

LETTER

Siderophores drive invasion dynamics in bacterial communities through their dual role as public good versus public bad

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Number: KR 5017/2-1**Editor:** Minus van Baalen**Abstract**

Microbial invasions can compromise ecosystem services and spur dysbiosis and disease in hosts. Nevertheless, the mechanisms determining invasion outcomes often remain unclear. Here, we examine the role of iron-scavenging siderophores in driving invasions of *Pseudomonas aeruginosa* into resident communities of environmental pseudomonads. Siderophores can be ‘public goods’ by delivering iron to individuals possessing matching receptors; but they can also be ‘public bads’ by withholding iron from competitors lacking these receptors. Accordingly, siderophores should either promote or impede invasion, depending on their effects on invader and resident growth. Using supernatant feeding and invasion assays, we show that invasion success indeed increased when the invader could use its siderophores to inhibit (public bad) rather than stimulate (public good) resident growth. Conversely, invasion success decreased the more the invader was inhibited by the residents’ siderophores. Our findings identify siderophores as a major driver of invasion dynamics in bacterial communities under iron-limited conditions.

KEYWORDSbacterial interactions, bacterial public goods, biological invasion, cheating, community ecology, competition, microbial ecology, *Pseudomonas*, pyoverdine, siderophores**INTRODUCTION**

Invasions by non-native species into established communities are a widespread phenomenon and have been extensively studied in eukaryotes (Lowe et al., 2000), where they often lead to biodiversity loss and deterioration of ecosystem functioning (Doherty et al., 2016; Ehrenfeld, 2010). More recently, invasions have also received attention in microbiology because diverse microbial communities populate almost all habitats on earth (Amalfitano et al., 2015; Thakur et al., 2019), and often perform essential functions such as the fixation of CO₂ and nitrogen, or the synthesis of amino acids (Bäckhed et al., 2005;

Sunagawa et al., 2015; Trivedi et al., 2020; Wagg et al., 2014). Microbial invasions can disturb such functions, for instance by reducing the diversity or altering the composition of the resident community (Acosta et al., 2015; Mallon, van Elsas, et al., 2015), and may thereby compromise ecosystem services or spur dysbiosis and disease in hosts (Bäumler & Sperandio, 2016).

The outcome of microbial invasions is often linked to properties of the resident community and its members. High diversity and ecological dissimilarity within a community, as well as high niche overlaps between residents and invaders, tend to reduce invasion success (Eisenhauer et al., 2013; Van Elsas et al., 2012; Li et al., 2019; Van Nevel

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et al., 2013). By contrast, high resource availability may either promote or hamper invasion, depending on who can better use the resources (Ma et al., 2015; Mallon, Poly, et al., 2015; Yang et al., 2017). Mechanistically, two broad classes of traits seem to predict invasion success. First, metabolic versatility and high growth potentials, which make invaders strong competitors over limited resources (Ma et al., 2015; Mächler & Altermatt, 2012). Second, the deployment of toxins or contact-dependent killing mechanisms, which allow invaders to subdue residents through interference competition (García-Bayona & Comstock, 2018; Libberton et al., 2015).

Here, we investigate the role of social interactions mediated by secreted iron-scavenging siderophores as an alternative mechanism driving invasion dynamics in bacterial communities. Siderophores are required for the uptake of environmentally bound iron and allow bacteria to grow under the iron-limited conditions prevailing in many natural habitats (Cornelis, 2010). We predict that siderophores are important drivers of invasion dynamics because (1) they are produced by most bacterial taxa (Hider & Kong, 2010); (2) many chemically different types exist, each requiring a specific receptor for iron uptake (Cornelis & Matthijs, 2002) and (3) bacteria typically possess additional receptors for the uptake of heterologous siderophores produced by other community members (Sexton et al., 2017). Consequently, siderophores can have two opposing social effects on community members: they can either be ‘public goods’, promoting iron acquisition and growth of members with matching receptors; or ‘public bads’, inhibiting the growth of members lacking matching receptors by locking iron away (Bruce et al., 2017; Butaitė et al., 2017; Gu, Wei, et al., 2020; Kramer et al., 2020; Niehus et al., 2017).

Here, we test whether siderophores, in their dual role as public good versus public bad, can predict invasion success of a bacterium into a resident community. We hypothesise that invasion success depends on the combined effect that invader and residents have on each other through their siderophores (Figure S1). Particularly, invasion success should be lowest when the resident produces a public bad for the invader and the invader a public good for the resident. Conversely, invasion success should be highest when the resident produces a public good and the invader a public bad. Moreover, we predict that invasion success should decline in more diverse resident communities, because siderophore diversity eventually exceeds the invader's receptor repertoire, which increases the likelihood that the residents' siderophores act as public bads for the invader. Finally, we expect invasion success to depend on the origin of residents, because different habitats often select for different siderophore types and production levels (Butaitė et al., 2018; Kümmerli et al., 2014).

To test our predictions, we used eight resident communities from pond and soil samples, each comprising 20 members of the genus *Pseudomonas*, and the

opportunistic human pathogen *P. aeruginosa* as the invader. Invader and residents are fluorescent pseudomonads that secrete pyoverdine as their primary siderophore (Butaitė et al., 2017; Schalk & Guillon, 2013; Schalk et al., 2020). Many structurally different pyoverdine types exist (Meyer et al., 2008). Each strain produces only one type, but typically has additional receptors to take up pyoverdines produced by other community members (Butaitė et al., 2017; Sexton et al., 2017). We first quantified growth and pyoverdine production of all 160 residents and the invader under iron-limited (pyoverdine-inducing) and iron-rich (control) conditions. Next, we fed pyoverdine-containing supernatants of all residents to the invader and vice versa. This allowed us to distinguish between pyoverdines acting as public good versus public bad, and to quantify their effects on growth. Finally, we conducted invasion-from-rare experiments, where we competed the invader against single residents or communities of 5, 10, or 20 residents.

We found that invasion success was shaped by both public good and public bad effects of pyoverdines. Specifically, invasion success decreased as the effect of resident pyoverdines became more inhibitory and when the invader competed against more diverse resident communities. Conversely, invasion success increased in competitions with single residents and when the invader produced an inhibitory pyoverdine for the residents. Our findings demonstrate that siderophore-mediated social interactions can have a major impact on bacterial community assembly and invasion dynamics under iron-limited conditions.

