Interspecific social interactions shape public goods production in natural microbial communities

One sentence summary – Interspecific exploitation shapes the evolution and ecology of public goods production

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Abstract

Some microbial public goods benefit conspecifics, as well as other species. Here, we use evolution and competition experiments to determine how exploitation of public goods by the wider microbial community shapes the production of an interspecific public good: metal-detoxifying siderophores. By simultaneously studying whole microbial communities and an embedded focal species, we show that interspecific exploitation results in both ecological selection against microbial taxa that produce relatively large amounts of siderophores, and evolution of reduced siderophore production within taxa over similar time scales. Our findings demonstrate the crucial role of interspecific interactions in shaping microbial social behaviours.

Keywords – ecology, evolution, microbial communities, public goods, siderophores, interspecific exploitation, Black Queen Hypothesis.

Main text

Microbes produce a range of metabolically costly public goods that improve their growth and survival, but that are open to exploitation by non-producing 'cheats' (*1-6*). Optimal levels of public goods therefore reflect a balance of the benefits received by the producer and individuals who share the same genes, and costs associated with enhancing the fitness of exploiting competitors. The evolution of public goods has typically been studied in the context of within-species interactions (*7*), but many microbial public goods, including antibiotic-degrading enzymes (*8, 9*), resource-scavenging molecules (*10, 11*) and immune-manipulating effectors (*12, 13*), potentially benefit not only conspecifics but

also other species. As a consequence, interspecific exploitation may play a key role in shaping the production of public goods, and may select for the loss of key metabolic genes (*14, 15*). However, experimental evidence for interspecific exploitation of community-wide public goods, and the resultant ecological and evolutionary consequences, is lacking.

Metal-detoxifying siderophores have the potential to act as a community-wide public good (*16-18*). The canonical function of siderophores is to bind and take up insoluble iron (*19*), but these extracellular agents can also bind to toxic heavy metals (*20, 21*) to prevent uptake by cells (*22*). Siderophores can therefore provide protection from toxic metals both to conspecifics (*23*) and to members of different species (*16*). There is good evidence for intraspecific exploitation of metal-detoxifying siderophores, as low siderophore-producers of *Pseudomonas aeruginosa* – an opportunistic bacterial pathogen – grow poorly alone but can invade isogenic wild-type producers (*16*). Here, we explore whether interspecific exploitation also plays an important role in shaping siderophore production, both through competition between species and through evolutionary change.

We previously determined how mean siderophore production and community composition changed as a function of copper pollution in natural soil and experimental compost microbial communities (*17*). We found that copper favours higher siderophore-producing microbial taxa, thereby increasing mean levels of siderophore production in polluted compared to non-polluted communities. This suggests there are direct and/or kin-selected benefits to producing siderophores (*24, 25*). Re-analysis of this dataset suggests that high siderophore-producers are also open to exploitation: copper-mediated increases in mean siderophore levels are accompanied by a reduction in the variation

(dispersion parameter in Gaussian double GLM with copper as explanatory variable: χ^2 = 22.85, df = 1, $P < 0.001$), demonstrating selection against high as well as low siderophore-producing taxa (Fig. 1A). Moreover, genera producing relatively large amounts of siderophores in non-polluted compost microcosms displayed lower production in copper-polluted compost, and *vice versa* for low siderophore-producing taxa (copper × genus interaction in 2-way ANOVA: $F_{5, 170} = 2.87$ and $P = 0.02$; Fig. 1B).

