1 **Ecological selection of siderophore-producing microbial taxa in response**

2 **to heavy metal contamination**

3

4 Elze Hesse[1$*], Siobhán O'Brien[1,2*], Nicolas Tromas[3], Florian Bayer[1¶], Adela Lujan[1,4], Eleanor van

5 Veen[5], Dave J. Hodgson[6], Angus Buckling[1‡]

6

7 [1] ESI & CEC, Biosciences, University of Exeter, Penryn Campus, Cornwall, TR10 9FE, UK. E-

8 mail: F.Bayer@exeter.ac.uk[¶], A.J.Buckling@exeter.ac.uk[‡]

9 [2] Institut für Integrative Biologie, ETH Zürich, Universitätstrasse 16, Zürich, 8092, Switzerland. E-

10 mail: siobhan.obrien@env.ethz.ch

11 [3] Département de sciences biologiques, Université de Montréal, 90 Vincent-d'Indy, Montréal, QC

12 H2V 2S9, Canada. E-mail: tromas.nicolas@gmail.com

13 [4] CIQUIBIC, Departamento de Química Biológica, Facultad de Ciencias Químicas, CONICET,

14 Universidad Nacional de Córdoba, Córdoba, Argentina. E-mail: adem.lujan@gmail.com

15 [5] Camborne School of Mines, CEMPS, University of Exeter, Penryn Campus, Cornwall TR10 9FE,

16 UK. E-mail: E.M.Van-Veen@exeter.ac.uk

17 [6] CEC, University of Exeter, Penryn Campus, Cornwall, TR10 9FE, UK. E-mail:

18 D.J.Hodgson@exeter.ac.uk

19

20 * These authors contributed equally to this work

21

22 [$] Correspondence: E-mail: elzehesse@gmail.com; Tel: +44 (0) 1326 259470; Fax: +44 (0) 1392

23 263434

24

25 Conflict of Interest: We declare no conflict of interest

26

27 Authorship: EH, SOB, AL, DJH, EvV, AB conceived and designed the experiment. DJH provided
28 new perspectives. EH, SOB, FB, AL collected the data. EH, FB, NT, DJH carried out the data
29 analyses. EH & AB wrote the first draft of the manuscript, and all authors contributed substantially
30 to revisions.

31

32 Data accessibility: Upon acceptance, data presented in the manuscript will be made available on
33 Dryad.

34

35 Running title: Metals select for siderophore production

36

37 Type of Article: Letter

38

39 Key words: Adaptation, Detoxification, Ecological species sorting, Evolution, Metal tolerance,
40 Public good dynamics, Remediation, Selection

41

42 Word count: Abstract (145), Main text (4994)

43

44 Number of: references (82), Figures (5), Tables (0), Text boxes (0)

Abstract

Some microbial public goods can provide both individual and community-wide benefits, and are open to exploitation by non-producing species. One such example is the production of metal-detoxifying siderophores. Here, we investigate whether the conflicting selection pressures on siderophore production by heavy metals – a detoxifying effect of siderophores, and exploitation of this detoxifying effect - results in a net increase or decrease. We show that the proportion of siderophore-producing taxa increases along a natural heavy metal gradient. A causal link between metal contamination and siderophore production was subsequently demonstrated in a microcosm experiment in compost, in which we observed changes in community composition towards taxa that produce relatively more siderophores following copper contamination. We confirmed the selective benefit of siderophores by showing that taxa producing large amount of siderophores suffered less growth inhibition in toxic copper. Our results suggest that ecological selection will favour siderophore-mediated decontamination, with important consequences for potential remediation strategies.

Angus Buckling 6/10/17 13:45
Deleted: imposed by heavy metals increases siderophore production in natural microbial communities

Angus Buckling 6/10/17 13:46
Deleted: or whether

Angus Buckling 6/10/17 13:46
Deleted: reduces siderophore production

64 **INTRODUCTION**

65 It is becoming increasingly apparent that many public goods benefit not only conspecifics but also
66 other species. For example, many bacterial proteases show extracellular activity, providing potential
67 nutritional benefits to neighbouring bacteria independent of taxonomy (Suleman 2016); and
68 immune-repressing molecules produced by parasitic nematodes provide a potential benefit to all co-
69 infecting parasites (Maizels *et al.* 2001). Regardless of whether public goods are solely conspecific
70 or also have interspecific benefits, there is potential for non-producers to outcompete producers
71 assuming public good production carries some metabolic cost (Hamilton 1964; Hamilton & Axelrod
72 1981; Frank 1994). Hence, the evolution of costly public goods is crucially dependent on the extent
73 to which benefits are reaped by producers, other individuals carrying the public good gene or non-
74 producers. While the evolution of public goods has been studied extensively within species, we
75 know very little about how ecological sorting influences interspecific public good production within
76 natural communities. Here we combine surveys and experiments to determine how ecological
77 selection acts on a microbial interspecific public good: siderophore-mediated heavy metal
78 decontamination.

79 Heavy metals are ubiquitous components of the Earth's crust, and large amounts have been
80 released into the environment as a result of human activities (Nriagu & Pacyna 1988). Heavy metals
81 are toxic to microbes to varying degrees (Giller *et al.* 1998) and their presence can greatly impact
82 natural communities (Gans *et al.* 2005). In the face of long-term selection, microbes have evolved
83 mechanisms to cope with metal toxicity, including metal reduction, reduced cell permeability and
84 extracellular sequestration (Nies 1999; Bruins *et al.* 2000; Valls & De Lorenzo 2002). One such
85 detoxification mechanism is the production of siderophores. While the canonical function of
86 siderophores is to scavenge insoluble iron (Ratledge & Dover 2000), bacteria also use these
87 secreted molecules to detoxify metals (Braud *et al.* 2010). Siderophore production can be induced
88 by the presence of non-iron metals (Hofte *et al.* 1993; Teitzel *et al.* 2006), which they bind with
89 various affinities (Braud *et al.* 2009). These siderophore-metal complexes are unable to enter

90 bacterial cells, thereby reducing free toxic metal concentrations in the environment (Schalk *et al.*
91 2011). This has led to the suggestion of adding siderophores or siderophore-producing microbes to
92 remediate metal-contaminated environments (Rajkumar *et al.* 2010; O'Brien & Buckling 2015).
93 However, to understand how siderophores may both contribute to natural decontamination and
94 long-term remediation efficacy, it is crucial to determine how metal toxicity affects selection for
95 siderophore production in natural communities.