MATERIALS AND METHODS

Strains

The residents used in our experiments were drawn from an established collection of characterised pseudomonads, isolated from soil and pond samples (20 isolates per sample). Sampling and identification procedures of these isolates are provided elsewhere (Butaitė et al., 2017, 2018). Here, we used a subset of 160 isolates from four soil and four pond samples [hereafter: community]. As the invader, we used *P. aeruginosa* PAO1 (ATCC 15692), a wound isolate that has become a laboratory model pathogen (Stover et al., 2000). To be able to distinguish invader from residents (all non-*P. aeruginosa* strains), we used PAO1-*mcherry*, a variant that constitutively expresses the fluorescent protein mCherry (chromosomal insertion attTn7::Ptac-*mcherry*) (Rezzoagli et al., 2019).

Growth and pyoverdine production measurements

We quantified growth and pyoverdine production of all strains under iron-limited and iron-rich conditions. First,

we grew bacterial pre-cultures in 96-well plates containing 200 μl lysogeny broth (LB) per well under shaking conditions (170 rpm) for 48h at 28°C. Subsequently, we diluted pre-cultures 100-fold into 96-well plates containing 200 μl medium per well. We used (1) iron-limited CAA medium (5 g casamino acids, 1.18 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ and 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per litre, supplemented with 25 mM HEPES buffer and 200 μM 2,2'-bipyridine, a strong iron-chelator), and (2) an iron-rich control medium, also consisting of CAA but supplemented with 40 μM FeCl_3 . We inoculated each plate with all 20 residents from one community, or the invader, in fourfold replication. After 24 h of static incubation at 28°C, we quantified growth (as optical density OD_{600} , measured at 600 nm) and pyoverdine production (as relative fluorescence units RFU_{pvd} , excitation/emission at 400/460 nm) of each replicate after 120 s of shaking, using an Infinite M200 Pro microplate reader (Tecan).

Supernatant assay

We explored interactions between invader and residents via secreted products under iron-limited and iron-rich conditions by exposing the invader to supernatants collected from the residents and vice versa. We first harvested supernatants from cultures generated in the above-described experiment by spinning them through 96-well filter plates with a 3 μm glass fiber/0.2 μm Supor membrane (AcroPrep Advance; Pall Corporation) and then collecting the sterile supernatants in 96-well plates. We sealed these plates and stored them at -20°C until further use.

Next, we diluted invader pre-cultures 100-fold into new 96-well plates and subjected them to the following four experimental conditions in four-fold replication. (1) $\text{SN}_{\text{limited}}$: invader growing in 160 μl iron-limited CAA medium supplemented with 40 μl of resident supernatant generated under iron-limited conditions (supernatants contain high pyoverdine concentration plus other secreted compounds). (2) $\text{SN}_{\text{replenished}}$: invader growing in 160 μl iron-rich CAA medium supplemented with 40 μl resident supernatant generated under iron-limited conditions (removes the effect of pyoverdine but retains the effect of other metabolites). (3) SN_{rich} : invader growing in 160 μl iron-rich CAA medium supplemented with 40 μl resident supernatant generated under iron-rich conditions (supernatants contain low pyoverdine concentration plus other secreted compounds). (4) $\text{SN}_{\text{control}}$: invader growing in 160 μl iron-limited or iron-rich CAA supplemented with 40 μl of 0.85% (w/v) NaCl instead of supernatant (control mimicking the addition of spent medium). We measured invader growth [OD_{600}] and overall pyoverdine production [RFU_{pvd}] of each replicate after 24 h of incubation at 28°C under static conditions.

Subsequently, we calculated each resident's supernatant effect on invader growth as growth

effect: $\text{GE}_{\text{treatment}} = (\text{SN}_{\text{treatment}}/\text{SN}_{\text{control}})$, where $\text{SN}_{\text{treatment}} = \text{SN}_{\text{limited}}$, $\text{SN}_{\text{replenished}}$, or SN_{rich} , with growth values being the median supernatant effects across the four replicates. Values smaller and greater than one indicate growth inhibition and stimulation respectively. From these measures, we calculated the net growth effect of the residents' pyoverdines as $\text{GE}_{\text{net}} = (\text{GE}_{\text{limited}} - \text{GE}_{\text{replenished}}) + 1$. This is possible because we used the exact same supernatants for $\text{SN}_{\text{limited}}$ and $\text{SN}_{\text{replenished}}$, but pyoverdines are only important for growth in the former and not in the latter condition, where iron is available in excess (Gu, Wei, et al., 2020). Although GE_{net} can also reflect the effects of other siderophore types, it is highly correlated with pyoverdine production in our isolates (Figure S2) and hence mainly reflects pyoverdine effects (see the Supplementary Material). Finally, we followed the exact same protocol to quantify the effects of invader supernatant on the growth of each resident.

Invasion-from rare experiments

We competed the invader against single residents and resident groups under iron-limited and iron-rich conditions. We set up competition experiments for each of our eight resident communities involving the invader and either an individual resident, groups of five residents, groups of 10 residents, or the entire community of 20 residents. For each community, we assembled four groups of five residents and two groups of 10 residents, whereby each of the 20 community members was randomly allocated to a single 5-resident and 10-resident group respectively. To ensure that our results are not biased by non-additive effects resulting from interactions between specific residents, we constructed a second set of four 5-resident and two 10-resident groups, by repeating the community assembly process (Bell et al., 2009). This resulted in 33 different competitions per resident community ($20 \times [1 \text{ resident}] + 2 \times [4 \times 5 \text{ residents}] + 2 \times [2 \times 10 \text{ residents}] + 1 \times [20 \text{ residents}]$).

We grew pre-cultures of residents and invader from freezer stocks in 24-well plates containing 1.5 ml LB per well at 28°C under shaking conditions (170 rpm). After 48 h of incubation, we washed cells in 0.85% NaCl, measured OD_{600} of each culture (1:10 dilution against a 0.85% NaCl blank), and then adjusted resident cultures to $\text{OD}_{600} = 0.2$ and invader cultures to $\text{OD}_{600} = 0.01$. Next, we assembled the resident mixes and inoculated them at a starting density of $\text{OD}_{600} = 0.01$ into 96-well plates containing 200 μl of iron-limited or iron-rich medium per well. We used a substitutive design, whereby overall starting density is constant across different mixes, while individual resident density decreases when resident number increases. We then inoculated the invader at $\text{OD}_{600} = 0.0001$ (1% frequency) into the resident mixes. We included invader monocultures as controls on each plate. We incubated plates at 28°C

under static conditions, and measured invader growth [as $\text{RFU}_{\text{mcherry}}$: excitation|emission at 582|620 nm] and pyoverdine production [RFU_{pvd}] after 16, 20 and 24 h. Note that the constitutively expressed mCherry signal is a reliable measure of growth in competitions (Leinweber et al., 2018). We carried out competitions either in eight-fold (single residents) or twelve-fold (multi-resident communities) replication.

