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► **To cite this version:**

Marion Chatelain, Adrien Frantz, Julien Gasparini, Sarah Leclaire. Experimental exposure to trace metals affects plumage bacterial community in the feral pigeon. 2016. hal-01274875

**HAL Id: hal-01274875**

**<https://hal.sorbonne-universite.fr/hal-01274875>**

Preprint submitted on 16 Feb 2016

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**Experimental exposure to trace metals affects plumage bacterial community in the feral  
pigeon**

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1 **Abstract**

2 Bacteria are fundamental associates of animals, and recent studies have highlighted their  
3 major role in host behaviour, immunity or reproductive investment. Thus, any environmental  
4 factor modifying bacterial community may affect host fitness. In birds, trace metals emitted  
5 by anthropogenic activities accumulate onto the plumage where they may alter bacterial  
6 community and ultimately affect bird fitness. Although trace metals are current major  
7 environmental issues in urban habitats, their effects on feather bacterial community have  
8 never been investigated. Here, we supplemented feral pigeons (*Columba livia*), an  
9 emblematic urban species, with zinc and/or lead in drinking and bath water. As expected,  
10 lead and zinc supplementations modified plumage bacterial community composition.  
11 Zinc decreased bacterial load, while lead decreased bacterial richness and the frequency  
12 of preening behaviour in birds, known to regulate feather bacteria. Our results  
13 demonstrate for the first time the effects of common urban trace metals on plumage  
14 bacterial community and shed light on one of the mechanisms by which trace  
15 metals can affect bird fitness. Further studies are now needed to investigate how this  
16 effect modulates avian life history traits known to depend on plumage bacterial community.

17

18 Key words: urban ecology, birds, bacteria

19

20 **INTRODUCTION**

21

22 Bacteria successfully colonize numerous and various habitats, including animal body parts  
23 that are in direct contact with the surroundings such as skin, feathers or fur, and the digestive  
24 tract. The normal flora, also called the microbiota, lives in symbiosis with its host, fulfilling  
25 essential functions for host metabolism, such as cellulose degradation, vitamins synthesis(Hill  
26 1997)and inhibition of pathogens proliferation (Olsson et al. 1992, Oh et al. 2006, Balcázar et  
27 al. 2007).Recent studies have also revealed that bacteria may have surprising effects such as  
28 shaping host behaviour and investment in reproduction (Ezenwa et al. 2012, Jacob et al.  
29 2015). However, we have just made the first steps in discovering the diversity and functions  
30 of these bacterial ecosystems, and it is now necessary to evaluate the factors that regulate  
31 microbiota.

32 The microbiota is influenced by numerous interconnected factors, including host  
33 behaviour,genotype and physiology(Mueller et al. 2006, Frank et al. 2011, Rosenthal et al.  
34 2011, Hildebrand et al. 2013, Leclaire et al. 2014a),as well as its environment(Dotterud et al.  
35 2008, Burkholder et al. 2008, Ruiz-de-Castañeda et al. 2011).Trace metals emitted by  
36 anthropogenic activities have well-known detrimental effects on animals (Jarup 2003, Hsu et  
37 al. 2006, Berglund et al. 2007, Eeva et al. 2009) andare thus of major environmental  
38 concernsin most urban environments(Azimi et al. 2005, Scheifler et al. 2006, Roux and Marra  
39 2007, Kekkonen et al. 2012). Some trace metals,such as lead, cadmium, zinc, copper, chrome  
40 and nickel, are known to have toxic effects onenvironmental microbial communities(Babich  
41 and Stotzky 1978, Giller et al. 1998). In contrast, although tracemetals can be naturally  
42 ingested or inhaled by animals, and deposited on integuments, their effects on animal  
43 microbiota have been poorly investigated (but seeHojberg et al. 2005, Vahjen et al. 2010,  
44 Breton et al. 2013, Liu et al. 2014).

45

46 Plumage is a key point of interaction between birds and the microbial world, and, like other  
47 integuments, is highly colonized by bacteria (Burt Jr and Ichida 1999, Muza et al. 2000,  
48 Shawkey and Hill 2004, Whitaker et al. 2005). Although several studies have shown that  
49 keratinolytic bacteria can alter feather structure *in vitro* (Burt Jr and Ichida 1999,  
50 Shawkey et al. 2007), the *in vivo* effects of feather bacteria on their host have been scarcely  
51 studied. A few correlative and experimental studies in captive and free-living birds have  
52 shown, however, that feather bacteria may influence sexual signalization such as feather  
53 colouration or plumage condition (Shawkey et al. 2007, Gunderson et al. 2009, Kilgas et  
54 al. 2012, Leclaire et al. 2014b; but see Jacob et al. 2014), and affect bird immune system  
55 (Leclaire et al. 2015) and investment in reproduction (Jacob et al. 2015). Any change in  
56 feather microbiota is thus likely to have a strong impact on bird fitness. The plumage and the  
57 uropygial gland secretions spread onto it can accumulate metals (Pilastro et al. 1993, Frantz  
58 et al. 2012), which can affect plumage bacterial community by direct contact. Ingested  
59 metals also circulate in the bloodstream and accumulate in organs and bones (Pattee 1984,  
60 Dauwe et al. 2002, Scheifler et al. 2006, Kekkonen et al. 2012, Reid et al. 2012), and may  
61 induce noxious (Redig et al. 1991, Snoeijs et al. 2004, Dauwe et al. 2005, Eeva et al. 2009) or  
62 beneficial (Mertz 1981, Prasad 1998) effects on bird physiology; therefore, metals can affect  
63 bird ability to regulate their microbiota (Piault et al. 2008, Moreno-Rueda 2010, Leclaire et al.  
64 2014b). Whatever the exact underlying mechanisms, further studies are clearly needed to  
65 evaluate the effects of trace metals on feather bacteria.