While selection against high levels of siderophore production could be a consequence of interspecific exploitation, this may also result solely from intraspecific exploitation or even not be driven by social interactions at all. To determine if interspecific exploitation shapes selection for siderophore production, we carried out all possible pairwise competitions between ten random compost isolates (i.e. isolated from experimental compost communities) spanning the observed continuum of siderophore production (Table S1), as well as growing each in isolation. By conducting all possible pairwise combinations, we tested for the effects of siderophores *per se* over and above any other competitive differences between isolates. In copper-contaminated compost, isolates that produced relatively large amounts of siderophore grew better in isolation than in competition, whereas non- or low-producing isolates benefitted from the presence of other taxa (linear model on $m_{\text{competition}} - m_{\text{isolation}}$: $F_{1, 88} = 10.95$, $P = 0.001$ for main effect of siderophore production; Fig. 2A). This demonstrates that high siderophore-producing taxa were exploited by low siderophore-producing taxa. The relationship between siderophore production and relative fitness in the presence *versus* absence of competitors was not significant in non-polluted compost, where the importance of

siderophores as an interspecific public good is likely to be greatly reduced (linear model: $F_{1, 88} = 0.05$, $P = 0.82$; Fig. 2B).

The above findings demonstrate that interspecific exploitation imposes selection on siderophore production, and that this can result in selection against high siderophore-producing taxa. Given that microbial evolution can occur on time scales concomitant with changes in community composition, we hypothesised that interspecific exploitation could result in selection for lower siderophore production in initially high-siderophore producing taxa in these communities. To test this, we conducted a similar experiment to our original study (*17*) but this time also followed the evolution of a focal taxon, *Pseudomonas fluorescens* SBW25 (*26*). This common soil bacterium produces a range of siderophores (*27*) known to chelate metals other than iron (*28*).

Changes in community-wide siderophore production in response to copper addition were qualitatively the same as we found before (*17*), with copper resulting in greater mean (LME with copper as fixed effect and random intercepts fitted for individual microcosms: $\chi^2 = 7.16$, df = 1, $P = 0.007$; Fig. 3A), but lower variation in siderophore production (Gaussian double GLM: $\chi^2 = 14.40$, df = 1, $P < 0.001$, Fig. 3B). Copper-mediated shifts in siderophore production were also associated with compositional changes in the microbial assemblage (PERMANOVA: $F_{1, 10} = 6.26$, $P = 0.003$, $R^2 = 0.38$; multivariate dispersion: $F_{1, 10} = 0.05$, $P = 0.82$; Fig. 3C), such that copper favoured microbial taxa that on average produced larger amounts of siderophore (Fig. 3D).

P. fluorescens – initially a very high siderophore-producing species – evolved to produce significantly less siderophore in copper-polluted compost (Fig. 4). However, evolutionary trajectories of *P. fluorescens* were the same whether the natural community

was present or absent (LME: $\chi^2 = 1.63$, df = 1, $P = 0.02$ for main community effect), with copper consistently reducing mean siderophore levels ($\chi^2 = 10.56$, df = 1, $P < 0.001$ for main copper effect: Fig. 4A–B). There are a number of interpretations of this finding. First, *P. fluorescens* siderophore production is not driven by exploitation, but may simply have been higher than needed to detoxify the environment. Second, exploitation occurred, but it is entirely driven by exploitation within species. These interpretations do not invoke interspecific exploitation, but the absence of an effect of interspecific exploitation on siderophore evolution seems surprising given its strong effect on ecological selection (Fig. 3).

A third interpretation is therefore that intraspecific exploitation compensates for interspecific exploitation when other species are not present. Given the community-wide nature of the public good, exploitation by competitors can theoretically have the same evolutionary consequences on a focal population regardless of whether the exploitation is by the same or different species (*20*). We hypothesised that in the presence of the community, *P. fluorescens* siderophore producers were exploited by both other taxa and evolved *P. fluorescens*, while in the absence of the community the greatly increased density of *P. fluorescens* (Fig. S1) resulted in comparable levels of exploitation from conspecifics alone.