We calculated invader success as $\text{CS}_{\text{invader}} = \text{Signal}_{\text{mix}} / \text{Signal}_{\text{mono}}$, where $\text{Signal}_{\text{mix}}$ and $\text{Signal}_{\text{mono}}$ are the median mCherry signals across all replicates in the competition mix and invader monoculture respectively. $\text{CS}_{\text{invader}}$ values smaller and greater than one indicate reduced and increased invader growth relative to the monoculture respectively. In competitions with a single resident, we directly compared $\text{CS}_{\text{invader}}$ to the pyoverdine-mediated growth effects GE_{net} . In competitions with multiple residents, we compared $\text{CS}_{\text{invader}}$ to the mean GE_{net} value across all residents in a mix.

Statistical analyses

We used linear mixed models (LMMs) for statistical analyses in R 4.0.3 (www.r-project.org). We implemented mixed models using the 'lme' function (nlme package; Pinheiro et al., 2021), and obtained the *p*-values of effects in these models using the *Anova* function (which uses type II sums of squares by default; Fox & Weisberg, 2019). We used the emmeans package (Lenth, 2021) to perform post hoc analyses and adjusted *p*-values for multiple testing ($n_{\text{test}} > 2$) using the false discovery rate. In a first set of LMMs, we tested whether growth or the (square root-transformed) pyoverdine production of the residents differed between experimental conditions (iron-rich vs. iron-limited) or resident habitat (soil vs. pond). The model analysing resident growth additionally contained pyoverdine production as a covariate. In a second set of LMMs, we tested for condition-dependent and habitat-dependent differences in supernatant effects ($\text{GE}_{\text{treatment}}$). Here, we used the *transformTukey* function (rcompanion package; Mangiafico, 2018) to find the power transformation that brings the supernatant effects closest to a normal distribution. In a third set of LMMs, we explored whether the net pyoverdine growth effect (GE_{net}) was predictive of the invader's success in invading resident communities. We first tested whether the (square-root transformed) success of the invader in competitions with single residents was affected by resident habitat, the effect of the invader's pyoverdine on the resident, and/or the effect of the resident's pyoverdine on the invader. Using the same model structure with resident number (5, 10 or 20; categorical) as an additional explanatory variable, we then explored the determinants of invasion success in competitions with multiple residents. In a final LMM, we assessed whether invasion

success depended on habitat, condition, resident number or the total pyoverdine production of the competition mix.

All models were initially fitted with all interaction terms. To account for the non-independence of measurements of residents from the same community, and to avoid pseudoreplication due to multiple measurements of each resident under different conditions, we initially fitted all models as random intercept models using resident community and—in case of repeated measurements—resident (mixture) identity nested within resident community as random effect(s). Each final model was then selected in a two-step procedure. First, we used the Akaike information criterion (AIC) to simplify the random effect structure of the model and to select an appropriate variance structure (using the *weights*-argument in the 'lme' function) where residual plots indicated a deviation from homogeneity (Zuur et al., 2009). Second, we simplified the fixed component by dropping non-significant interaction terms ($p > 0.05$). All final model structures are detailed in Table S4. Unless otherwise stated, model estimates were re-transformed to the original scale of the response for graphical display. Note that one resident hardly grew under iron limitation, but benefitted enormously from the invader's pyoverdine, resulting in a greatly inflated pyoverdine effect GE_{net} . This caused problems in our statistical models on invasion success. Consequently, we excluded this outlier from our analysis, and set its $\text{GE}_{\text{net}} = 1$ for the analysis involving multi-resident communities.

RESULTS

Growth and pyoverdine production profiles

We first quantified the growth of all 160 resident pseudomonads and the invader in monoculture (Figure 1a). We found that growth was higher under iron-rich (mean \pm SD: 0.799 ± 0.305) than iron-limited (0.522 ± 0.324) conditions ($t_{153} = 13.598$, $p < 0.001$; Table S1; Figure 1a). Most residents grew worse than the invader both under iron-limited (85.5%) and iron-rich (84.3%) conditions.

Next, we measured the pyoverdine production of all residents and invader. Consistent with our previous findings (Butaitė et al., 2018), we observed that pyoverdine production levels varied tremendously and were significantly higher among pond than soil residents ($\chi^2_1 = 45.02$, $p < 0.001$). Moreover, pyoverdine production was higher under iron-limited than iron-rich conditions ($\chi^2_1 = 369.55$, $p < 0.001$; Figure 1b), showing that residents and invader both down-regulate pyoverdine production when iron is more readily available. We further found that growth was positively linked to pyoverdine production under iron-limited conditions among residents (soil: $t_{153} = 9.462$, $p < 0.001$; pond: $t_{153} = 4.378$,