66

67 The feral pigeon (*Columba livia*) is an emblematic urban bird living in high density  
68 populations, with potentially elevated plumage bacteria transmission between individuals  
69 (reviewed in Archie and Theis 2011). Mate choice is crucial for this species which mate for

70 life (Johnston and Janiga 1995). In this species, bacterial load affects immunity (Leclaire et  
71 al. 2015), as well as feather condition and colouration (Leclaire et al. 2014b) which are two  
72 of the main criteria used in mate choice (Johnston and Janiga 1995). Consequently,  
73 trace metals, besides their direct effects on bird physiology, may further impact pigeon  
74 reproduction and fitness through their effects on plumage bacterial load and composition.  
75 Here we investigated the effects of an experimental exposure to lead and/or zinc on two  
76 traits that can affect each other: plumage bacteria composition and frequency of  
77 preening in the feral pigeon. Preening is a costly antimicrobial behaviour that can be  
78 directly influenced by feather bacterial load (Leclaire et al. 2014b) and bird health status  
79 (Piault et al. 2008, Moreno-Rueda 2010), which are both potentially affected by trace metals.

80

81

## 82 **METHODS**

83

### 84 **Subjects and housing**

85 Ninety six (48 males and 48 females) free-living adult feral pigeons (*Columba livia*) were  
86 caught during winter 2013 (February/March) in several pigeons' flocks within the Parisian  
87 agglomeration. Birds were all considered as adults because of their well-formed caruncle, the  
88 absence of juvenile plumage and the presence of iridescent neck feathers (Johnston and Janiga  
89 1995). Pigeons were kept in 8 outdoor aviaries (3.10 m x 2.00 m x 2.40 m) at the CEREEP field  
90 station (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, UMS 3194,  
91 Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). They were evenly distributed  
92 among aviaries according to their flock, sex and plumage eumelanin level (see below) in such a  
93 way that there was no confounding effect between aviaries and these variables (i.e. no  
94 statistically significant link between aviary and flock:  $\chi^2=71.09$ ,  $df=70$ ,  $P=0.441$ ; sex: 6

95 males and 6 females per aviary; or plumage eumelanin level:  $F_{1,80}=0.38$ ,  $P=0.537$ ). Birds were  
96 fed *ad libitum* with a mix of maize, wheat and peas and water was provided in a trough. The  
97 aviaries were enriched with a bowl of water used for bathing and with branches as perches. Birds  
98 were individually identified with a numbered plastic ring. Birds were genetically sexed  
99 following the protocol described by Griffiths et al. (1998). Before onset of treatment, birds  
100 were kept 2 to 7 weeks for acclimation. Alongside this experiment, we measured the effects  
101 of treatments (see below) on bird body mass condition, reproduction and immunity. At the  
102 end of the breeding season, all birds were released back to the wild at their site of capture. All  
103 experiments were carried out in strict accordance with the recommendations of the “European  
104 Convention for the Protection of vertebrate Animals used for Experimental and Other  
105 Scientific Purposes” and were conducted under the authorizations of the “Ministère de  
106 l’éducation nationale, de l’enseignement supérieur et de la recherche” (authorization  
107 N\_00093.02) and the “Direction Départementale des Services Vétérinaires de Seine et-  
108 Marne” (authorization N\_77-05).

109

### 110 **Measurement of plumage colouration**

111 At capture, birds were first categorised as eumelanic (grey to black pigmented) or  
112 pheomelanic (red pigmented) which define their melanin type. Then, eumelanic birds were  
113 individually photographed in order to measure their eumelanin level. Eumelanin level was  
114 calculated as the percentage of black on the wing surface (number of black pixels/number of  
115 white pixels x 100) using the Gimp image retouching and editing software. This measure has  
116 been shown to be a reliable and repeatable estimation of melanin concentration in pigeons  
117 (Jacquin et al. 2011). In eumelanic pigeons, plumage eumelanin level ranged from 4.2 to  
118 95.9%. Because of the small amount of pheomelanic birds (14 over 96) the measure of a  
119 pheomelanin level was not relevant.