96 Given their detoxifying effect, increasing metal toxicity might be expected to result in
97 ecological species sorting in favour of species with greater siderophore production. However, the
98 production of detoxifying siderophores not only benefits the producer (or its close relatives), but
99 potentially also neighbouring cells, both con- and hetero-specific, in the community. Siderophore
100 production – which is up-regulated in response to heavy metals (Hofte *et al.* 1993; Teitzel *et al.*
101 2006) – is often associated with a fitness cost, hence selection may favour cells that produce fewer
102 siderophores, but still receive the same detoxifying benefits of siderophore production from
103 neighbours (West *et al.* 2007; O'Brien *et al.* 2014). This can result in a 'tragedy of the commons',
104 whereby mean siderophore production levels are actually reduced in the presence of toxic metals,
105 despite the benefits that siderophores provide to the group as a whole (O'Brien *et al.* 2014).
106 moreover, the (almost) complete loss of public goods production, and the resultant decline in group
107 productivity, has been observed in various experimental set ups, including siderophore production
108 under iron-limited conditions (Griffin *et al.* 2004). Limited diffusion of public goods (Kummerli *et*
109 *al.* 2009; Kummerli *et al.* 2014) and positive assortment of producing cells resulting from spatial
110 structure (Hamilton 1964; West *et al.* 2007; Mitri & Foster 2013; Ghoul & Mitri 2016; Pande *et al.*
111 2016) may, however, limit community-wide benefits and prevent overexploitation of siderophores
112 by non-producing cells (Oliveira *et al.* 2014), potentially resulting in stable coexistence of
113 producing and non-producing taxa (Cordero *et al.* 2012; Morris *et al.* 2012; Morris 2015; Estrela *et*
114 *al.* 2016). The situation is further complicated by the iron-scavenging function of siderophores,
115 which is also open to exploitation within (Griffin *et al.* 2004; Buckling *et al.* 2007; Lujan *et al.*

Angus Buckling 6/10/17 14:20
Deleted: may then

Angus Buckling 6/10/17 14:20
Deleted: would

Angus Buckling 6/10/17 14:21
Deleted: .

Angus Buckling 6/10/17 12:26
Moved (insertion) [1]

Angus Buckling 6/10/17 12:27
Deleted: T

Angus Buckling 6/10/17 12:27
Deleted: is

Angus Buckling 6/10/17 12:28
Deleted: in the context of

Angus Buckling 6/10/17 12:26
Moved up [1]: This has been observed in various experimental set ups, including in the context of siderophore production (Griffin *et al.* 2004).

126 2015) and between species (Barber & Elde 2015; Galet *et al.* 2015). Given that siderophores
127 provide direct benefits (and indirect benefits through helping kin), but may also benefit non-kin and
128 other species, it is unclear if net siderophore production will increase or decrease in natural
129 communities as a function of metal toxicity.

130 To investigate how metal contamination affects ecological selection for siderophore
131 production, we first confirmed that siderophores act as interspecific public goods in an *in vitro*
132 siderophore-addition experiment. We then conducted a survey along a natural contamination
133 gradient. We correlated total metal content and soil acidity with species composition and estimates
134 of siderophore production determined from the proportion of bacteria that show detectable
135 extracellular iron-chelation *in vitro*. Soil acidity is an important environmental factor determining
136 metal solubility and thereby toxicity. We then conducted an experimental study in compost
137 communities to determine causal links between metal contamination and siderophore production.
138 Note that we do not simultaneously address within species selection alongside ecological selection,
139 largely because the genetic resolution of our sequencing methods is only at the genus level.
140

141 **METHODS**

142 **Siderophores as interspecific public goods**

143 To test whether siderophores can act as interspecific public goods we quantified whether the
144 presence of heterospecific siderophores – produced by taxonomically diverse soil-dwelling
145 microbes – ameliorates growth of non-producing *Pseudomonas aeruginosa* in copper-contaminated
146 broth. We inoculated ~10^4 colony forming units (CFUs) of a producing *P. aeruginosa* strain (PA01)
147 and an isogenic non-producing mutant (PA01*ΔpvdDΔpchEF*) in isolation into 3-4 replicate micro-
148 centrifuge tubes, containing 900 μl of copper-contaminated KB broth (final morality 0.6 mM
149 $CuSO_4$), which reduces relative non-producer fitness (O'Brien *et al*. 2014). In addition, ~10^4 CFUs
150 of either strain were inoculated in copper broth containing 0.6 mM of yersiniabactin (*P. stutzeri*),
151 ornibactin (*Burkholderia vietnamiensis*), ferrioxamine E (*Streptomyces olivaceus*) or schizokinen

152 (*Bacillus megaterium*). Copper is a common heavy metal (Nriagu & Pacyna 1988), including at our
153 field site (Fig. 2A); hence, we used $CuSO_4$ in all *in vitro* assays. Bacterial cultures were horizontally
154 shaken at 37°C for 24 hours (h), after which culture was plated onto agar to obtain cell densities and
155 calculate Malthusian growth rate: $m=\ln(N_f/N_0)/\Delta t$, where N_0 and N_f are initial and final bacterial
156 densities, and $\Delta t=24$h.
157 To confirm that non-producer growth was lower in toxic copper compared to the siderophore-
158 producing strain, we used a one-way ANOVA. We tested whether heterospecific siderophores can
159 ameliorate non-producer growth using a one-tailed t-test comparing mean growth differences
160 between strains in control and siderophore-supplemented copper broth.
161
162 **Natural microbial communities**
163 *Soil collection and characterization*
164 Soil samples were collected in a former poly-metallic mining area situated in the Poldice Valley (N:
165 50°14.56; W: 5°10.10) in Cornwall (UK). The valley is rich in heavy metals, as apparent from the
166 significant production of heavy metals during the 18-19th centuries (Burt 1998). The area is no
167 longer worked leaving a legacy of untreated mining waste. 94 samples were collected by pushing
168 sterile bulb planters into the ground near chimneys, slag heaps and regenerated areas, representing a
169 wide contamination range. The upper part of the soil core was discarded to rule out possible ground
170 surface contamination. Samples were then transferred to sterile 50 millilitre (ml) falcon tubes and
171 stored at 4°C until further processing. Prior to DNA extraction and soil characterization, samples
172 were sieved using individual plastic sterile sieves with 1 millimetre mesh size.
173 Quantification of heavy metals and metalloids (e.g., Fe, Cd, Cr, Cu, Mn, Hg, Ni, Ti, V, Zn,
174 Pb, Sn, As) was carried out by ALS global (Loughrea, Ireland), using an aqua regia digest (EPA
175 3050b). To assess the total content of these determinants, samples were analysed using emission
176 spectroscopy (ICP-OES). For each sample, we quantified pH by suspending 1 gram (g) of soil in 5

177 ml of 0.01M $CaCl_2$ (Hendershot & Lalande 2008), which was shaken for 30 minutes (min) and left
178 to stand for 1h, after which pH was measured using a Jenway 3510 pH meter (Stone, UK).
179
180 *Siderophore production*
181 The relationship between siderophore production, soil acidity and metal contamination was tested
182 by screening a subset of clones for siderophore production. Siderophore production was necessarily
183 measured under common garden conditions to avoid confounding effects of environmental variation
184 if conducted *in situ*, causing both differential siderophore induction and soil metal-chelating
185 activities, which could directly affect the siderophore assay. For each sample, 1 g of soil was
186 transferred to 6 ml of M9 solution in 30 ml glass vials, which were shaken for 2h at 28°C and 180
187 rpm, after which supernatant was plated onto LB agar. Thirty colonies per sample were randomly
188 selected and grown for 48h independently in 200 microliter (μl) KB broth at 28°C. A 2 μl sample
189 from each colony was then spotted on blue-tinted iron-limited CAS agar plates (Schwyn & Neilands
190 1987) using a pin replicator. Plates were incubated at 28°C for 48h, after which we scored the
191 presence of orange halos, a qualitative indicator of siderophore secretion, to obtain an estimate of
192 the proportion of siderophore-producing clones in each community.
193
194 *DNA extractions and real time PCR*
195 To determine how community abundance and composition varied across soils we extracted genomic
196 DNA from 250 milligram (mg) soil per sample, using MoBio Powerlyzer PowerSoil© DNA
197 isolation kits (Carlsbad, CA, USA), following the manufacturer's protocol with the bead beating
198 parameter set to 4500 rpm for 45 seconds (s). The integrity of DNA was confirmed using 1% TAE
199 agarose gels stained with 1x Redsafe DNA Stain (20 000X); 5 samples were subsequently
200 discarded, yielding 89 DNA samples in total.
201 Community density was quantified using real-time PCR (StepOnePlus Real-Time PCR,
202 Applied Biosystems, Foster City, CA, USA) on 1:10 and 1:100 diluted samples with primers 16S