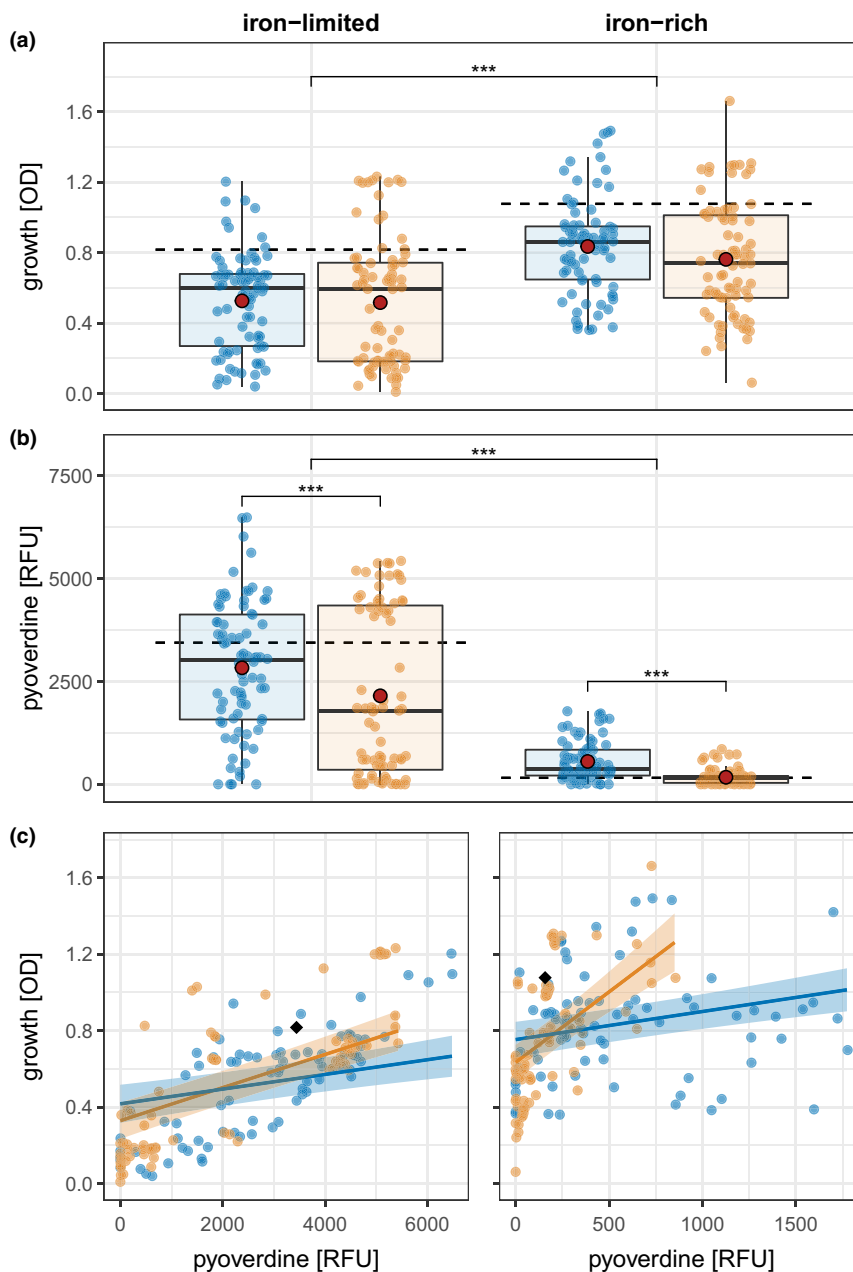


FIGURE 1 Growth and pyoverdine production of the 160 residents and the invader. Shown are the (a) growth, (b) pyoverdine production and (c) relationship between these two measures among pond (blue) and soil (orange) residents under iron-limited and iron-rich conditions. Boxplots show the median (bold line), mean (red point), 1st and 3rd quartile (box) and 5th and 95th percentile (whiskers). The dashed horizontal lines in (a) and (b) show the growth and pyoverdine production of the invader respectively. The solid lines and shaded areas in (c) are regression lines and 95% confidence intervals respectively (orange = soil-specific relationship; blue = pond-specific relationship). The black diamonds in (c) show invader values for comparison. Datapoints are the medians of four replicates obtained for each isolate

$p < 0.001$; difference soil vs. pond: $t_{153} = 3.793$, $p < 0.001$; Table S1; Figure 1c), suggesting that pyoverdine production promotes growth in this environment. Under iron-rich conditions, these positive association persisted (difference soil: $t_{153} = -7.443$, $p < 0.001$; difference pond: $t_{153} = -3.706$, $p < 0.001$; Table S1; Figure 1c), possibly because the low baseline production of pyoverdine fosters the accumulation of higher pyoverdine levels in cultures of residents with higher growth (Gu, Wei, et al., 2020).

The residents' pyoverdines are predominantly public bads for the invader

To quantify to what extent resident pyoverdines serve as public goods (stimulate growth) or public bads (inhibit growth) for the invader, we fed supernatants from all 160 residents to the invader under iron-limited, iron-replenished and iron-rich conditions, and then compared its growth relative to a control treatment. We found that resident supernatant effects

were independent of habitat ($\chi^2_1 = 0.66$, $p = 0.418$), but differed among conditions ($\chi^2_1 = 126.79$, $p < 0.001$). Under iron-limitation, supernatant effects varied from mild stimulation to strong inhibition (Figure 2a). When repeating the assay with the same supernatants, but under iron-replenished conditions to cancel out pyoverdine effects, we found that most inhibitory effects were gone. Overall, supernatant effects were significantly lower (i.e. more inhibitory) under iron-limited (0.881 ± 0.265) than under iron-replenished

(1.035 ± 0.106) conditions ($t_{316} = -10.74$, $p < 0.001$). By contrast, supernatant effects were close to neutral for both iron-replenished and iron-rich conditions (1.030 ± 0.092), suggesting a low baseline production of toxic compounds. When examining the net pyoverdine effect calculated from these supernatant effects, we found that resident pyoverdines typically had a neutral ($n = 76$) or inhibitory (public bad; $n = 82$), but rarely a promoting (public good; $n = 1$) effect on invader growth (Table S2, Figure 2a).