120

## 121 **Treatments**

122 Two weeks before the onset of treatment, aviaries were divided into 4 metal-exposure  
123 treatments; this means that there were 2 aviaries per treatment with 12 pigeons each (24  
124 pigeons in total per treatment). For each treatment, the two aviaries were purposely spatially  
125 separated from one another. Aviaries were in direct contact along a linear transect and  
126 numbered from 1 to 8 (*lead* (1), *zinc* (2), *control* (3), *lead+zinc* (4), *lead* (5), *control* (6),  
127 *zinc*(7), *lead+zinc* (8)). Side-by-side aviaries were separated by wire mesh. Treatments  
128 consisted of water supplemented with lead (*lead* group; 1ppm lead acetate; aviaries 1 and 5),  
129 zinc (*zinc* group; 10 ppm zinc sulphate; aviaries 2 and 7), lead and zinc (*lead+zinc* group;  
130 1ppm lead acetate and 10ppm zinc sulphate; aviaries 4 and 8), or control(*control* group; tap  
131 water with no metal added; aviaries 3 and 6). We chose these concentrations based on both  
132 lead blood concentrations measured in urban birds (ranging from 0,053 to 0,264ppm; Roux  
133 and Marra 2007) and the gastrointestinal absorption rate of lead in zebra finches  
134 (<10%)calculated from (Dauwe et al. 2002). Zinc concentrations were approximated using the  
135 zinc/lead concentration ratio in the environment and in bird feathers (on average, zinc was 10  
136 times more concentrated than lead;Azimi et al. 2005, Frantz et al. 2012, Chatelain et al.  
137 2014).Drinking troughs and baths were filled with the corresponding treated water every other  
138 day. Our supplementation treatments were validated by measuring lead and zinc  
139 concentrations in blood and feathers of the birds. Blood was sampled 10 weeks after the start  
140 of the experiment. Moreover, the fifth secondary remige of each bird was removed a first time  
141 and the regrowth feather was used for metal measurements. Both blood and feathers were  
142 digested using a previouslydescribed protocol (Chatelain et al. 2014) and lead and zinc  
143 concentrations were measured by mass spectrometry (ICP-MS) and by optic emission  
144 spectrometry (ICP-OES) respectively. Validity of analytical methods was checked by means



145 of a standard biological reference material (TMDA-64.2. Environment Canada). Lead and  
146 zinc concentrations in blood were the highest among birds exposed to lead (*lead* and  
147 *lead+zinc* groups) and birds exposed to zinc (*zinc* and *lead+zinc* groups) respectively (Table  
148 1). These results ensured that metals added to water were ingested by the birds. In feathers,  
149 while lead concentrations were significantly the highest among birds exposed to lead (*lead*  
150 and *lead+zinc* groups), the increase in zinc concentration among birds exposed to zinc (*zinc*  
151 and *lead+zinc* groups) was not significant (Table 1). Zinc and lead measured in the feathers  
152 were respectively 80 and 1.5 times less concentrated than the ones measured in feathers of  
153 urban pigeons (Nam et al. 2004, Adout et al. 2007, Hoff Brait and AntoniosiFilho 2011,  
154 Frantz et al. 2012, Chatelain et al. 2014), suggesting that our experimental exposure  
155 corresponded to the lower range of urban exposure.

156

#### 157 **Measurement of plumage bacterial load**

158 Plumage bacterial load was measured 20 weeks after onset of treatment. 4 hours after  
159 renewing the water of the bowls used for bathing, 10 birds (5 males and 5 females) were  
160 randomly sampled in each treatment. Each sampled bird was caught with a net that had been  
161 previously sprayed with 70% ethanol. Then, a whole flora agar slide (Hygialim, 3026091,  
162 Plate Count Agar +triphenyltetrazoliumchloride+Neutralizing) was put flat against the back of  
163 the bird for 10 seconds. The slides were then incubated at 37°C for 24h. Feather bacterial load  
164 was expressed as the number of bacterial colonies per slide.

165

#### 166 **Molecular analysis of plumage bacterial communities**

167 Fifteen weeks after the start of the experiment, 91 adults were caught with a net previously  
168 sprayed with 70% ethanol (n.b. 5 pigeons died for unknown reasons before this measure, 2  
169 from the *zinc* group, 2 from the *lead+zinc* group and 1 from the *control* group). After washing

170 her hands with alcohol, the experimenter cut a clump (10 feathers on average) of back  
171 feathers with sterilized scissors and pliers, avoiding the outermost feathers. The feathers were  
172 immediately placed in sterile 2ml plastic tubes and stored at -20°C until analysis.

173 We extracted DNA using the QiagenDNeasy® Blood and Tissue Kit and the standard  
174 protocol designed for the purification of total DNA from Gram-positive bacteria (Qiagen,  
175 Venlo, Netherlands; July 2006).

176 To characterize the bacterial communities present in each sample, we performed automated  
177 ribosomal intergenic spacer analyses (ARISA; Ranjard et al. 2000). This DNA fingerprinting  
178 method is based on the amplification of the internal transcribed spacer (ITS) region lying  
179 between the 16S and 23S ribosomal RNA genes in the ribosomal operon. The ITS region is  
180 extremely variable, in both sequence and length, for different bacterial species. Therefore,  
181 the DNA amplification profile obtained with ARISA allows straightforward estimation of  
182 bacterial diversity, avoiding biases inherent in classical culture-based techniques (Ranjard et  
183 al. 2000). We amplified the ITS using the FAM (6-carboxyfluorescein)-labeled primer S-D-  
184 Bact-1522-b-S-20 (5'-[6FAM] TGCGGCTGGATCCCCTCCTT-3') and the unlabeled primer  
185 L-D-Bact-132-a-A-18 (5'-CCGGGTTTCCCCATTCGG-3') (Ranjard et al. 2000). We  
186 performed the PCR amplification in 10 µL mixtures containing 200 µM each deoxynucleotide  
187 triphosphate, 0.20 µM each primer, 1.25 units of PerfectTaq DNA polymerase, 1× PCR  
188 buffer (5 Prime, GmbH, Hamburg, Germany), and 1 µL DNA extract, using the  
189 following protocol: initial denaturation at 94 °C for 3 min, 40 cycles consisting of denaturation  
190 at 94 °C for 30 s, annealing at 55 °C for 45 s, elongation at 72 °C for 1 min, and a final  
191 elongation at 72 °C for 10 min. We then mixed 1 µL of the PCR products with 15 µL of highly  
192 deionized formamide and 0.2 µL of Genescan 1200 LIZ size standard (Applied Biosystems,  
193 Foster City, CA). The mixtures were separated with a 24-capillary 3500XL DNA Analyzer  
194 (Applied Biosystems) using POP-7 polymer and the manufacturer's default electrophoresis