203 rRNA 338f (ACT CCT ACG GGA GGC AGC AG) and 518r (ATT ACC GCG GCT GCT GG)
204 (Øvreås & Torsvik 1998). Triplicates of each sample were run along gDNA standards (5 x 10^{2-6} 16S
205 rRNA genes of *Pseudomonas fluorescens*) and non-template controls. All assays were based on 15
206 µL reactions, using 1x Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent
207 technologies, Santa Clara, CA, USA), 150nM 338f and 300nM 518r primers, 300nM ROX and
208 100ng/µL BSA. Thermal conditions were set to 3 min at 95ºC for initial denaturation, followed by
209 40 cycles of 5s at 95ºC and 10s at 60ºC (collection of fluorescent data), followed by a melting curve
210 at 95ºC for 15s, 60ºC for 1 min ramping up to 95ºC in steps of +0.3ºC for 15s. Melting curves and
211 confirmation of non-template controls was analysed using StepOne Software 2.3 (Applied
212 Biosystems). Baseline corrections, Cq values and efficiencies (1.89 ± 0.07 and 1.89 ± 0.08 for
213 standards and samples) were determined using LinRegPCR version 2016.0 (Ruijter *et al.* 2009).
214 16S rRNA gene quantities were calculates using the one point calibration method (Brankatschk *et*
215 *al.* 2012), corrected for variation in dry weight. Bacterial cell counts were estimated using
216 'CopyRighter' (Angly *et al.* 2014), which corrects for variation in lineage-specific 16S gene copy
217 numbers across samples. Note that this method does not account for unassigned OTUs.
218
219 *Statistical analyses*
220 Because of strong collinearity among heavy metals, we carried out a principal component analysis
221 (PCA) on centred and scaled data. Most metals loaded positively on the first principal component
222 (PC1; Fig. 2A), which was subsequently used as proxy for total contamination. To test how PC1
223 and pH affect siderophore production we used individual generalized linear models (GLMs) with a
224 quasi-binomial error structure. The effect of these environmental variables on bacterial densities
225 was tested using individual GLMs on $\log_{10}$-transformed data.
226
227 *Sequencing, OTU picking and diversity analyses*
228 Library preparation and sequencing was performed by the Center for Genomic Research (University

229 of Liverpool, Supplementary Methods).

230 Base-calling and de-multiplexing of indexed reads was performed using CASAVA (Illumina,
231 San Diego, CA, USA) to produce 89 samples from the 1st lane of sequence data, which were
232 trimmed to remove Illumina adapter sequences using Cutadapt (Martin 2011) and to remove low
233 quality bases, using Sickle 1.200 with a minimum quality score of 20. After trimming, reads <10 bp
234 were removed. If both reads from a pair passed this filter, each was included in the R1 (forward
235 reads) or R2 (reverse reads) file. If only one of a read pair passed this filter, it was included in the
236 R0 (unpaired reads) file.

237 Sequences were processed using default parameters of the SmileTrain pipeline
238 (https://github.com/almlab/SmileTrain/wiki/), including reads quality and chimera filtering, paired-
239 end joining, de-replication and *de novo* distribution-based clustering using USEARCH (Edgar 2010;
240 htpp://www.drive5.com/usearch), Mothur (Schloss *et al.* 2009), Biopython, dbOTUcaller
241 algorithms (Preheim *et al.* 2013; https://github.com/spacocha/dbOTUcaller) and custom scripts. We
242 generated an OTU table that was filtered to minimize false OTUs using QIIME (Caporaso *et al.*
243 2010; http://qiime.org/) by removing OTUs observed <10. We assigned taxonomy, post-clustering,
244 using the 97% reference OTU collection of the GreenGenes database (http://greengenes.lbl.gov).
245 Taxonomy information was added to the OTU table using biom add-metadata scripts (http://biom-
246 format.org/). A total of 8 604 074 sequences were obtained, ranging from 39 253 to 192 455 reads
247 per sample, with a median of 91 646. This dataset was clustered into 45 891 OTUs.

248 Diversity calculations were based on non-rarefied OTU tables. β-diversity was calculated
249 using Jensen-Shannon Divergence (JSD) metrics (Fuglede & Topsoe 2004; Preheim *et al.* 2013),
250 which are robust to sequencing depth variation. The R ‘phyloseq’ package (McMurdie & Holmes
251 2013) was used to transform the OTU table into relative abundances, which were square-root-
252 transformed into Euclidean metrics (Legendre & Gallagher 2001). Finally, we used Nonmetric
253 Multidimensional Scaling (NMDS) plots (Shepard 1962; Kruskal 1964) to order bacterial
254 community composition. Differences in community structure were tested using PERMANOVA

255 (Anderson 2001), implemented using *adonis*() from the R 'vegan' package with 999 permutations.

256 To confirm that pH and PC1 shape community structure, we used K-means partitioning
257 algorithms (MacQueen 1967) implemented with *cascadaKM*() from the 'vegan' package with 999
258 permutations. K-means is a completely independent way of binning samples. We Hellinger-
259 transformed (Rao 1995) the OTUs table using *decostand*(x. method="hellinger") and tested whether
260 our samples naturally clustered into 2-10 groups based on their composition using the Calinski-
261 Harabasz index (Caliński & Harabasz 1974).

262 To investigate how environmental variables contributed towards explaining variation in
263 community composition, we used a multivariate regression tree analysis (MRT; Breiman *et al.*
264 1984; De'Ath 2002) for pH and PC1 separately, using the R 'mvpart' package (De'Ath 2007;
265 Therneau *et al.* 2015). The OTU table was first Hellinger-transformed (Rao 1995) before carrying
266 out the analyses (Ouellette *et al.* 2012). After 200 cross-validations (Breiman *et al.* 1984), we
267 plotted and pruned the tree using the 1-SE rule (Legendre & Legendre 2012) to select the least
268 complex model. We used *rpart.pca*() from the 'mvpart' package to plot a PCA of the MRT.

269 α-diversity was estimated using Shannon (Oksanen *et al.* 2010; 'vegan' package) and Chao1
270 (Vavrek & Larsson 2010; 'fossil' package) indices. We used *resample_estimate*() from the R
271 'breakaway' package (Willis & Bungle 2014) to account for sample size variability, setting the
272 number of bootstraps to 500 with replacement. The relationship between α-diversity and
273 environmental variables was tested using *betta*() from the 'breakaway' package, which accounts for
274 statistical errors associated with estimating these indices.

275

276 **Copper-addition experiment**

277 *Experimental design*

278 To infer a causal relationship between toxic metals and siderophore production, we set up
279 experimental compost communities. We isolated the community from fresh compost (Verve John
280 Innes No. 1) by adding 40g to 200 ml of M9 solution and incubating at 150 rpm at 28°C for 24h.