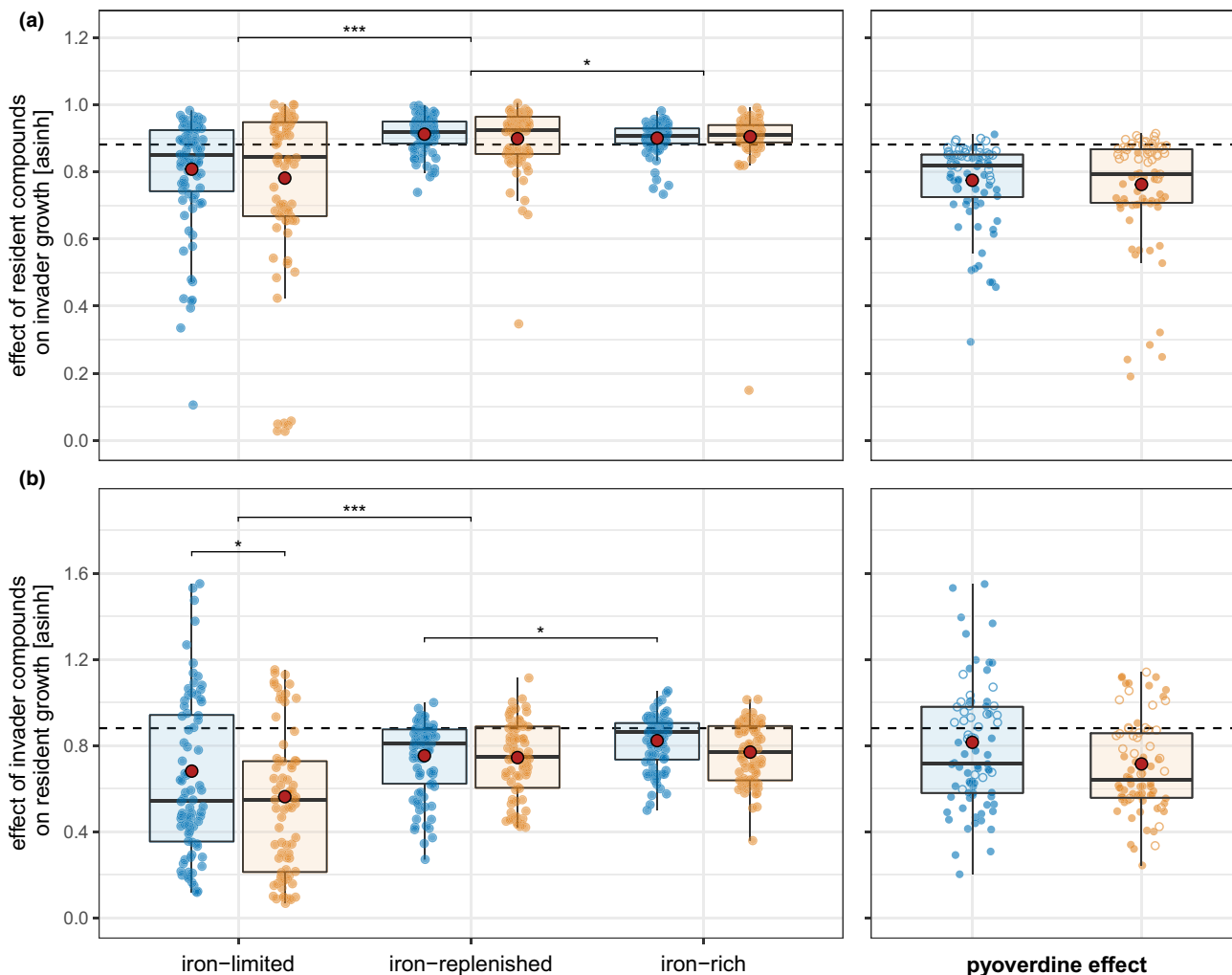


FIGURE 2 Supernatant and pyoverdine effects. Shown are (a) the effects of compounds in the resident supernatants on invader growth and (b) the effects of compounds in the invader supernatants on resident growth. Boxplots show the median (bold line), mean (red point), 1st and 3rd quartile (box) and 5th and 95th percentile (whiskers). Datapoints are the median of four replicates obtained for each isolate. The growth effects under iron-limitation were measured by feeding supernatant generated in iron-limited medium (supernatants contain high pyoverdine concentration + other secreted compounds) to receivers growing in iron-limited medium. Conversely, the growth effects under iron-replenished conditions were measured by feeding supernatant generated in iron-limited medium to receivers growing in iron-rich medium (such that the effect of pyoverdine is removed while the effect of other metabolites is retained). Finally, the growth effects under iron-rich conditions were measured by feeding supernatant generated in iron-rich medium (supernatants contain low pyoverdine concentration + other secreted compounds) to receivers growing in iron-rich medium. The dashed horizontal lines indicate the null line where compounds in the supernatants have no effect on growth. The net effect caused by pyoverdine alone (right column) was obtained by subtracting the growth effect of the iron-replenished condition from the growth effect of the iron-limited condition (followed by the addition of 1 to keep the null line comparable across treatments). Net pyoverdine values deviating or not deviating significantly from neutral are indicated as filled and hollow circles, respectively. Note that we performed an inverse hyperbolic sine (arsinh) transformation to display our data, as the high range of observed effects would have otherwise prevented a meaningful data presentation

The invader's pyoverdine can be a public good or a public bad for residents

We repeated the above assay but fed supernatants of the invader to each of the 160 residents. We found that the effect of the invader's supernatant on resident growth depended on an interaction between condition and resident habitat (condition: $\chi^2_2 = 91.17$, $p < 0.001$; habitat: $\chi^2_1 = 2.55$, $p = 0.110$; interaction: $\chi^2_2 = 6.67$, $p = 0.036$). Under iron-limitation, the supernatant effect varied between strong stimulation to strong inhibition, and was lower for soil than for pond residents (soil: 0.594 ± 0.404 ; pond: 0.736 ± 0.502 ; $t_6 = -2.581$, $p = 0.042$; **Figure 2b**). As before, these growth effects diminished when replenishing the supernatant with iron (soil: 0.816 ± 0.228 , $t_{314} = 6.569$, $p < 0.001$; pond: 0.827 ± 0.217 , $t_{314} = 2.953$, $p = 0.005$), and rendered the supernatant effect similar to the effect under iron-rich conditions (soil: 0.849 ± 0.180 ; pond: 0.920 ± 0.164). Examinations of the resulting net pyoverdine effect revealed that the invader's pyoverdine indeed served as a public good for some residents ($n = 23$), and as a public bad for others ($n = 87$; the effect was neutral for $n = 49$ residents; Table S2, **Figure 2b**).

Interactions between pyoverdine effects drive invasion success in one-to-one competitions

To test whether pyoverdine effects predict invasion success under iron-limited conditions where siderophores are important for growth, we quantified to what extent the invader can increase in abundance from rare in competitions against each of the 160 residents. In support of our hypothesis, we found that invasion success was shaped by an interaction between the effects of the resident's and the invader's pyoverdines (**Figure 3a+b**; **Table 1A**). Specifically, invasion success increased when the resident's pyoverdine had neutral rather than an inhibitory (public bad) effect on the invader, and this increase was reinforced when the invader's own pyoverdine inhibited (public bad) rather than stimulated (public good) resident growth (**Figure 3a+b**). Notably, invasion success increased with the resident's pyoverdine effect in both habitats, albeit steeper in soil as compared to pond (soil: $t_{147} = 9.468$, $p < 0.001$; pond: $t_{147} = 5.327$, $p < 0.001$; difference: $t_{147} = 1.986$, $p = 0.049$; **Table 1A**).

Effects of resident pyoverdines predict invasion success in complex communities

We then quantified invasion success into multi-resident communities, where the invader has to cope with many residents producing a cocktail of different pyoverdines. Here, we expected the effect of the invader's pyoverdine to diminish and invasion success to be predominantly determined by the aggregate effect of the residents'

pyoverdines. Our analysis supports this prediction (**Figure 3**): invasion success increased when the average effect of the residents' pyoverdines on the invader was neutral rather than inhibitory (public bad) (**Table 1B**; **Figure 3c**). By contrast, the average effect of the invader's pyoverdine on the residents had no longer an impact on invasion success, and there was no longer an interaction between the effects of the residents' and invader pyoverdines (**Figure 3d**; **Table 1B**). Notably, all these effects were independent of resident number (**Table 1B**).