To determine whether or not intra- and interspecific exploitation played a role in shaping *P. fluorescens* evolutionary trajectories, we conducted a series of short-term (week-long) growth rate assays of wild-type SBW25 and an isogenic pyoverdine knock-out mutant strain (*29*) in copper-polluted compost. This mutant does not produce the main siderophore pyoverdine (which reduces total siderophore production to 21% of the

wild-type), and displays levels of siderophore production similar to the average compost community member. In the absence of the community, the *P. fluorescens* wild-type and mutant grew equally well as monocultures (Fig. 5A), demonstrating that there is no abiotic selection against high siderophore production. However, the pyoverdine mutant outcompeted the wild-type in co-culture in a frequency dependent manner (growth rate: $F_{2, 56} = 31.67$, $P < 0.001$ for strain × frequency interaction; selection coefficient: $F_{2, 27} = 179.87$, $P < 0.001$ for effect of frequency; Fig. 5B). Such dynamics are indicative of intraspecific exploitation of high siderophore producers (10, 35). We next determined how the producer and mutant each independently fared in the presence *versus* absence of the community. As above, in the absence of the community the siderophore producing wild-type and pyoverdine mutant grew equally well as monocultures (Fig. 5C). The presence of the community did not notably affect the growth rate of the mutant, suggesting little or no net exploitation of detoxifying siderophores by other community members. By contrast, the growth rate of the wild-type was markedly reduced by the community, strongly suggesting exploitation of its siderophores (2-way ANOVA: social background × strain = $F_{1, 39} = 9.42$, $P < 0.01$; Fig. 5C). As a net result, the wild-type had a significantly lower fitness compared to the mutant when growing together with the natural compost community (ANOVA: $F_{1, 19} = 12.65$, $P < 0.01$; Fig. 5D). Taken together, these results suggest both intra- and interspecific exploitation played an important role in driving the evolution of reduced siderophore production in *P. fluorescens*.

To conclude, we show that interspecific exploitation plays a key role in shaping community-wide levels of public goods, selecting for and against microbial taxa that differ in their mean siderophore production. Moreover, interspecific (in addition to

intraspecific) exploitation plays a key role in the driving within-taxon evolutionary changes. While both ecological and evolutionary processes operate over similar time frames, we anticipate that evolutionary effects resulting from interspecific interactions will become increasingly important relative to ecological selection once community composition has reached equilibrium. Indeed, this is precisely the scenario envisaged by the Black Queen Hypothesis (*14*), where selection favours intermediate levels of public goods because of differential benefits and selection to lose traits as a result of interspecific exploitation. Given that microbial public goods with community-wide benefits are extremely common (*10, 30*), our results suggest interspecific exploitation likely plays a key role in shaping microbial community composition and evolution in nature.

Acknowledgments

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material availability: Sequences have been deposited as ENA Project PRJEB29924 (https://www.ebi.ac.uk/ena/data/search?query=PRJEB29924). Experimental data will be uploaded on Dryad.

Supplementary Materials

Material and Methods

Table S1 – Siderophore production of compost isolates used in pairwise competitions.

Table S2 – Pairwise treatment contrasts accompanying Figure 5 in the main text.

Figure S1 – The effects of copper and interspecific competition on *Pseudomonas fluorescens* population densities following six weeks of evolution.

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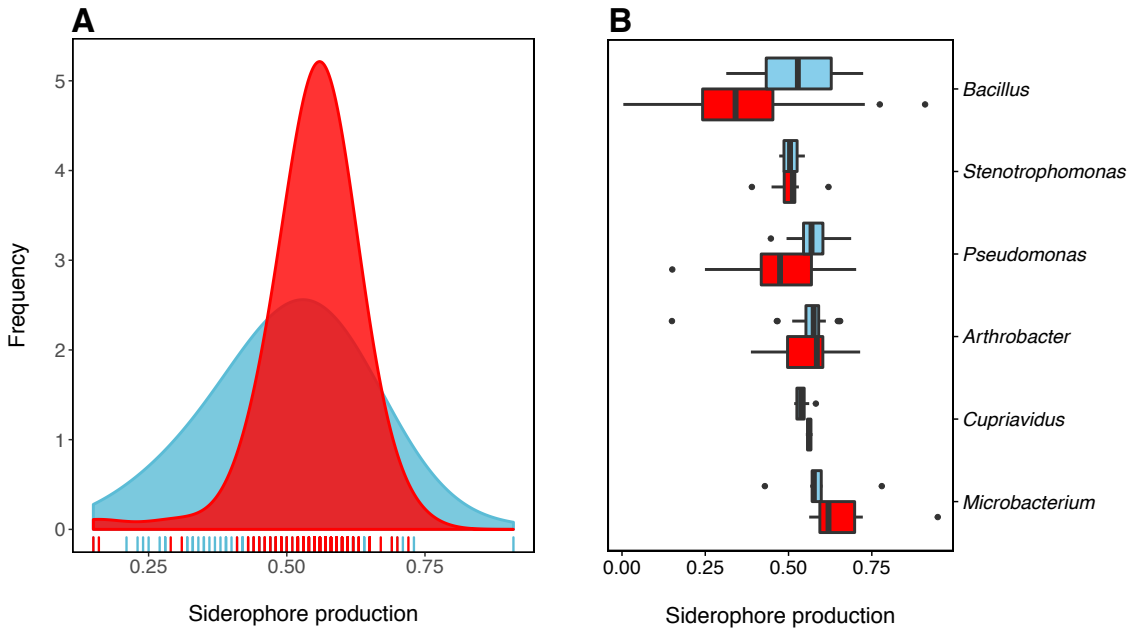