195 run settings. Data analysis and genotyping were performed with GeneMapper software  
196 (Applied Biosystems). For each sample, the sequencer produced an ARISA profile in which  
197 each peak corresponds to 1 phylotype or operational taxonomic unit (OTU). In the various  
198 samples, the sequencer detected ITS fragments ranging in size from 300 to 950base pairs.  
199 For each individual, we calculated bacterial richness as the number of different OTUs.  
200 Because the probability to detect an OTU is likely affected by the amount of feathers used for  
201 DNA extraction, feathers were dried overnight and weighted to the nearest mg after DNA  
202 extraction. We estimated bacterial community dissimilarities between individuals using  
203 Jaccard distance based on presence/absence of OTUs.

204

### 205 **Observations of preening behaviour**

206 An observer recorded a total of 95 independent behavioural sessions of 5 minutes each. The  
207 observer remained outside the aviary and waited a few minutes before starting her  
208 observations not to influence bird behaviour. The observed birds were chosen randomly but  
209 the observer switched to a new treatment for each new session to have a similar number of  
210 observations for all treatments: 25 observations (corresponding to 21 different individuals)  
211 among the *control* group, 29 observations (24 individuals) among the *lead* group, 22  
212 observations (20 individuals) among the *zinc* group and 19 observations (16 individuals)  
213 along the *lead+zinc* group. The behaviour recording was performed with the JWatcher  
214 software. We recorded the time birds spent preening; during this behaviour, the plumage may  
215 be spread with preen secretions (Mardon et al. 2011), which have antimicrobial and  
216 antiparasite properties (e.g. in feral pigeons, house sparrows, eastern bluebirds and hoopoes;  
217 Moyer et al. 2003, Shawkey et al. 2007, Ruiz-Rodriguez et al. 2009, Waite et al. 2012,  
218 Czirják et al. 2013). Because preening is costly (Piault et al. 2008, Moreno-Rueda 2010), the  
219 time allocated to preening has been shown to be adjusted to bacterial load (Leclaire et al.

220 2014b),and to reflect birds'health status. For instance, juvenile apapanes  
221 (*Himationesanguinea*) experimentally infected with *Plasmodium relictum* spent less time  
222 preening (Yorinks and Atkinson 2000).

223

## 224 **Statistical analyses**

225 Statistical analyseswere performedusingR (R.3.0.2; R Development Core Team). Final  
226 models were retained based on their AIC.

227 To test the effects of metal exposure on the composition of bacterial communities, we  
228 performed a PERMANOVA with 5000 permutations (i.e. nonparametric multivariate analysis  
229 of variance, Adonis function, VEGAN package in R(Oksanen et al. 2007), based on Jaccard  
230 distance for OTU presence/absence data. Zinc exposure, lead exposure, sex and their  
231 interactions were included as explanatory variables. Because spatial proximity may influence  
232 bacterial communitysimilarity between individuals, we added the aviary as a covariate. Then,  
233 we ran similar analyses between each pair of treatments.Finally, we tested the differences of  
234 bacterial communities between aviaries among each metal treatment.

235 To investigate more precisely the effect of spatial proximity on bacterial communities'  
236 similarities, we compared a matrix of bacterial Jaccard distances between individuals to a  
237 matrix of spatial distances (scored as 0 for individuals inhabiting the same aviary, to 7 for the  
238 most distant individuals), considering a matrix of treatment membership (scored as 0 for  
239 individuals submitted to the treatment and 1 for individuals of different treatments) using  
240 partial mantel test with 5000 permutations.

241 We graphically represented similarities between individuals using a constrained redundancy  
242 analysis (RDA function in R) based on the Jaccard distance matrix.

243

244 We also tested plumage bacterial richness using a generalized linear model for Poisson  
245 distribution with zinc exposure, lead exposure, sex and their interactions as explanatory  
246 variables, the weight of feathers used for the analysis as a covariate and aviary as random  
247 effect.

248

249 To test the effects of metal exposure on plumage bacterial load, we performed Wilcoxon tests  
250 because our sample size was low (n=10 per treatment). First, we tested the effects of zinc and  
251 the effects of lead in two different tests; then, we performed Wilcoxon tests between each pair  
252 of treatments to test the effects of the interaction between zinc and lead exposure.