281 Two ml (~5 x 10^7 CFUs) of supernatant was subsequently used to seed twelve microbial
282 communities in 90 millimetre Petri dishes containing 30g of twice-autoclaved compost. Hence, all
283 treatments started off with the same community and level of siderophore production.
284 Microcosms were incubated at 26°C and 75% humidity for 24h, after which we supplemented
285 six microcosms with 2 ml of filter-sterilised 0.25M $CuSO_4$ or ddH_20. This concentration of $CuSO_4$
286 hindered bacterial growth. Microcosms were incubated for 6 weeks. After three weeks, another 2 ml
287 dose of $CuSO_4$ or ddH_2O was added where appropriate. Samples of the community were taken prior
288 to copper amendment and 3-6 weeks post-inoculation by transferring 1g compost to 6 ml of M9
289 solution in 30 ml glass vials. Vials were shaken for 2h at 28°C at 180 rpm, after which supernatants
290 were frozen at -80° C in 25% glycerol.
291
292 *Siderophore and copper resistance assays*
293 To quantify siderophore production, 24 individual clones per treatment-time combination were
294 isolated by incubating supernatant on LB plates at 28°C for 48h. Individual colonies were then
295 transferred to 2 ml of KB broth and grown for 48h at 28°C, after which the supernatant was assayed
296 for the extent of iron chelation. Siderophore production was quantified using the liquid CAS assay
297 described by Schwyn and Neilands (1987), with the modification that one volume of ddH_20 was
298 added to the assay solution (Harrison & Buckling 2005). We used the following quantitative
299 measure to obtain an estimate of siderophore production per clone: $[1 - (A_i/A_{ref})] / [OD_i)]$, where
300 OD_i = optical density at 600 nanometre (nm) and A_i = absorbance at 630 nm of the assay mixture *i*
301 or reference mixture (KB+CAS; A_{ref}). Note that CAS assays performed in iron-limited KB
302 (supplemented with 20 mM $NaHCO_3$ and 100 μg ml^{-1} human apotransferrin) provided qualitatively
303 similar results (data not shown).
304 All final time-point clones were grown at 28°C for 24h, after which ~10^4 CFUs were
305 inoculated into 96-well plate wells containing 200 μl of KB broth supplemented with or without a
306 toxic dose of $CuSO_4$ (6.17 mM). Clones were incubated statically at 28°C for 48h, and their OD was

307 measured at 600 nm every 8-12h to quantify growth (Varioskan Flash plate reader, Thermo
308 Scientific, Waltham, MA, USA).
309
310 *Sanger sequencing of 16S rRNA*
311 The 16S rRNA gene of all assayed final-time point clones was sequenced to confirm genus-level
312 identity: PCRs were performed in 25µL reactions containing 1x DreamTaq Green PCR Master Mix
313 (2X) (Thermo Scientific), 200 nM of the 27F and 1492R primers and 3 µL of 1:100 diluted culture
314 that had undergone 3 freeze-thaw cycles. The thermal cycling parameters were set to 94ºC for 4
315 min, followed by 35 cycles of 1 min at 94ºC, 30s at 48ºC and 2 min at 72ºC, and a final extension of
316 8 min at 72ºC. Following Exo-AP clean-up, high quality samples were Sanger sequenced using the
317 27F primer (Core Genomic Facility, University of Sheffield).
318 The quality of all sequences was assessed using *plotQualityProfile*() from the R 'dada2'
319 package (Callahan *et al.* 2016). Based on the obtained plots, sequences were trimmed in Genious to
320 achieve an overall quality score >35. Using Mother, sequences longer then 300bp were aligned to
321 the Silva.Bacteria.Fasta database, and taxonomy was classified using the RDP trainset 14 032015 as
322 reference database.
323
324 *Statistical analyses*
325 The effects of copper and time on mean siderophore production was tested using a linear mixed
326 effects (LME; 'lme4' R package; Bates *et al.* 2014) model with copper x time (3-6 weeks post
327 inoculation) as fixed categorical effects and random intercepts fitted for each community (n = 12),
328 and individual clones nested within communities (n = 24), to account for temporal dependencies.
329 We used NMDS ordination plots to depict pair-wise Bray-Curtis dissimilarities in genus-level
330 composition between microcosms. To test whether treatments differed significantly in their
331 composition we used PERMANOVA with 999 permutations, and tested for equality of between-
332 treatment variance using permutation tests for homogeneity of multivariate dispersion.

333 To test for the effect of copper on metal tolerance, we used LME with $ln(OD_{Cu}/OD_{KB})$ as
334 response variable, copper background as fixed effect and a random slope fitted for mean-centred
335 hours: random=~(Hours)|Community/Clone. The model thus accounts for intrinsic differences
336 between communities, and nested clones, in their ability to tolerate toxic copper over time, and
337 explicitly tests whether pre-adaptation to copper increases mean copper tolerance. To test whether
338 tolerance was directly mediated by variation in siderophore production, we replaced 'copper
339 background' with clone-specific siderophore production.
340 In general, full models were simplified by sequentially eliminating non-significant terms ($P >$
341 0.05), after which the significance of the explanatory variables was established using likelihood
342 ratio tests. In case of significant differences, Tukey contrasts were computed using the 'multcomp'
343 package (Hothorn *et al.* 2008), with $\alpha < 0.05$. We used *R* Version 3.1.3 for all analyses
344 (http://www.r-project.org).
345
346 **RESULTS**
347 **Foreign siderophores restore non-producers fitness in toxic copper broth**
348 Non-producer growth was significantly lower in toxic copper compared to that of the producing
349 wild type strain of *Pseudomonas aeruginosa* ($F_{1,6} = 10.97$, $P = 0.02$; Fig. 1). Crucially, the addition
350 of heterospecific siderophores significantly reduced mean growth differences between strains (one-
351 tailed t-test: $t = 3.67$, *d.f.* = 3, $P = 0.035$; Fig. 1).
352
353 **Microbial diversity, abundance and siderophore production along a natural metal gradient**
354 We found that the proportion of siderophore-producing isolates was significantly greater in more
355 contaminated soils (PC1: $\chi^2 = 4.42$; d.f. = 1, $P = 0.04$ Fig. 2C). Because contamination co-varied
356 with soil acidity (Pearson's correlation: $r = 0.61$, d.f. = 86 and $P < 0.001$; Fig. 2B), siderophore
357 production also increased as a function of pH ($\chi^2 = 28.16$; d.f. = 1, $P < 0.001$; Fig. 2C). Neither pH
358 nor PC1 significantly affected microbial abundance (GLM: $F_{1,87} = 0.01$, $P = 0.99$ for PC1 and pH;