Figure 1. Variation in siderophore production in compost communities and across individual microbial taxa. (A) Density plot demonstrating that copper (red) selects against very low and very high siderophore-producing taxa following six weeks of evolution in compost. Variation in siderophore production was lower in polluted compared non-polluted (blue) communities. **(B)** Box–whisker plot depicting variation in siderophore production in six common culturable microbial genera as a function of copper pollution (red = polluted and blue = non-polluted communities) following six weeks of evolution. Boxes depict the upper and lower quartiles of the treatment-specific raw data with the centre line showing the median and whiskers providing a measure of the 1.5x interquartile range. Siderophore production was quantified for multiple isolates (n = 13–62) per genus using individual liquid CAS assays (*31*), corrected for variation in cell density as measured by OD_{600}.

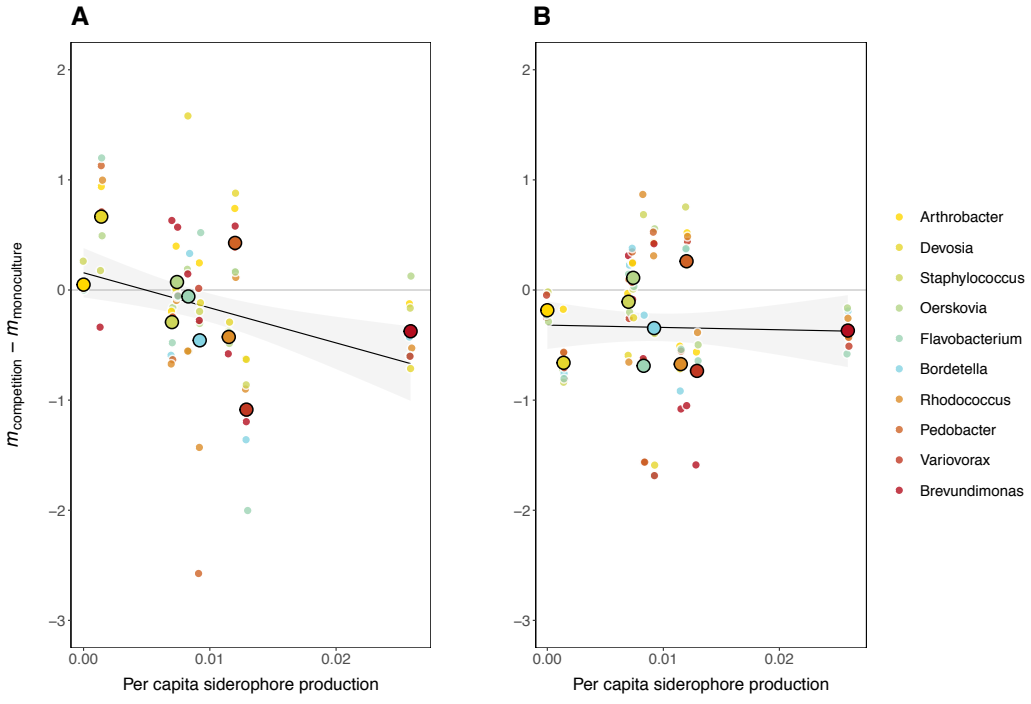

Figure 2. Copper pollution changes the relationship between siderophore production and the relative performance of compost isolates during pairwise competition. Species performance (n = 10) was calculated as: $d = m_{\text{competition}} - m_{\text{isolation}}$. In scenarios where $d > 0$, compost isolates benefitted from the presence of other species, whereas $d < 0$ indicates isolates grew better in isolation. Small dots depict d for all possible pairwise competitions (n = 9 per isolate) and large dots provide a measure of across-species mean performance in **(A)** copper-polluted and **(B)** non-polluted microcosms. Line and shaded area depict the fitted relationship ± 95% CI for copper (d = 0.16[-0.07, 0.38] – 31.85[-50.96, -12.73] × siderophores) and control microcosms (d = -0.32[-0.54, -0.10] – 2.11[-20.58, 16.37] × siderophores). Siderophore production was quantified using individual liquid CAS assays (*31*), corrected for variation in OD_{600}.