Invasion success is habitat-specific and declines in more diverse resident communities

While the above analyses directly related invasion success to public good versus public bad properties of pyoverdines under iron-limited conditions, we now explore the effects of resident number, iron availability, habitat type and total community pyoverdine production on invasion success (**Figure 4**). We found that invasion success was shaped by all these factors (**Table 2**). In particular, the invader's success was higher in competitions with single residents (0.48 ± 0.36) than in competition with groups of 5 (0.27 ± 0.25), 10 (0.25 ± 0.24) or 20 (0.18 ± 0.15) residents (single vs. five: $t_{252} = 6.661$, $p < 0.001$; 5 vs. 10: $t_{252} = 0.598$, $p = 0.550$; 10 vs. 20: $t_{252} = 1.231$, $p = 0.329$; **Figure 4a**). This diversity effect occurred in both iron-limited and iron-rich conditions and independently of resident habitat. Overall, invasion success was higher under iron-limited as compared to iron-rich conditions (0.48 ± 0.36 vs. 0.30 ± 0.30 , $t_{257} = 8.003$, $p < 0.001$; **Table 2**; **Figure 4b**). Moreover, invasion success decreased with the overall pyoverdine production of the mixed culture both under iron-limited (soil: $t_{257} = -7.213$, $p < 0.001$; pond: $t_{257} = -2.006$, $p = 0.046$; difference: $t_{257} = -4.236$, $p < 0.001$; **Figure 4c**) and iron-rich conditions (soil: $t_{257} = -5.825$, $p < 0.001$; pond: $t_{257} = -4.319$, $p < 0.001$; difference: $t_{257} = -4.365$, $p < 0.001$; **Figure 4d**).

An in-depth analysis of this latter effect reveals that residents that grow well and produce high levels of pyoverdine in mono-culture have the lowest (i.e. most inhibitory) pyoverdine effects (**Figure S3**), and are most efficient in compromising invasion success under iron-limitation (**Figure 3a+c**). Since both public good versus public bad effects and pyoverdine production levels seem to drive invasion success, we finally examined which of the two effects is more important. We thus repeated our analyses of invasion success using a pyoverdine effect that is corrected for the amount of pyoverdine produced by residents (**Figure S4**). We found that the corrected pyoverdine effects still positively affected invasion success in competitions with soil residents, but no longer in competitions with pond residents (**Supplementary Analyses and Table S3**). These results suggest that socio-ecological factors (e.g. pyoverdine production and resident number) and abiotic

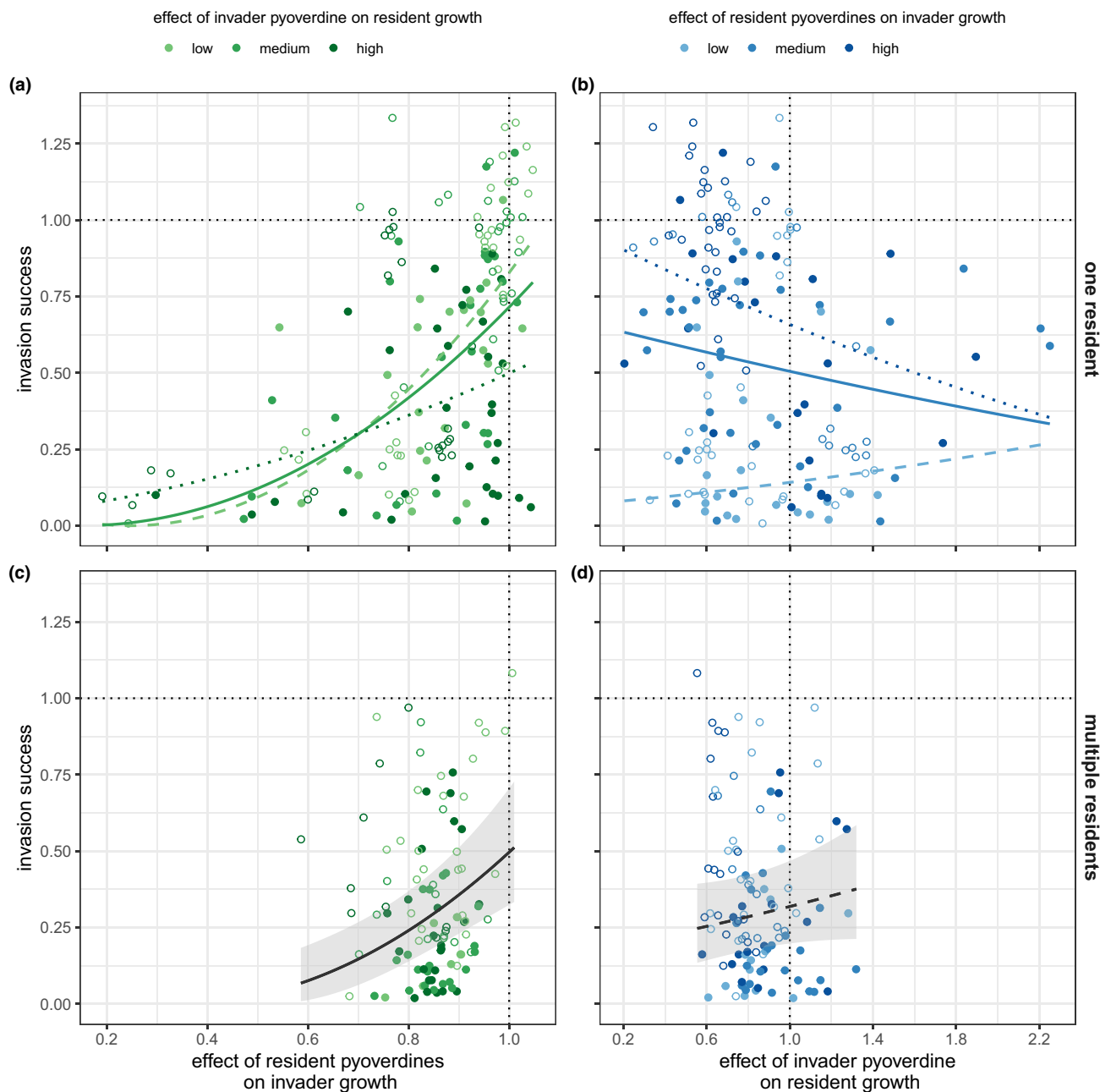


FIGURE 3 Pyoverdine-based effects on invasion success in competitions with one or multiple residents. Shown are the effects of resident pyoverdines on invader growth (a+c) and the effects of invader pyoverdine on resident growth (b+d), detected in invasion-from-rare experiments between the invader (starting frequency 1%) and residents. (a) and (b) show the interaction between the two pyoverdine effects in competitions between the invader and a single resident with the effect of the resident pyoverdines and the effect of the invader pyoverdine as the focal predictor respectively. (c) Main effect of the residents' pyoverdines in competitions between the invader and multi-resident communities (5, 10 or 20 members). (d) Main effect of the invader's pyoverdine in competitions between the invader and multi-resident communities. To illustrate the interactions in (a) and (b), regression lines are provided for a low (dashed line; mean of first tercile), medium (solid line; mean of second tercile), and high (dotted line; mean of third tercile) value of the effect of invader pyoverdine on resident growth and the effect of resident pyoverdines on invader growth respectively. The solid and dashed regression lines in (c) and (d) indicate significant and non-significant relationships respectively. Grey shaded areas are 95% confidence intervals. Datapoints are the medians of four replicates obtained for each (set of) isolates. They are coloured according to which tercile of the effect of invader pyoverdine on resident growth (a+c; shades of green) or the effect of resident pyoverdines on invader growth (b+d, shades of blue) they fall in. Filled and empty points represent pond and soil residents respectively. The dotted horizontal and vertical lines are reference lines showing invader growth in the absence of a competitor (y-axis) and invader (a+c) and resident (b+d) growth in the absence of supernatants