359 Fig. 2D). Note that total iron content neither co-varied with pH (Pearson's correlation: $r = 0.03$, d.f.
360 = 86 and $P = 0.09$) nor affected siderophore producers (Fe: $\chi^2 = 0.45$; d.f. = 1, $P = 0.50$; Fig. 3).
361 Both pH and PC1 predicted community structure: samples with similar range values of pH
362 (PERMANOVA: $R^2 = 0.087$, $P < 0.001$) or PC1 ($R^2 = 0.065$, $P < 0.001$) had similar community
363 composition. Because the explanatory power of these variables was relatively low (Fig. S1 in
364 Supplementary Information), we performed a K-means analysis, which showed that samples were
365 naturally divided into 2-3 groups differing significantly in their PC1 or pH, respectively (Fig. S2 in
366 Supplementary Information). We used MRT to confirm these findings and observed that R^2 was
367 highest when pH was used as explanatory variable (pH: $R^2 = 0.183$ and PC1: $R^2 = 0.085$; Fig. 4).
368 Alpha diversity was largely independent of PC1, but varied as a function of pH (Fig. S3 in
369 Supplementary Information; $P < 0.001$ for both indices).
370
371 **The effect of copper on siderophore production in experimental communities**
372 Our assay of siderophore production along a natural gradient showed that siderophore production
373 was greater in more contaminated soils. However, it remains unclear whether metals are a
374 significant driver explaining variation in siderophore production. Notably, pH is an important
375 predictor of soil bacterial diversity and composition (e.g., Fierer & Jackson 2006; Griffiths *et al.*
376 2011), and correlated positively with contamination, making any interpretation ambiguous. To
377 determine a causal link between metals and siderophore production, we carried out an experiment
378 and characterised and measured siderophore production of multiple clones as well as their metal
379 tolerance. We found that mean siderophore production was significantly greater in communities
380 subjected to copper contamination (LME: copper effect: $\chi^2 = 6.91$; d.f. = 1; $P < 0.01$; Fig. 5A). Note
381 that overall siderophore production decreased through time (time effect: $\chi^2 = 16.02$; d.f. = 1; $P <$
382 0.001) independent of treatment (time x treatment effect: $\chi^2 = 0.001$; d.f. = 1; $P = 0.98$). Soil acidity
383 marginally increased following copper contamination (mean pH ± SE after 3 and 6 weeks of

384 incubation in control = 7.13 ± 0.05, 7.09 ± 0.02 and in copper = 6.90 ± 0.04, 6.60 ± 0.05), indicating
385 that siderophore production was greater in more acidic compost.

386 We identified clones at the genus-level to explore the role of ecological sorting in driving
387 siderophore production. Community composition varied significantly between treatments
388 (PERMANOVA: $F_{1, 11} = 3.88$, $P = 0.015$; multivariate dispersion: $F_{1, 11} = 0.021$, $P = 0.91$; Fig. 5B),
389 with siderophore-producing genera being selectively favoured in copper-contaminated compost
390 (Fig. 5C and Table S2). Crucially, clones isolated from copper-contaminated communities were
391 significantly less inhibited when grown in toxic copper broth compared to those from non-
392 contaminated communities (LME: $\chi^2 = 6.80$; d.f. = 1; $P < 0.01$; Fig. 5D), which was mediated by
393 increased siderophore production (LME: $\chi^2 = 16.68$; d.f. = 1; $P < 0.001$).
394
395 **DISCUSSION**

396 In this study, we investigated how heavy metals affected ecological selection for siderophore
397 production – an interspecific microbial public good – across a natural contamination gradient and
398 during a controlled experiment in compost. We hypothesised there could be selection for both
399 increased and decreased siderophore production, because of the detoxifying effect of siderophores
400 and the potential for interspecific exploitation, respectively. Our findings suggest that the presence
401 of toxic metals resulted in net ecological selection for taxa that produced large amounts of
402 siderophore, although this doesn't rule out the possibility that some exploitation occurs. We also
403 confirmed that bacteria producing more siderophores suffered less growth inhibition in toxic copper
404 broth.

405 Ecological selection for increased siderophore production contrasts with previous *in vitro*
406 within-species (*P. aeruginosa*) results, in which non-producing 'cheats' were able to outcompete
407 siderophore producers in copper-contaminated broth (O'Brien *et al.* 2014), resulting in a net
408 reduction in siderophore production in the presence of toxic metals. A key reason for this difference
409 is likely to be the spatial structure in soil/compost resulting in localised detoxification, such that

410 producers and their immediate neighbours gain the most from siderophores (Hamilton 1964; West
411 & Buckling 2003; Buckling *et al.* 2007; West *et al.* 2007; Lujan *et al.* 2015). Hence, low
412 siderophore producers should experience more of the toxic metal effect. Limited dispersal would
413 also lead to immediate neighbours having a higher probability of being conspecifics - a likely
414 reason as to why taxa that typically produce more siderophores dominated metal-contaminated
415 communities. Direct comparison of intra- and inter-specific changes in siderophore production in
416 soil would tease apart the differing roles of spatial and community structure in determining these
417 results.

418 Siderophore production decreased in all our experimental communities over time, which is
419 likely caused by novel abiotic selection pressures resulting from laboratory conditions. We also
420 cannot rule out the possibility that non-producers did in fact benefit from siderophores produced by
421 other community members. However, as the decrease occurred in both copper and non-copper
422 environments, this reduction cannot be explained by exploitation of detoxifying siderophores. That
423 is not to say that this exploitation does not play a role in the observed levels of siderophore
424 production, but that the beneficial effects of siderophores to the producers outweigh these costs.
425 This is analogous to the evolution of collective antibiotic resistance in microbial populations (Lee *et*
426 *al.* 2010; Vega & Gore 2014), where resistant cells enhance the survival capacity of the overall
427 population by allowing 'weaker' cells to endure more antibiotic stress than they could in isolation.

428 In our survey of a former mining area, soil acidity and total contamination positively co-
429 varied, with both prolonged metal leaching in acidic soils and precipitation in more basic soils
430 likely contributing to this pattern (Alloway 1990; Adriano 2001). This covariance may well have
431 contributed to the observed patterns. First, acidity is a major determinant of microbial community
432 composition (e.g., Fierer & Jackson 2006; Griffiths *et al.* 2011), hence pH-mediated selection may
433 have indirectly favoured taxa that produce siderophores in larger amounts. Second, acidity affects
434 metal speciation and bio-availability to microbes in variable ways (Lofts *et al.* 2004; Gobran &
435 Huang 2011), with iron becoming largely insoluble at pH > 6.5 (Guerinot 1994). As such, increased

436 siderophore production in basic soils, which also had the highest metal concentrations, may have
437 been driven by selection imposed by iron limitation. However, our experimental manipulations,
438 where the same compost community was propagated with and without copper, strongly suggest a
439 direct effect of metal-imposed selection on siderophore production. This manipulation did have a
440 small effect on pH (copper decreased pH from approximately 7.1 to 6.6), but in this case there was
441 negative, rather than positive, covariance.

442 It was initially surprising to find that microbial densities were similar along the contamination
443 gradient; several studies have demonstrated that toxic metals reduce microbial abundance (reviewed
444 in Giller *et al.* 1998). These differences may perhaps reflect relatively low concentrations of
445 biologically available metals in our study; we only measured total metal content. Moreover, given
446 the mining history of our focal site, microbes are likely to be relatively well adapted to toxic metals:
447 selection of taxa with increased copper tolerance occurred very rapidly in our experiment. Note that
448 other more heavy metal resistance mechanisms, in addition to siderophore production, such as metal
449 reduction, reduced cell permeability (Nies 1999; Bruins *et al.* 2000; Valls & De Lorenzo 2002)
450 were not investigated here, and hence their importance relative to siderophores in determining metal
451 resistance is unknown.