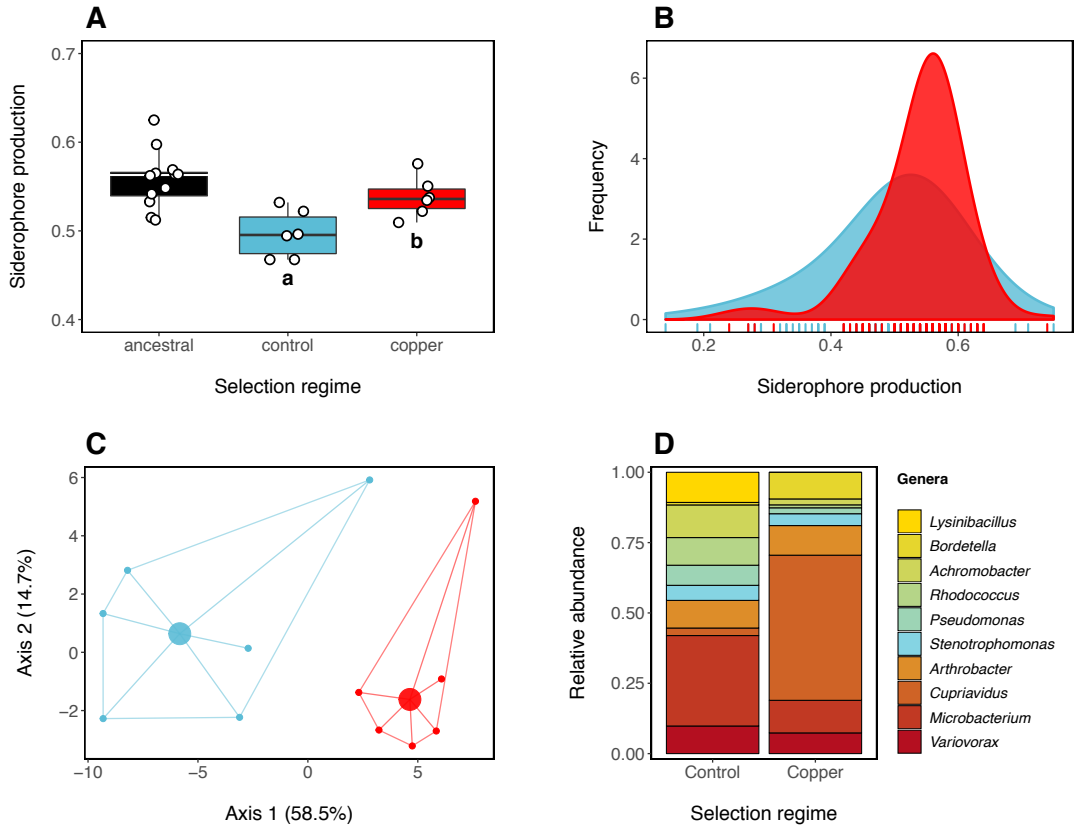

Figure 3. The effect of copper pollution on microbial community composition and siderophore production in the presence of *P. fluorescens*. (A) Copper-polluted communities produced larger amounts of siderophore compared to non-polluted communities following six weeks of evolution. Boxes depict upper and lower quartiles of treatment-specific raw data with the centre line showing the median and whiskers providing a measure of 1.5x interquartile range. Points represent mean values per community. Letters denote significant contrasts, with $\alpha < 0.05$. **(B)** Copper (red) selects against very low and very high siderophore-producing taxa, thereby reducing variation in siderophore production compared to non-polluted (blue) communities. **(C)** Principal Coordinate Analysis (PCoA) plot based on Bray-Curtis dissimilarities between communities. The percentage of variation explained is shown on each axis, calculated

from the relevant eigenvalues. Communities belonging to the same treatment are joined with straight coloured lines (copper = red and control = blue), with large points representing treatment-specific centroids and small points individual microcosms. **(D)** Relative abundance of ten common culturable genera, listed in order of their mean across-treatment siderophore production, increasing from top to bottom, such that orange-red taxa are intermediate-high producers. Siderophore production was quantified using individual CAS assays (*31*), corrected for variation in OD_{600}.