253

254 Finally, we investigated the amount of time birds allocated to preening by performing a  
255 generalized linear mixed model for Poisson distribution with zinc exposure, lead exposure,  
256 sex and their interactions as explicative variables and bird identity and aviary as random  
257 effects.

258

259 All the previously described models were performed on the totality of the birds, whatever  
260 their plumage colouration (i.e. eumelanic and pheomelanic birds). The same models were  
261 performed on eumelanic birds only (i.e. excluding pheomelanic pigeons). In these models,  
262 plumage eumelanin level and its interaction with the other considered parameters were added  
263 as explanatory variables.

264

265

266 **RESULTS**

267

268 The composition of bacterial communities depended on the interaction between lead and zinc  
269 exposure( $F_{1,82}=3.47$ ,  $P<0.001$ ; Fig. 1) and on aviaries( $F_{1,82}=3.91$ ,  $P<0.001$ ). Each pairwise test  
270 between metal exposures was significant (Table 2). The composition of bacterial communities  
271 differed significantly between aviaries among each metal treatment but was less dissimilar  
272 among the *lead* group (zinc-exposure:  $F_{1,19}=3.48$ ,  $P<0.001$ , lead-exposure:  $F_{1,21}=1.70$ ,  $P=0.018$ ,  
273 zinc and lead-exposure:  $F_{1,19}=4.48$ ,  $P<0.001$ , control:  $F_{1,20}=7.59$ ,  $P<0.001$ ). Moreover, there  
274 was a highly significant positive correlation between bacterial distance and spatial distance  
275 ( $r=0.30$ ,  $P<0.001$ ; Fig. 2).

276  
277 Plumage bacterial richness depended on the interaction between zinc and lead-exposure  
278 ( $\text{Chi}^2=9.09$ ,  $df=80$ ,  $P=0.003$ ; Fig. 3): the *lead* group had lower bacterial richness than  
279 the *control* group ( $\text{Chi}^2=5.58$ ,  $df=42$ ,  $P=0.018$ ) and the *zinc+lead* group ( $\text{Chi}^2=12.63$ ,  $df=41$ ,  
280  $P<0.001$ ). Moreover, the *lead+zinc* group tended to have higher bacterial richness than the *zinc*  
281 group ( $\text{Chi}^2=3.63$ ,  $df=40$ ,  $P=0.057$ ).

282  
283 Plumage bacterial load was significantly lower among birds exposed to zinc (*zinc* and  
284 *lead+zinc* groups) than among the others (*lead* and *control* groups;  $W=280.5$ ,  $P=0.029$ ; Fig. 4).  
285 Although there was no significant difference between each pair of treatments ( $P>0.067$ ),  
286 the *zinc* group tended to have lower plumage bacterial loads than the *control* group ( $W=29$ ,  
287  $P=0.072$ ) and the *lead* group ( $W=25$ ,  $P=0.067$ ). Lead did not significantly affect plumage  
288 bacterial load ( $W=181$ ,  $P=0.623$ ).

289  
290 Finally, the time birds spent preening depended on the interaction between zinc and  
291 lead exposure ( $\text{Chi}^2=3.97$ ,  $df=92$ ,  $P=0.04$ ). We performed partial models to compare each pair  
292 of treatments. Although there was no significant difference between each pair of treatments

293 ( $P > 0.101$ ), our results suggest that time spent preening was shorter among the *lead* group than  
294 among the other groups (*control*:  $65.00 \pm 15.18$ s, *lead*:  $34.21 \pm 9.69$ s, *zinc*:  $53.86 \pm 13.79$ s,  
295 *lead+zinc*:  $60.84 \pm 15.30$ s).

296

297 Sex and plumage eumelanin level were retained in none of the tested models.

298

299

## 300 **DISCUSSION**

301

302 As expected, the composition of plumage bacterial community varied with metal exposure. The  
303 exposure to lead alone appears to induce the strongest effect. Plumage bacterial composition  
304 was more similar amongst the two aviaries hosting birds exposed to lead only than expected if  
305 considering a spatial effect only. In addition, the plumage bacterial composition of birds  
306 exposed to lead only were the most distant from the communities of the other  
307 treatments. Moreover, birds of the *lead* group had reduced plumage bacterial richness  
308 compared to birds of the *control* group. These results suggest that lead may select for lead-  
309 tolerant plumage bacteria. To the best of our knowledge, our study is the first to show that lead  
310 exposure has effects on plumage bacterial community. It is consistent with a previous study  
311 showing that lead alters the intestinal microbiome of mice (Breton et al. 2013). Moreover,  
312 birds of the *lead* group tended to preen less frequently than birds of the *control* group. Lead  
313 exposure decreases bird immunity (unpublished results) and reproductive success (but there is  
314 no effect of metal exposure on bird breeding success; Chatelain et al. in press). Lead, by  
315 decreasing bird condition, may affect bird ability to preen, a costly behaviour that helps to  
316 regulate feather microbiota (Piault et al. 2008, Moreno-Rueda 2010, Leclaire et al. 2014b).  
317 Because preening is adjusted to feather bacterial load (Leclaire et al. 2014b), the change in

318 preening frequency observed in our study may also non-exclusively result from the change in  
319 feather bacterial community caused by trace metal exposure. Whatever the mechanism  
320 underlying the differences in preening frequency, the tendency of lead to reduce bird control on  
321 its plumage bacterial community may change the dominant status of bacteria species and  
322 therefore induce the proliferation of species that were previously sensitive to preen secretions.  
323 High-throughput DNA sequencing would help identifying lead-tolerant bacteria species and  
324 therefore inferring their potential pathogenicity and propensity to degrade feathers. More  
325 analyses should also be conducted to identify the proximal mechanisms involved in lead  
326 toxicity. For instance, *in vitro* exposure of feathers to these metals would allow us to  
327 disentangle the direct and indirect effects that may induce these metals.

