LETTER

Elevated success of multispecies bacterial invasions impacts community composition during ecological succession

Damian W. Rivett,[1,2]* Matt L. Jones,[1] Josep Ramoneda,[1†] Shorok B. Mombrikotb,[1] Emma Ransome[1] and Thomas Bell[1]

Abstract

Successful microbial invasions are determined by a species' ability to occupy a niche in the new habitat whilst resisting competitive exclusion by the resident community. Despite the recognised importance of biotic factors in determining the invasiveness of microbial communities, the success and impact of multiple concurrent invaders on the resident community has not been examined. Simultaneous invasions might have synergistic effects, for example if resident species need to exhibit divergent phenotypes to compete with the invasive populations. We used three phylogenetically diverse bacterial species to invade two compositionally distinct communities in a controlled, naturalised *in vitro* system. By initiating the invader introductions at different stages of succession, we could disentangle the relative importance of resident community structure, invader diversity and time pre-invasion. Our results indicate that multiple invaders increase overall invasion success, but do not alter the successional trajectory of the whole community.

Keywords

Bacteria, community assembly, community stability, Invasion, next generation sequencing, species sorting, timing.

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Introductions of alien species rarely occur in isolation, with some regions receiving high numbers of invasive species (Dawson *et al.* 2017; Turbelin *et al.* 2017). The arrival of foreign species into established communities can alter the composition and functioning of the resident community, with the potential for negative impacts on ecosystem services and on the economy (Paini *et al.* 2016; Russell & Blackburn 2017). The frequency and impacts of invasions are likely to increase, with discovery rates of species outside of their native ranges rapidly increasing for both macro- and microscopic taxa and on almost every continent (Johnson *et al.* 2009; Seebens *et al.* 2017). For example, a recent study indicated that ~4% of vascular plant species have become naturalised outside their natural ranges (van Kleunen *et al.* 2015); given the ubiquity of species invasions, it is unlikely that any invasion happens in isolation. Despite the recognition that invasive species pose a threat to established ecosystems, and that multiple introductions are occurring simultaneously in most ecosystems, little is known about the interaction between the two.

Successful invasions are dependent on the interaction between the invader(s), the resident community and the environment. These include the ability of the invader to exploit the niches available in the new habitat (Chesson 2000; Vall-llosera *et al.* 2016), to resist exclusion due to resource competition and other antagonistic interactions (Alba *et al.* 2017), and in so doing, to integrate into the interactions within the resident community (Padrón *et al.* 2009). Integration of an invader into a resident community can disrupt the existing network of interactions among resident species (Sanders *et al.*

2003; Alba *et al.* 2017), giving the invader the opportunity to establish in the community. Experimental manipulations have demonstrated that invasion success is also improved by providing additional niches (e.g. higher resource diversity and availability) (Catford *et al.* 2009; Symons & Arnott 2014; Mallon *et al.* 2015), and by disrupting the resident community (e.g. through habitat disturbance) (Mächler & Altermatt 2012).

Invasions themselves can be highly disruptive to communities (Báez & Collins 2008), so have the potential to impact simultaneous or subsequent invasions in a similar way to any other type of perturbation. However, the effect of multiple simultaneous invasions has received limited attention in ecology (Johnson *et al.* 2009). Past studies, predominantly on freshwater ecosystems, generally show competitive interactions amongst invaders and between invaders and the resident community (Burič *et al.* 2009; Hudina *et al.* 2011). In these systems, direct competitive interactions predominate between invaders due to overlapping resource utilisation (Hudina *et al.* 2011), so coexistence of multiple invaders is dependent on resource heterogeneity (Burič *et al.* 2009). In homogenous environments, multiple invaders were more detrimental to the resident community than single invaders (Johnson *et al.* 2009). There are, however, some examples of invaders facilitating other invaders. For example, Johnson *et al.* (2009) reported that an initial invader (rusty crayfish, a snail predator) aided the establishment of a second invader (Chinese mystery snails) at a later date, because the crayfish was able to reduce the abundance of the resident snail community,

[1]*Department of Life Sciences, Imperial College London, Silwood Park Campus, Ascot, UK*

[2]*Division of Biology and Conservation Ecology, School of Science and the Environment, Manchester Metropolitan University, Manchester, UK*

[†]*Present address: Department of Environmental Systems Science, Group of Plant Nutrition, ETH Zürich (Swiss Federal Institute of Technology), Switzerland*

Correspondence: E-mail: d.rivett@imperial.ac.uk

potentially freeing the second invader from competition (Montgomery *et al.* 2012). Such findings suggest that the impact of multiple invasions is dependent on the balance between competition amongst the invaders themselves and between invaders and the resident community (Preston *et al.* 2012).