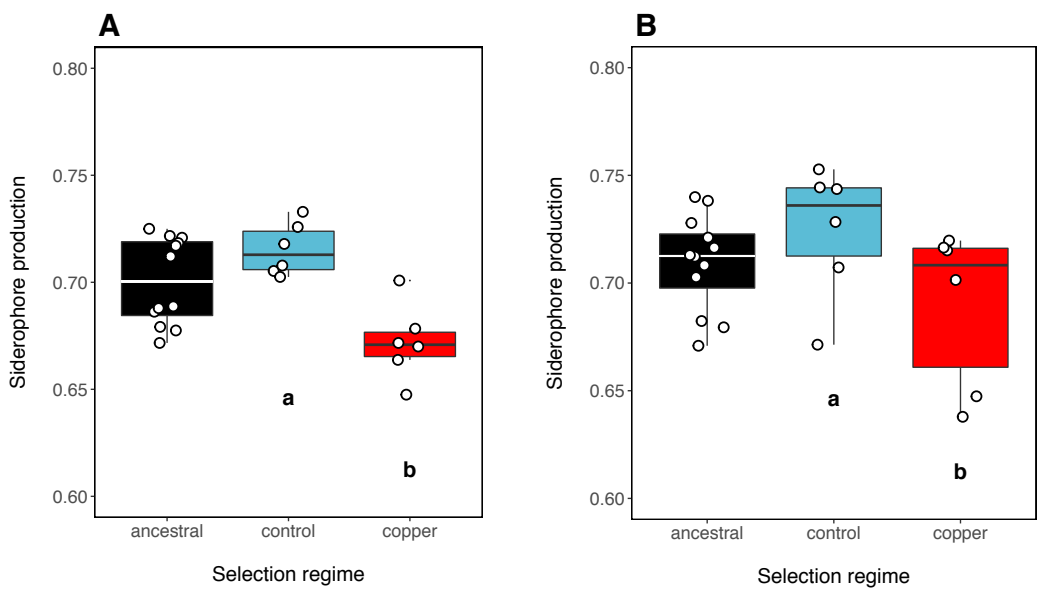

Figure 4. Toxic copper selects against high siderophore-producers in focal *Pseudomonas fluorescens.* Box–whisker plots demonstrating that mean siderophore levels in *P. fluorescens* populations are reduced under copper stress when evolving in the presence **(A)** and absence **(B)** of the compost microbial community. Boxes depict the upper and lower quartiles of treatment-specific raw data with the centre line showing the median and whiskers providing a measure of 1.5x interquartile range, where black, blue and red depict ancestral (i.e. prior to copper amendment; $n = 12$), non-polluted ($n = 6$) and copper-polluted evolved populations ($n = 6$), respectively. Points represent the mean value per population based on twenty-four clones. Siderophore production per clone was quantified using individual liquid CAS assays (*31*), corrected for variation in OD_{600}. Letters denote significant contrasts calculated based on the full LME model testing the interactive effects of copper × social background on mean siderophore levels by averaging across social backgrounds (copper – control contrast: t ratio = -3.47, P = 0.002).