328

329 Like lead exposure, zinc exposure had toxic effects on the plumage bacterial community with  
330 birds exposed to zinc exhibiting lower bacterial load than control birds. Similarly, high doses  
331 of zinc decrease bacterial load and change bacterial community in the gastrointestinal tract of  
332 piglets (Hojberg et al. 2005, Vahjen et al. 2010), and inhibit bacterial growth in sludge and  
333 sediment (Cabrero et al. 1998, Vega-López et al. 2007). Zinc is known to be essential to  
334 several metabolic functions of bacteria (Sugarman 1983). At high concentrations, zinc can  
335 however reduce protein and ATP content, interact with nucleic acids and enzyme active sites,  
336 decrease membrane health and eventually lead to cell necrosis (Martinez-Tabche and Gutierrez  
337 2000, Vega-López et al. 2007). Although the concentration of zinc we used is within the  
338 natural range found in cities, it may be high enough to negatively affect feather bacteria and to  
339 decrease bacterial load. Zinc may also affect feather bacterial load indirectly through its  
340 immunostimulating effect (Smith 2003). In feral pigeons, zinc has a positive effect on the  
341 production of specific antibodies (unpublished data), and might, therefore, increase the  
342 bactericidal capacity of uropygial secretions.



343

344 Bacterial community composition, bacterial richness and the time birds spent preening  
345 depended on the interaction between lead and zinc exposure. More precisely, the toxic effects  
346 of lead exposure was not detected in birds exposed to both lead and zinc, suggesting that zinc  
347 may compensate lead toxicity. Zinc is known to reduce the absorption and retention of  
348 ingested lead (Cerklewski and Forbes 1976, El-Gazzar et al. 1978, Prasanthi et al. 2010),  
349 which may therefore reduce the negative effects of lead on bird condition. In addition, the  
350 negative effect of zinc exposure on bacterial load would be higher in birds exposed to zinc  
351 only than in birds exposed to both lead and zinc, suggesting again an interaction between lead  
352 and zinc exposure.

353

354 Our results showed a strong effect of spatial proximity on bird plumage bacterial community,  
355 with birds in closer aviaries showing more similar bacterial communities. In accordance with  
356 other studies (Bisson et al. 2007, 2009, Saag et al. 2011), they point out the relatively small  
357 spatial scale transmission of plumage bacteria. While bacteria are likely transmitted through  
358 close contacts (Kulkarni and Heeb 2007) and reciprocal delousing, some bacteria may be able  
359 to survive on non-feather substrates and, therefore, be transmitted through bathwater, perches,  
360 soil and the grids separating the aviaries (Bisson et al. 2007). Because pigeons live in high  
361 density but have limited movements within their local environment (Frantz et al. 2012), the  
362 plumage bacterial community of wild pigeons may, therefore, greatly vary between  
363 populations, which may lead to local coevolution and co-adaptation between the host and its  
364 bacterial community. The strong effect of spatial proximity on plumage bacterial community  
365 detected in our study may have decreased and increased bacterial community similarities  
366 between and within aviaries respectively; ideally, future studies should increase the number of  
367 replicates (aviaries) or house pigeons in individual and spatially distant cages.

368

369 Our experimental exposure of feral pigeons to naturally occurring concentrations of lead  
370 and/or zinc highlights, for the first time, the effects of some trace metals commonly  
371 encountered in urban areas on plumage bacterial community. The birds used in our study were  
372 captured in Paris, and had therefore been previously exposed to trace metals in their natural  
373 urban habitat. Consequently, plumage bacterial communities at the start of the experiment  
374 might have already been shaped by past metal exposure. Because there was no significant  
375 correlation between bird capture site (i.e. pigeon flock) and aviary, the potential initial  
376 differences in bacterial community between the birds would, however, have reduced the  
377 power of our analysis, and the significant differences between treatments observed in our  
378 study are therefore conservative.

379 Although our knowledge on plumage bacterial community composition and function is  
380 scarce, feather bacteria seem to play a role in bird immunity, reproduction and feather  
381 colouration and condition (Clayton 1999, Shawkey et al. 2007, Gunderson et al. 2009,  
382 Leclaire et al. 2014, 2015, Jacob et al. 2015 but see Jacob et al. 2014). Through their effects  
383 on plumage bacteria, trace metals may, for instance, affect thermoregulation and visual  
384 signals potentially involved in dominant status assessment and mate choice (Wolf 2000, Hill  
385 and McGraw 2006). Future studies should now investigate if and how the changes in plumage  
386 bacterial communities induced by trace metals affect bird fitness.