452 Human-imposed metal contamination is a major problem for natural ecosystems. Several
453 studies have noted that addition of siderophores or siderophore-producing microbes could aid in
454 detoxifying contaminated soils, particularly when combined with the use of hyper-accumulating
455 plants, which commonly extract metals more efficiently when they are bound to siderophores
456 (Lebeau *et al.* 2008; Dimkpa *et al.* 2009). Crucially, hyper-accumulating plants take up
457 siderophore-metal complexes before metals flow back in the system following siderophore decay.
458 Our results provide some key insights into the optimal use of siderophores for phytoremediation.
459 The addition of high siderophore-producing bacteria following recent contamination events is likely
460 to be effective, because these organisms should have a selective advantage and hence contribute to
461 increasing community-level siderophore production. However, siderophore addition is unlikely to

Angus Buckling 6/10/17 13:52
Deleted: ,

Angus Buckling 6/10/17 13:53
Deleted: through

Angus Buckling 6/10/17 13:55
Deleted: direct

Angus Buckling 6/10/17 13:55
Deleted: not investigated here

Angus Buckling 6/10/17 13:55
Deleted: ,

Angus Buckling 6/10/17 13:56
Deleted: in addition to siderophore production. Selection of taxa with increased copper tolerance occurred very rapidly in our experiment, although

Angus Buckling 6/10/17 13:57
Deleted: we cannot rule out a role of additional resistance mechanisms positively co-varying with siderophore production, and

474 significantly improve phytoremediation of historically contaminated sites, in which siderophore
475 production will already have been stabilised by selection. The direct addition of siderophores, while
476 providing a short-term benefit, may actually result in longer-term negative effects on
477 phytoremediation regardless of length of time since contamination, as selection for siderophore
478 production is relaxed. More generally, our results highlight that interspecific public goods
479 production can be maintained at high levels in natural microbial communities, despite the potential
480 of exploitation by cheating non-producers.
481
482 **ACKNOWLEDGEMENTS**
483 This work was funded by the AXA Research Fund, BBSRC and NERC to AB. SOB was funded by
484 a "Bridging the Gaps" award and PhD scholarship from the University of Exeter. NT was funded by
485 the EU's Horizon 2020 programme under the Marie Sklodowska-Curie grant agreement (656647).
486 AML was supported by Marie Curie International Incoming Fellowships within the EU Seventh
487 Framework Programme. AB acknowledges support from the Royal Society.

488 **REFERENCES**

489 1.

490 Adriano, D.C. (2001). *Trace elements in terrestrial environments*. Spinger-Verlag, New York, NY,
491 USA.

492 2.

493 Alloway, B.J. (1990). *Heavy metals in soil*. John Wiley & Sons, New York, NY, USA.

494 3.

495 Anderson, M.J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral*
496 *Ecol.*, 26, 32-46.

497 4.

498 Angly, F.E., Dennis, P.G., Skarshewski, A., Vanwonterghem, I., Hugenholtz, P. & Tyson, G.W.
499 (2014). CopyRighter: a rapid tool for improving the accuracy of microbial community profiles
500 through lineage-specific gene copy number correction. *Microbiome*, 2, 11.

501 5.

502 Barber, M.F. & Elde, N.C. (2015). Buried treasure: evolutionary perspectives on microbial iron
503 piracy. *Trends Genet.*, 31, 627-636.

504 6.

505 Bates, D., Maechler, M., Bolker, B. & Walker, S. (2014). lme4: Linear mixed-effects models using
506 Eigen and S4.

507 7.

508 Brankatschk, R., Bodenhausen, N., Zeyer, J. & Bürgmann, H. (2012). Simple absolute
509 quantification method correcting for quantitative PCR efficiency variations for microbial
510 community samples. *Appl. Environ. Microbiol.*, 78, 4481-4489.

511 8.

512 Braud, A., Geoffroy, V., Hoegy, F., Mislin, G.L.A. & Schalk, I.J. (2010). Presence of the
513 siderophores pyoverdine and pyochelin in the extracellular medium reduces toxic metal

514 accumulation in *Pseudomonas auruginosa* and increases bacterial metal tolerance. *Environ.*
515 *Microbiol. Rep.*, 2, 419-425.
516 9.
517 Braud, A., Hoegy, F., Jezequel, K., Lebeau, T. & Schalk, I.J. (2009). New insights into the metal
518 specificity of the *Pseudomonas aeruginosa* pyoverdine–iron uptake pathway. *Environ.*
519 *Microbiol.*, 11, 1079-1091.
520 10.
521 Breiman, L., Friedman, J., Stone, C.J. & Olshen, R.A. (1984). *Classification and regression trees*.
522 Wadsworth International Group, Belmont, CA, USA.
523 11.
524 Bruins, M.R., Kapil, S. & Oehme, F.W. (2000). Microbial resistance to metals in the environment.
525 *Ecotoxicol. Environ. Saf.*, 45, 198-207.
526 12.
527 Buckling, A., Harrison, F., Vos, M., Brockhurst, M.A., Gardner, A., West, S.A. *et al.* (2007).
528 Siderophore-mediated cooperation and virulence in *Pseudomonas aeruginosa*. *FEMS*
529 *Microbiol. Ecol.*, 62, 135-141.
530 13.
531 Burt, R. (1998). History of metalliferous mining. In: *The geology of Cornwall* (eds. Sellwood, EB,
532 Durrance, EM & Bristow, CM). University of Exeter Press Exeter, pp. 211-225.
533 14.
534 Caliński, T. & Harabasz, J. (1974). A dendrite method for cluster analysis. *Communications in*
535 *statistics: theory and methods*, 3, 1-27.
536 15.
537 Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. & Holmes, S.P. (2016).
538 DADA2: high-resolution sample inference from Illumina amplicon data. *Nature methods*, 13,
539 581-583.

540 16.

541 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. *et al.*
542 (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature*
543 *Methods*, 7, 335-336.

544 17.

545 Cordero, O.X., Ventouras, L.-A., DeLong, E.F. & Polz, M.F. (2012). Public good dynamics drive
546 evolution of iron acquisition strategies in natural bacterioplankton populations. *Proc. Natl.*
547 *Acad. Sci. U. S. A.*, 109, 20059-20064.

548 18.

549 De'Ath, G. (2002). Multivariate regression trees: a new technique for modeling species–
550 environment relationships. *Ecology*, 83, 1105-1117.

551 19.

552 De'Ath, G. (2007). mvpart: Multivariate partitioning.

553 20.

554 Dimkpa, C., Weinand, T. & Asch, F. (2009). Plant–rhizobacteria interactions alleviate abiotic stress
555 conditions. *Plant Cell Environ.*, 32, 1682-1694.

556 21.

557 Edgar, R.C. (2010). Search and clustering orders of magnitude faster in BLAST. *Bioinformatics*,
558 26, 2460-2461.

559 22.

560 Estrela, S., Morris, J.J. & Kerr, B. (2016). Private benefits and metabolic conflicts shape the
561 emergence of microbial interdependencies. *Environ. Microbiol.*, 18, 1415-1427.

562 23.

563 Fierer, N. & Jackson, R.B. (2006). The diversity and biogeography of soil bacterial communities.
564 *Proc. Natl. Acad. Sci. U. S. A.*, 103, 626-631.

565 24.