	(A) One-to-one competitions			(B) One-to-many competitions		
	χ^2	df	<i>p</i>	χ^2	df	<i>p</i>
Habitat (soil or pond)	1.71	1	0.191	5.15	1	0.023
Resident number (5, 10 or 20)	–	–	–	2.46	2	0.292
Effect of invader pyoverdine [EoIP]	4.03	1	0.045	2.09	1	0.148
Effect of resident pyoverdine(s) [EoRP]	106.65	1	<0.001	20.97	1	<0.001
Habitat: EoRP	3.94	1	0.047	0.02 ^a	1 ^a	0.884 ^a
EoIP: EoRP	3.87	1	0.049	3.36 ^a	1 ^a	0.067 ^a

^aValues before removal of the interaction from the model.

factors (e.g. iron availability and habitat type) jointly shape invasion success.

DISCUSSION

Although microbial invasions are increasingly receiving attention due to their drastic effects on community composition and functioning, it often remains unclear which microbial traits help invaders to successfully establish themselves in a community (Kinnunen et al., 2016; Litchman, 2010; Mallon, van Elsas, et al., 2015). Here, we tested whether social interactions mediated by iron-scavenging siderophores predict the success of the opportunistic pathogen *P. aeruginosa* to invade environmental resident communities assembled from 160 *Pseudomonas* isolates from soil and pond samples. We found that pyoverdines—the main siderophores produced by fluorescent pseudomonads—take on a dual role during invasion: they act either as ‘public goods’ by delivering iron to individuals possessing the matching receptors required for uptake, or as ‘public bads’ by locking iron away from competitors lacking these receptors. We show that these pyoverdine-based interactions are a strong predictor of invasion success under iron-limited conditions. Particularly, invasion success increased when the invader could use its siderophores to inhibit the residents (public bad effect) rather than to stimulate (public good effect) their growth. This pattern was reinforced in one-to-one competitions when the resident's pyoverdine had neutral rather than inhibitory effects for the invader. Conversely, invasion success decreased when residents produced inhibitory and/or high amounts of pyoverdines, and in more diverse communities.

At the mechanistic level, receptor (in)compatibility should determine whether a pyoverdine acts as a public good or public bad. We previously investigated this aspect through the analysis of 24 resident genomes (Butaitė et al., 2017). We found that residents had on average 5.1

(range: 1–19) different pyoverdine receptor homologues. Moreover, we found that residents typically produce structurally different pyoverdine types and that the degree of receptor compatibility between residents predicted the level of stimulation versus inhibition (Butaitė et al., 2017). Given this rich receptor repertoire, it is understandable that several residents could exploit the invader's pyoverdine and use it as a growth-stimulating public good in our current setup. The situation looks different for our invader *P. aeruginosa* PAO1, which has only two pyoverdine receptors (Ghysels et al., 2004). This limited receptor repertoire could explain why most resident pyoverdines had an inhibitory public bad effect on the invader.

In addition to receptor compatibilities, we found that invasion success under iron-limitation was influenced by the resident(s) pyoverdine production levels (Figure 4c). Our finding that residents with high pyoverdine production are better at resisting invasion makes intuitive sense because resident pyoverdines are predominantly public bads for the invader, with higher production levels leading to stronger inhibition (Figure S3). The effect of pyoverdine production levels on reducing invasion success was particularly pronounced in pond communities (while receptor compatibility seemed more important in soil communities; Figure S4). This is likely a direct consequence of our observation that pond residents produce more pyoverdine than soil residents (Figure 1b), presumably because iron is more limited in the pond habitat (Butaitė et al., 2018).

Our findings highlight the role of siderophores in driving invasion patterns under iron limitation, but how do they relate to other factors determining invasion outcomes? Consistent with previous work (Acosta et al., 2015; Bonanomi et al., 2014), we found that invasion success decreased as resident number increased. Under iron-limited conditions, we expected this effect because pyoverdine diversity increases with resident number, while the invader receptor repertoire remains

TABLE 1 Determinants of invasion success in competitions with (A) one or (B) multiple residents. Significant *p*-values in bold