387

388

### 389 **ACKNOWLEDGMENTS**

390

391 We thank the “Mairie de Paris” (Thomas Charachon) for allowing the capture of birds and the  
392 Centre de Recherche en Ecologie Expérimentale et Prédictive (CEREEP) which provided

393 logistic support for the field work of this study. We are very thankful to T. Gayet, S. Pollet, S.  
394 Hasnaoui, F. Lorente, S. Perret and B. Decencière for their help all along the field work.  
395 Molecular analyses were partly performed at the technical facilities of the labexCeMEB and  
396 were funded by an “ANR PDOC” grant (to SL) (No. ANR-13-PDOC-0002).

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635 **FIGURE LEGENDS**

636

637 **Fig. 1** Constrained redundancy analysis (RDA function in R) on bacterial community  
638 dissimilarities (estimated by Jaccard distances) between *lead*, *zinc*, *lead and zinc* and  
639 *control* groups. Both aviaries of a same treatment are distinguished using either filled or empty  
640 circles.

641

642 **Fig. 2** Mean  $\pm$  SE plumage bacterial community dissimilarities (estimated by Jaccard distance)  
643 in dyads of pigeons according to the spatial distance between them (0 means that the  
644 individuals belonged to the same aviary, 1 means that they were in side-by-side aviaries, 2  
645 means that 1 aviary was between them, etc.).

646

647 **Fig. 3** Mean  $\pm$  SE plumage bacterial richness (number of different OTUs) according to metal  
648 exposure. a and b were significantly different (p-value < 0.05) while ab was not different from  
649 a or from b.

650

651 **Fig. 4** Mean  $\pm$  SE plumage bacterial load (number of bacterial colonies per slide) according to  
652 metal exposure. Tests were performed for each pair of treatment. a and b tended to be  
653 different (p-value < 0.072) while ab was not different from a or from b.



Table 1. Lead blood and feathers concentrations (mean±se, ppb) in lead-exposed and lead non-exposed birds, zinc blood and feathers concentrations (mean±se, ppm) in zinc-exposed and zinc non-exposed birds and ANOVAs with lead or zinc concentrations in blood or feathers as dependent variable and zinc or lead exposure as explanatory variable.

		Lead exposed	Lead non-exposed	F	P
Lead	Blood	55.49±6.54	35.73±6.77	4.47	0.040
	Feathers	402.31±35.68	255.81±49.75	19.61	<0.001
		Zinc exposed	Zinc non-exposed	F	P
Zinc	Blood	4.69±0.15	4.20±0.18	5.52	0.022
	Feathers	96.62±2.15	92.31±2.25	2.13	0.149

Table 2. PERMANOVAs with 5000 permutations based on Jaccard distance for OTU presence/absence data with the metal exposure as the explicative variable. Bacterial communities' similarities were compared between each pair of metal treatment.

	Control	Zinc-exposure	Lead-exposure
Zinc-exposure	$F_{1,40}=2.74, P=0.002$	-	-
Lead-exposure	$F_{1,42}=3.44, P<0.001$	$F_{1,41}=3.16, P<0.001$	-
Zinc and lead-exposure	$F_{1,40}=4.40, P<0.001$	$F_{1,39}=2.10, P=0.006$	$F_{1,41}=5.13, P<0.001$