566 Frank, S.A. (1994). Genetics of mutualism: the evolution of altruism between species. *J. Theor. Biol.*, 170, 393-400.
567
568 25.
569 Fuglede, B. & Topsoe, F. (2004). Jensen-Shannon divergence and Hilbert space embedding. In: *Proceedings of the IEEE International Symposium on Information Theory* p. 31.
570
571 26.
572 Galet, J., Deveau, A., Hôtel, L., Frey-Klett, P., Leblond, P. & Aigle, B. (2015). *Pseudomonas fluorescens* pirates both ferrioxamine and ferricoelichelin siderophores from *Streptomyces ambofaciens*. *Appl. Environ. Microbiol.*, 81, 3132-3141.
573
574
575 27.
576 Gans, J., Wiolinsky, M. & Dunbar, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science*, 309, 1387-1390.
577
578 28.
579 Ghoul, M. & Mitri, S. (2016). The ecology and evolution of microbial competition. *Trends Microbiol.*, 24, 833-845.
580
581 29.
582 Giller, K.E., Witter, E. & Mcgrath, S.P. (1998). Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biol. Biochem.*, 30, 1389-1414.
583
584 30.
585 Gobran, G.R. & Huang, P. (2011). *Biogeochemistry of trace elements in the rhizosphere*. Elsevier, Amsterdam, The Netherlands.
586
587 31.
588 Griffin, A.S., West, S.A. & Buckling, A. (2004). Cooperation and competition in pathogenic bacteria. *Nature*, 430, 1024-1027.
589
590 32.

591 Griffiths, R.I., Thomson, B.C., James, P., Bell, T., Bailey, M. & Whiteley, A.S. (2011). The
592 bacterial biogeography of British soils. *Environ. Microbiol.*, 13, 1642-1654.
593 33.
594 Guerinot, M.L. (1994). Microbial iron transport. *Annu. Rev. Microbiol.*, 48, 743-772.
595 34.
596 Hamilton, W.D. (1964). The genetical evolution of social baviour. I. *J. Theor. Biol.*, 7, 1-16.
597 35.
598 Hamilton, W.D. & Axelrod, R. (1981). The evolution of cooperation. *Science*, 211, 1390-1396.
599 36.
600 Harrison, F. & Buckling, A. (2005). Hypermutability impedes cooperation in pathogenic bacteria.
601 *Curr. Biol.*, 15, 1968-1971.
602 37.
603 Hendershot, W.H. & Lalande, H. (2008). Soil reaction and exchangeable acidity. In: *Soil sampling*
604 *and methods of analysis* (eds. Carter, MR & Gregorich, EG). CRC Press Boca Raton, USA, pp.
605 173-178.
606 38.
607 Hofte, M., Buysens, S., Koedam, N. & Cornelis, P. (1993). Zinc affects siderophore-mediated high
608 affinity iron uptake systems in the rhizosphere *Pseudomonas aeruginosa* 7NSK2. *Biometals*, 6,
609 85-91.
610 39.
611 Hothorn, T., Bretz, F. & Westfall, P. (2008). Simultaneous interference in general parametric
612 model. *Biometrical Journal*, 50, 346-363.
613 40.
614 Kruskal, J.B. (1964). Nonmetric multidimensional scaling: a numerical method. *Psychometrika*, 29,
615 115-129.
616 41.

617 Kummerli, R., Griffin, A.S., West, S.A., Buckling, A. & Harrison, F. (2009). Viscous medium
618 promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*. *Proceedings of*
619 *the Royal Society of London. Series B, Biological sciences*, 276, 3531-3538.
620 42.
621 Kummerli, R., Schiessl, K.T., McNeill, K. & Ackermann, M. (2014). Habitat structure and the
622 evolution of diffusible siderophores in bacteria. *Ecol. Lett.*, 17, 1536-1655.
623 43.
624 Lebeau, T., Braud, A. & Jézéquel, K. (2008). Performance of bioaugmentation-assisted
625 phytoextraction applied to metal contaminated soils: a review. *Environmental Pollution*, 153,
626 497-522.
627 44.
628 Lee, H.H., Molla, M.N., Cantor, C.R. & Collins, J.J. (2010). Bacterial charity work leads to
629 population-wide resistance. *Nature*, 467, 82.
630 45.
631 Legendre, P. & Gallagher, E.D. (2001). Ecologically meaningful transformations for ordination of
632 species data. *Oecologia*, 129, 271-280.
633 46.
634 Legendre, P. & Legendre, L.F. (2012). *Numerical ecology*. Elsevier, Oxford, UK.
635 47.
636 Lofts, S., Spurgeon, D.J., Svendsen, C. & Tipping, E. (2004). Deriving soil critical limits for Cu,
637 Zn, Cd, and Pb: a method based on free ion concentrations. *Environ. Sci. Technol.*, 38, 3623-
638 3631.
639 48.
640 Lujan, A.M., Gomez, P. & Buckling, A. (2015). Siderophore cooperation of the bacterium
641 *Pseudomonas fluorescens* in soil. *Biol. Lett.*, 11, 20140934.
642 49.

643 MacQueen, J. (1967). Some methods for classification and analysis of multivariate observations. In:
644 *Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability*, pp.
645 281-297.
646 50.
647 Maizels, R.M., Blaxter, M.L. & Scott, A.L. (2001). Immunological genomics of *Brugia malayi*:
648 filarial genes implicated in immune evasion and protective immunity. *Parasite Immunol.*, 23,
649 327-344.
650 51.
651 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads.
652 *EMBnet.journal*, 17, 10-12.
653 52.
654 McMurdie, P.J. & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis
655 and graphics of micobiome census data. *PLoS One*, 8, e61217.
656 53.
657 Mitri, S. & Foster, K.R. (2013). The genotypic view of social interaction in microbial communities.
658 *Annu. Rev. Genet.*, 47, 247-273.
659 54.
660 Morris, J.J. (2015). Black Queen evolution: the role of leakiness in structuring microbial
661 communities. *Trends Genet.*, 31, 475-482.
662 55.
663 Morris, J.J., Lenski, R.E. & Zinser, E.R. (2012). The Black Queen hypothesis: evolution of
664 dependencies through adaptive gene loss. *MBio*, 3, 2 e00036-00012.
665 56.
666 Nies, D.H. (1999). Microbial heavy-metal resistance. *Appl. Microbiol. Biotechnol.*, 51, 730-750.
667 57.

668 Nriagu, J.O. & Pacyna, J.M. (1988). Quantitative assessment of worldwide contamination of air,
669 water and soils by trace metals. *Nature*, 333, 134-139.
670 58.
671 O'Brien, S. & Buckling, A. (2015). The sociality of bioremediation. *EMBO reports*, e201541064.
672 (59.
673 O'Brien, S., Hodgson, D.J. & Buckling, A. (2014). Social evolution of toxic metal bioremediation
674 in *Pseudomonas aeruginosa*. *Proceedings of the Royal Society of London. Series B, Biological*
675 *sciences*, 281, 20140858.
676 60.
677 Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., O'Hara, R.B., Simpson, G.L. *et al.* (2010).
678 Vegan: Community Ecology Package.
679 61.
680 Oliveira, N.M., Niehus, R. & Foster, K.R. (2014). Evolutionary limits to cooperation in microbial
681 communities. *Proc. Natl. Acad. Sci. U. S. A.*, 111, 17941-17946.
682 62.
683 Ouellette, M.H., Legendre, P. & Borcard, D. (2012). Cascade multivariate regression tree: a novel
684 approach for modelling nested explanatory sets. *Methods Ecol. Evol.*, 3, 234-244.
685 63.
686 Øvreås, L. & Torsvik, V. (1998). Microbial diversity and community structure in two different
687 agricultural soil communities. *Microb. Ecol.*, 36, 303-315.
688 64.
689 Pande, S., Kaftan, F., Lang, S., Svatoš, A., Germerodt, S. & Kost, C. (2016). Privatization of
690 cooperative benefits stabilizes mutualistic cross-feeding interactions in spatially structured
691 environments. *ISME J.*, 10, 1413-1423.
692 65.