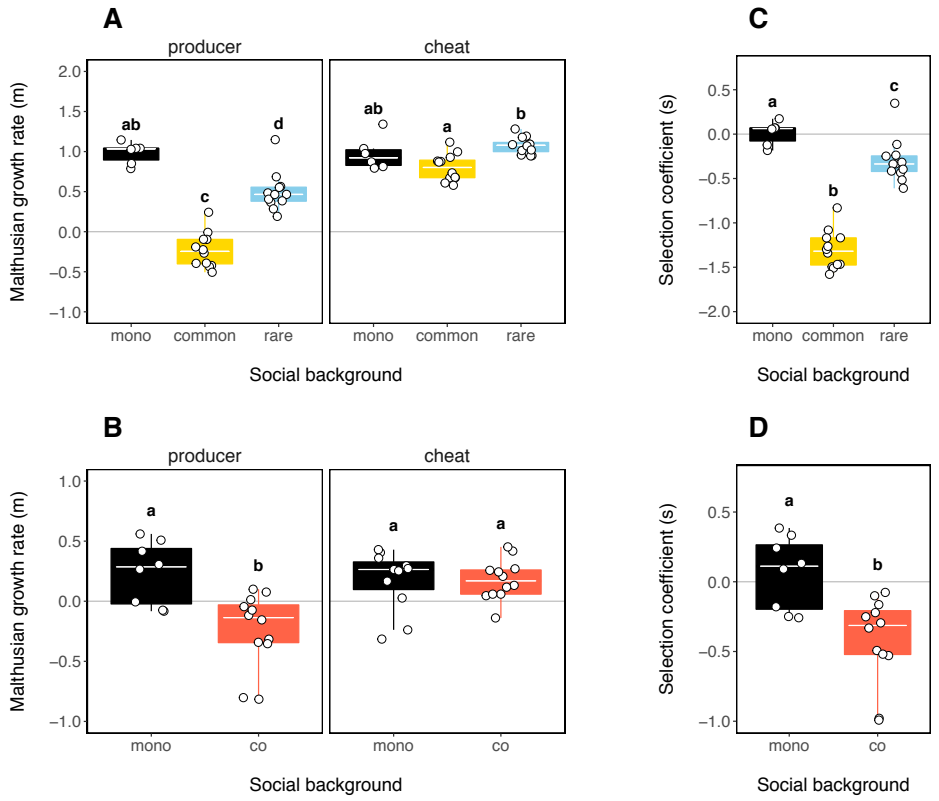

Figure 5. Public goods exploitation decreases the relative fitness of high siderophore producers in copper-polluted compost. Box–whisker plots depicting **(A)** Malthusian growth rates (*m*) of a wild-type pyoverdine producer and isogenic knockout mutant of *Pseudomons fluorescens* when grown as monocultures (black) or together at high (yellow) or low (blue) frequencies. In a separate experiment **(B)**, strains were individually grown in copper-polluted compost in the presence (red) or absence (black) of the compost community. Boxes depict upper and lower quartiles of treatment-specific raw data with the centre line showing the median and whiskers providing a measure of the 1.5x interquartile range. Points represent individual microcosms (n = 6 – 12 per treatment). Box–whisker plots depicting the relative fitness of the wild-type producer **(C–D)** under different social conditions (see above for colour coding). A selection coefficient of zero indicates the wild-type and mutant have equal fitness. Different letters denote significant

1 treatment effects based on pairwise Bonferroni-adjusted contrasts, with $\alpha < 0.05$ (Table

2 S2).