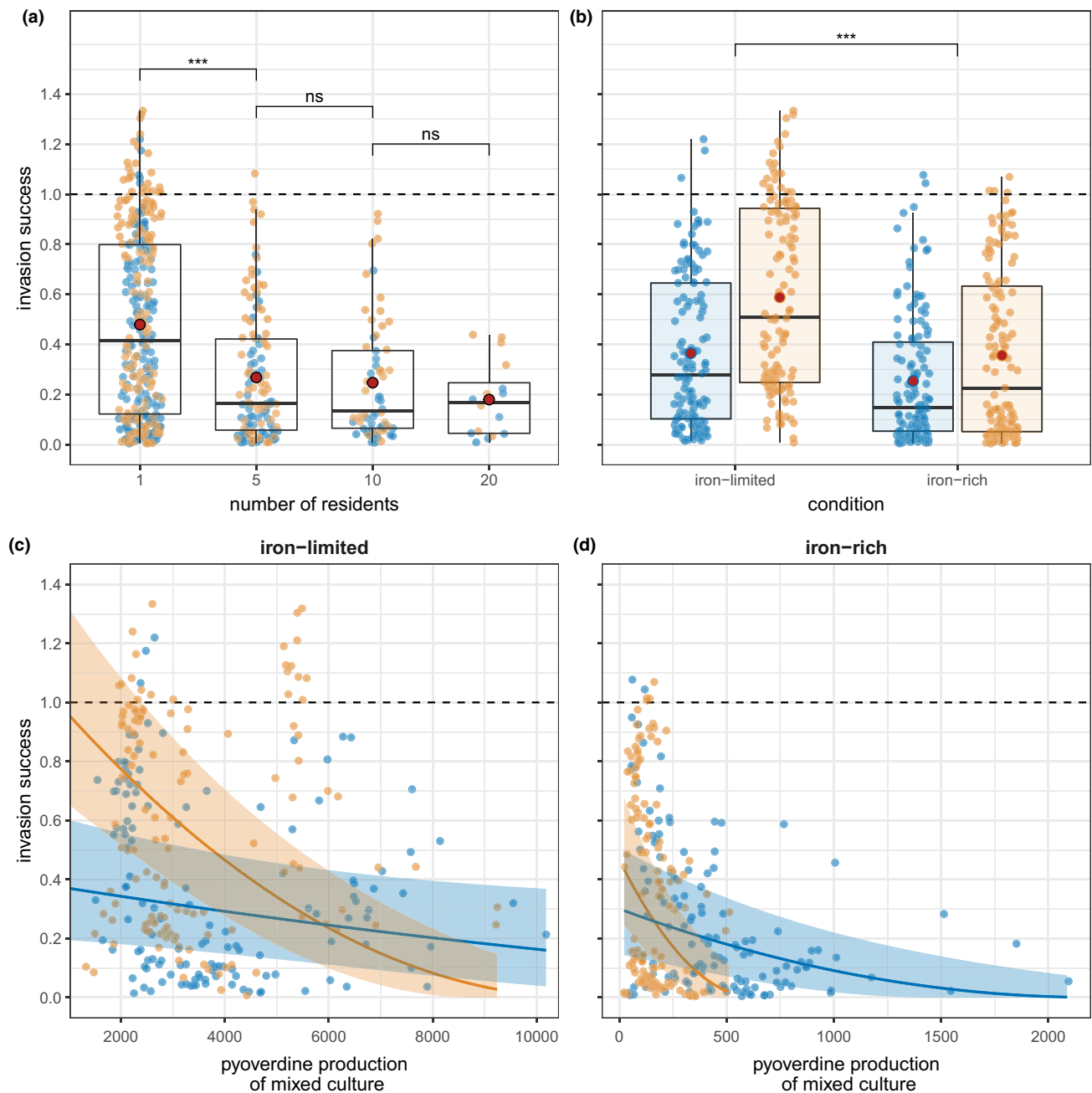


FIGURE 4 General determinants of the invader's competitive success. Shown are associations between the invader's competitive success and (a) the number of soil (orange) and pond (blue) residents, (b) the origin of residents under iron-limited and iron-rich conditions, and the relative pyoverdine production of the mixed culture under (c) iron-limited and (d) iron-rich conditions. Boxplots show the median (bold line), mean (red point), 1st and 3rd quartile (box) and 5th and 95th percentile (whiskers). The dashed horizontal lines indicate the null line, where the invader's growth is not affected by the presence of residents (i.e. monoculture growth). The solid lines and shaded areas in (c) and (d) are regression lines and 95% confidence intervals respectively (orange = soil-specific relationship; blue = pond-specific relationship). Datapoints are the medians of four replicates obtained for each (set of) isolates

small (i.e. two receptors), such that the fraction of public-bad producing residents likely increases with higher resident numbers. Under iron-rich conditions, this explanation does not hold, as pyoverdines are not required for growth and therefore not produced in high amounts. Instead, we propose that resource and interference competition could jointly contribute to this effect. While competition under iron limitation solely

revolves around iron, CAA (a blend of amino acids and peptides) supplemented with iron offers many different nutrient niches. Niche occupancy likely increases with resident number, thereby reducing open niche space for invaders. Similarly, interference competition might further reduce invasion success as resident number increases, because it might become more likely that the invader faces competitively superior residents.

TABLE 2 General determinants of invasion success. Significant *p*-values in bold

	χ^2	df	<i>p</i>
Habitat (soil or pond)	0.83	1	0.362
Condition (iron-limited or iron-rich)	103.81	1	<0.001
Resident number (1, 5, 10, or 20)	74.73	3	<0.001
Pyoverdine production	32.12	1	<0.001
Habitat: condition	39.71	1	<0.001
Habitat: pyoverdine production	15.58	1	<0.001
Condition: pyoverdine production	28.98	1	<0.001
Habitat: condition: pyoverdine production	16.28	1	<0.001

This effect could occur despite our supernatant assays revealing little evidence for inhibitory effects. This is because toxins and contact-dependent killing systems are often only deployed upon direct contact between competitors (Mavridou et al., 2018; Melvin et al., 2017). Such direct contact is prevented in supernatant assays, but occurs in our invasion assays, especially in iron-rich environments where cultures grow to higher densities. While siderophores can have neutral or positive effects on the invader, resource and interference competition effects are always negative, which could explain why invasion success was overall lower under iron-rich than iron-limited conditions.

One could envision siderophores as mediators of both resource and interference competition through their role as public bads. The resource competition side is clear, as siderophores necessarily withhold iron from competitors lacking matching receptors. The interference competition side is less established, and the main question to be addressed is whether producers are selected to secrete siderophores beyond of what is required for iron scavenging to specifically combat competitors. A recent theoretical study showed that siderophore secretion can indeed evolve to inhibit other strains even under iron-rich conditions (Niehus et al., 2017). Moreover, bacteria have been observed to upregulate siderophore production in the presence of competing species (Harrison et al., 2008; Leinweber et al., 2018). To elucidate the role of siderophores in interference competition, future studies should focus on the fitness consequences of such excessive siderophore production.

While we focused on pyoverdines in *Pseudomonas* communities, a key question is whether our findings can be extrapolated to microbial consortia with more diverse siderophore repertoires. A recent study supports this notion. Gu, Wei, et al. (2020) competed 2150 bacterial isolates from rhizosphere microbiomes against the plant

pathogen *Ralstonia solanacearum*. They found that the effects of rhizosphere siderophores on pathogen growth vary from strong growth inhibition to promotion. While no community effects were examined and the type of siderophores and receptors produced remained unknown, this study supports the dual role of siderophores in driving social interactions not only among pseudomonads, but also in diverse microbial communities.

In conclusion, we show that the dual role of siderophores as public good versus public bad predicts microbial invasions under iron limitation, a common feature of many natural habitats (Boyd & Ellwood, 2010; Colombo et al., 2014). Our results could have broad implications on two fronts. From a medical perspective, they suggest that siderophore and receptor repertoires of pathogens affect their potential to establish reservoirs in natural communities after escaping from patients or hospitals (Loveday et al., 2014; Quick et al., 2014). From an ecological disease management perspective, our results highlight that knowledge on siderophore and receptor repertoires could be leveraged to engineer microbial communities that are resistant to pathogen invasion and can thereby protect their plant and animal hosts (Gu, Yang, et al., 2020).

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CONFLICT OF INTEREST

We have no conflict of interests.

AUTHORSHIP

ARTF, ÖÖ, RK and JK designed the research. ARTF, ÖÖ and JK collected the data. JK performed the statistical analyses. ARTF, ÖÖ, RK and JK wrote the manuscript.

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