693 Preheim, S.P., Perrotta, A.R., Martin-Platero, A.M., Gupta, A. & Alm, E.J. (2013). Distribution-
694 based clustering: using ecology to refine the operational taxonomic unit. *Appl. Environ.*
695 *Microbiol.*, 79, 6593-6603.
696 66.
697 Rajkumar, M., Ae, N., Prasad, M.N.V. & Freitas, H. (2010). Potential of siderophore-producing
698 bacteria for improving heavy metal phytoextraction. *Trends Biotechnol.*, 28, 142-149.
699 67.
700 Rao, C.R. (1995). A review of canonical coordinates and an alternative to correspondence analysis
701 using Hellinger distance. *Qüestiió*, 19, 23-63.
702 68.
703 Ratledge, C. & Dover, L.G. (2000). Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.*,
704 54, 881-941.
705 69.
706 Ruijter, J., Ramakers, C., Hoogaars, W., Karlen, Y., Bakker, O., Van den Hoff, M. *et al.* (2009).
707 Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data.
708 *Nucleic Acids Res.*, 37, e45-e45.
709 70.
710 Schalk, I.J., Hannauer, M. & Braud, A. (2011). New roles for bacterial siderophores in metal
711 transport and tolerance. *Environ. Microbiol.*, 13, 2844-2854.
712 71.
713 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B. *et al.* (2009).
714 Introducing Mothur: open-source, platform-independent, community-supported software for
715 describing and comparing microbial communities. *Appl. Environ. Microbiol.*, 75, 7537-7541.
716 72.
717 Schwyn, B. & Neilands, J.B. (1987). Universal chemical assay for the detection and determination
718 of siderophores. *Anal. Biochem.*, 160, 47-56.

719 73.
720 Shepard, R.N. (1962). The analysis of proximities: multidimensional scaling with unknowns
721 distance function. I. *Psychometrika*, 27, 125-140.
722 74.
723 Suleman, L. (2016). Extracellular bacterial proteases in chronic wounds: a potential therapeutic
724 target? *Adv. Wound Care*, 5, 455-463.
725 75.
726 Teitzel, G.M., Geddie, A., De Long, S.K., Kirisits, M.J., Whiteley, M. & Parsek, M.R. (2006).
727 Survival and growth in the presence of elevated copper: transcriptional profiling of copper-
728 stressed *Pseudomonas aeruginosa*. *J. Bacteriol.*, 188, 7242-7256.
729 76.
730 Therneau, T., Atkinson, B. & Ripley, B. (2015). rpart: Recursive Partitioning and Regression Trees.
731 77.
732 Valls, M. & De Lorenzo, V. (2002). Exploiting the genetic and biochemical capacities of bacteria
733 for the remediation of heavy metal pollution. *FEMS Microbiol. Rev.*, 26, 327-338.
734 78.
735 Vavrek, M.J. & Larsson, H.C.E. (2010). Fossil: palaeoecological and palaeogeographical analysis
736 tools.
737 79.
738 Vega, N.M. & Gore, J. (2014). Collective antibiotic resistance: mechanisms and implications. *Curr.*
739 *Opin. Microbiol.*, 21, 28-34.
740 80.
741 West, S.A. & Buckling, A. (2003). Cooperation, virulence and siderophore production in bacterial
742 parasites. *Proceedings of the Royal Society of London. Series B, Biological sciences*, 270, 37-
743 44.
744 81.

745 West, S.A., Diggle, S.P., Buckling, A., Gardner, A. & Griffin, A.S. (2007). The social lives of
746 microbes. *Annu. Rev. Ecol. Syst.*, 38, 53-77.
747 82.
748 Willis, A. & Bungle, J. (2014). breakaway: Species Richness Estimation and Modeling.
749
750

1 **FIGURE LEGENDS**

2 **Figure 1. Siderophores act as an interspecific public good in toxic copper broth.** Mean
3 Malthusian growth rate (m) ± SE of a siderophore-producing (black bars) and non-
4 producing (white bars) strain of *Pseudomonas aeruginosa* in toxic copper broth ($CuSO_4$).
5 The addition of heterospecific siderophores (ferrioxamine, ornibactin, schizokinen and
6 yersiniabactin) significantly reduces mean growth differences between producing and non-
7 producing strains.

8

9 **Figure 2. The effect of soil acidity and heavy metal contamination on microbial**
10 **abundance and siderophore production in natural soils. (A)** Heavy metal loadings on
11 the first principal component (PC1), which explained 27% of the observed environmental
12 variation; **(B)** Positive correlation between soil acidity (pH) and heavy metal
13 contamination (PC1); **(C)** Proportion of siderophore producers and **(D)** microbial density
14 (log_{10}-transformed bacterial cells g^{-1} soil) as a function of heavy metal contamination and
15 soil acidity. Lines and shaded area depict the fitted relationships ± standard error.

16

17 **Figure 3. Relationship between soil acidity, iron and siderophore production.** (**A**) Soil
18 acidity (pH) and total iron content (%) do not co-vary and (**B**) variation in total iron
19 availability does not affect the proportion of siderophore producers along a natural heavy
20 metal gradient associated with historical mining activity. Line and shaded area depict the
21 fitted relationships ± standard error.

22

23 **Figure 4. Community composition variation changes as a function of soil acidity.**
24 Multivariate regression tree (MRT) analysis was used to estimate the impact of soil acidity
25 (pH) and heavy metals (PC1) on community structure, indicating that pH is the main
26 environmental driver explaining variation in community structure. The most parsimonious

tree (**A**) shows that the community could be divided into 3 different leaves (colored symbols) based on microbial abundance and composition. The composition within leaves is represented in a PCA plot (**B**), where small points represent individual samples and large points represent the group mean (within leaf). The most important taxa in each leaf are summarized in Supplementary Table S1.

Figure 5. The effect of copper contamination on experimental microbial communities in compost. (A) Copper addition results in a net increase in mean per capita siderophore production ± SE over time, where open circles and black circles represent non-contaminated and copper-contaminated experimental communities, respectively; **(B)** NMDS ordination plot depicting the pair-wise Bray-Curtis dissimilarity between soil microcosms after six weeks of incubation (stress = 0.096). Points represent individual microcosms belonging to the non-contaminated (open circles) and copper-contaminated (black circles) treatment, such that microcosms similar in their genus-level composition are ordinated closer together; **(C)** Relative abundance of the ten most common genera and their mean siderophore production. Genera are listed in order of their mean across-treatment siderophore production, increasing from top to bottom, such that blue- and red genera are non-producers and producers, respectively. See Table S2 in Supplementary Tables for more details; **(D)** The effect of copper background (filled and open symbols are presence and absence of copper contamination, respectively) on metal tolerance, where more negative values indicate a stronger inhibitory effect of $CuSO_4$ on bacterial growth. Bars denote 1 SE.