overall community composition of the nests. Similar trends have been found earlier, noting the general importance of specific temperatures on bacterial growth rates and composition in animals (Bestion et al., 2017; Cook et al., 2005; Sepulveda and Moeller, 2020).

It is important to note that microbes detected in the nest materials may partially overlap with faecal microbiota (van Veelen et al., 2017), despite of the parents' tendency to remove faecal sacs from the nest to maintain nest hygiene and reduce the bacterial load (Azcárate-García et al., 2019). Consequently, the microbiota detected in the nest samples may respond to pollution-induced alterations in the nestling diet (Eeva et al., 2005). Furthermore, the effect is likely bidirectional, as pollution-altered microbiota (Kiikkilä, 2003; Li et al., 2017; Zhao et al., 2020) originating from the nest materials may disrupt the sensitive gut colonization phase of nestlings after hatching. Further investigation of this aspect falls beyond the scope of our study and should be examined more in the future. In summary, investigation of the bird nests provides a non-invasive approach for studying the effects of environmental factors on bird-associated microbiota, including the presence of potential pathogens.

5. Conclusions

In our observational approach, we found that pollution-related effects on three wild passerine birds largely depend on the species, regarding both microbiota and fitness. Especially *P. major* and *C. caeruleus* appeared to be susceptible to pollution-induced disturbances affecting their nest microbiota and fitness. In contrast, *F. hypoleuca* and their nest microbiota showed fewer responses to pollution, suggesting higher tolerance for the pollution-induced changes under moderate levels of pollution. Metal pollution either directly or indirectly via habitat change increased the abundance of Enterobacteriales, *Escherichia/Shigella*, and *Catellicoccus* in the nests, all those taxa having negative implications for the bird fitness-related parameters, especially in the parids. In summary, our study reveals species-specific, pathogen-promoting effects of metal pollution on the nest microbiota, potentially impairing bird survival. This previously unrecognized indirect mechanism of metal pollution emphasizes the necessity for further investigation through experimental study setups.

CRediT authorship contribution statement

Lyydia I. Leino: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Eero J. Vesterinen:** Writing – review & editing, Software, Methodology, Investigation, Data curation. **Pablo Sánchez-Virosta:** Writing – review & editing, Resources. **Pere Puigbò:** Writing – review & editing, Supervision. **Tapio Eeva:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Miia J. Rainio:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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

During the preparation of this work the author(s) used GPT-3.5 and Microsoft Copilot in order to improve readability and generate select illustrations featured in the graphical abstract. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) 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.

Data availability

Our data can be accessed via Mendeley Data. The link to data is provided both within the manuscript and at the \"attach files\" step.

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Appendix A. Supplementary data

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