Fig. 1

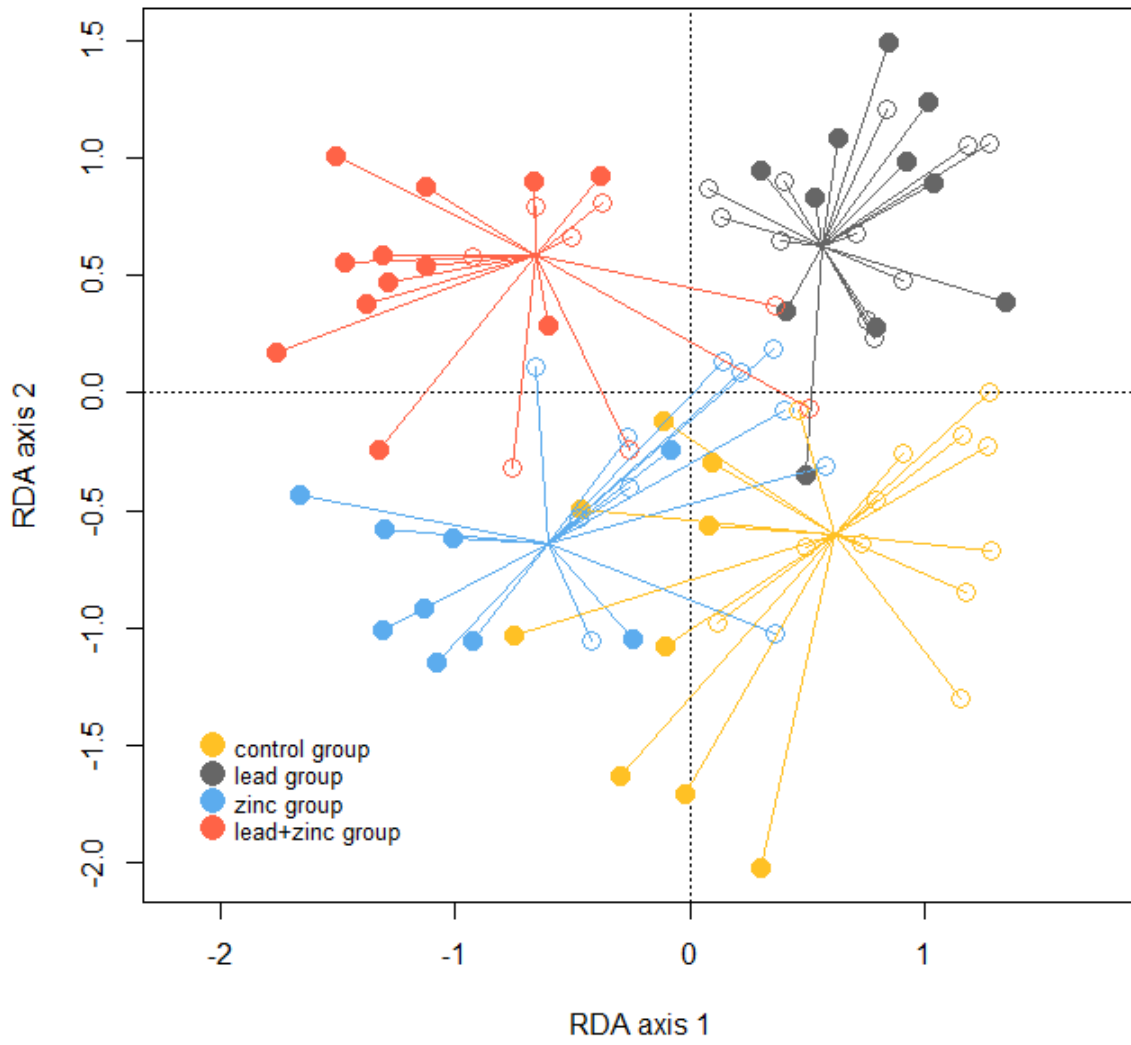


Fig. 2

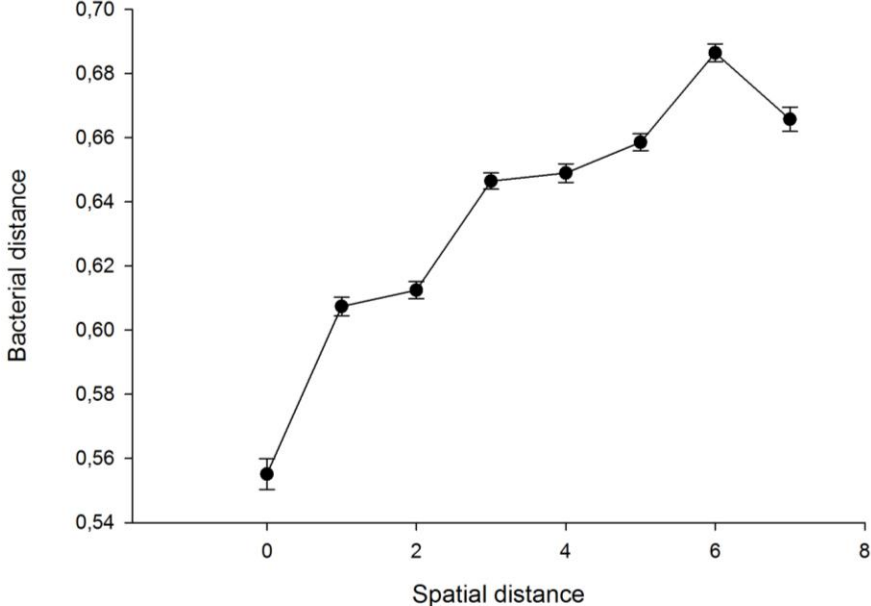


Fig. 3

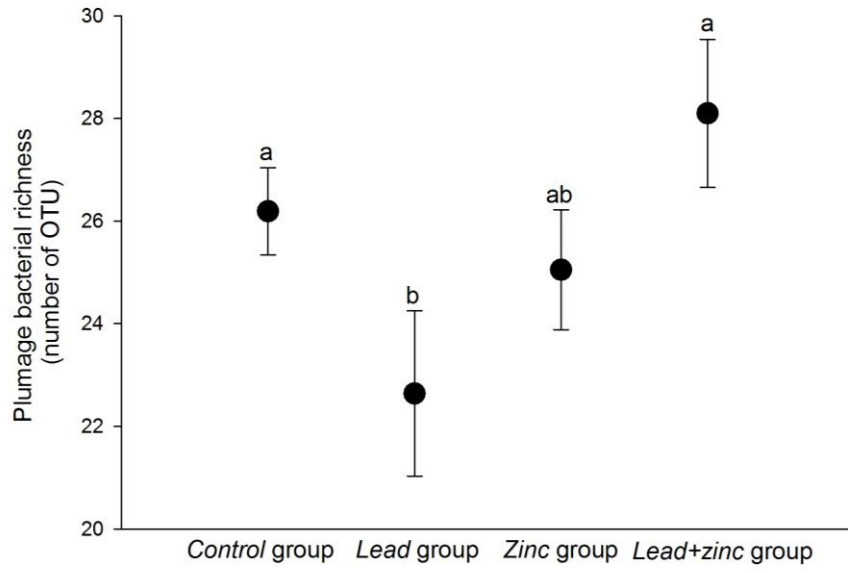


Fig. 4