While past studies have demonstrated the potential for interactions among multiple invaders, the studies have been highly selective in the invaders being studied, often predicated on invaders that were known to interact directly or indirectly (Buřič *et al.* 2009; Johnson *et al.* 2009; Hudina *et al.* 2011). It is less clear whether these findings offer us a more general understanding of the success and impact of multiple invasions compared to invasions of species in isolation (Livingston *et al.* 2013). We suggest there are two main reasons to expect interactions among invaders *a priori*. First, if multiple species invade simultaneously, one of those species is more likely to be a successful invader, due to an increased chance that one species has a high competitive ability or possesses unique traits that allow it to colonise a novel niche (i.e. a selection effect; Loreau & Hector 2001). Secondly, multiple invader species can facilitate each other's growth and persistence (i.e. a complementarity effect), for example by cross-feeding or by disturbing the resident community. These two predictions are not mutually exclusive and likely work together. These principles have long been established in biodiversity-ecosystem functioning research, but studies of multiple invasions lack such a framework. Utilising such a framework, and contrasting with many of the existing studies we cite above, we predicted that simultaneous multispecies invasions may result in higher invasion success and also greater impact on the community.

In this study, we tested the ability of three phylogenetically diverse, aquatic bacterial isolates to separately and simultaneously invade, and impact, resident communities during succession. Microbial invasion ecology is an emerging field within invasion ecology, not just because microbial systems are often simple to experimentally manipulate, but also because microbial invasions are likely to have relevance to understanding pathogenesis, bioremediation and industrial contamination (Stecher & Hardt 2011; Lewis *et al.* 2015; O'Brien & Buckling 2015; Wei *et al.* 2015). Additionally, multiple simultaneous introductions are likely to be the norm for microbial invasions given their passive dispersal and small size (Lindström & Östman 2011; Rillig *et al.* 2015). We used bacterial communities and invaders taken from water-filled tree holes (Bell *et al.* 2005). The invaders were selected and isolated based on their cosmopolitan distribution across tree holes and aerobic culturability. Previous work using the same system (Fiegna *et al.* 2015; Rivett *et al.* 2016; Jones *et al.* 2017) has demonstrated shifting resource usage during community succession. We predicted a decline in the importance of complementarity among invader species during succession because declines in resource availability would provide fewer opportunities for complementarity. In contrast, we predicted an increase in the importance of selection effects during succession because early-stage communities contain more vacant resource niches and more transient interspecific interactions than those of a more progressed successional stage (Rivett *et al.* 2016), and these permissive

conditions allow invasion of less-fit invader species. Later-stage communities only allow invaders with more specialised traits, resulting in an increased importance of selection effects in these communities. Successful multi-species invasions in stable or late successional communities might result from synergistic impacts between invaders (e.g. by sharing extracellular enzymes or by displacing a resident, competing species) or because they have a higher probability of including one successful generalist invader.

MATERIALS AND METHODS

Bacterial sampling, isolation and identification

Water samples were collected from rain-filled depressions in the roots of beech trees at four locations across South England during October and November 2014. Detritus was filtered from the samples (pore size 20–25 μm, Whatman plc, Maidstone, UK) and the remaining bacteria were used to inoculate beech leaf tea (Lawrence *et al.* 2012), supplemented with 200 μg mL^{-1} of the anti-fungal agent cycloheximide (Sigma-Aldrich, Gillingham, UK) for 7 days at 22 °C. These communities were stored at −80 °C in 30% v/v glycerol (final concentration; Sigma-Aldrich) with 0.85% NaCl (Sigma-Aldrich), until required.

Bacterial isolates were cultured from different communities, identified and stored as previously described (Rivett *et al.* 2016). Briefly, isolates were picked from colonies grown from filtered communities on R2A agar plates (Sigma-Aldrich), and stored at −80 °C in ProtectTube Cryobead Systems (Technical Consultants Ltd, Heywood, UK). DNA was extracted using the ZR-96 Fungal/Bacterial DNA extraction kit (Zymo Research, Irvine, CA, USA) and a fragment of the 16S rRNA gene was amplified using primers 27f and 1492r, as previously described (Rivett *et al.* 2016). We selected for spontaneous rifampicin resistant mutants so that invasion success could be quantified using selective plating within communities lacking rifampicin resistant populations. To select the invaders, bacterial isolates were revived from storage and grown overnight in 1 mL lysogeny broth (LB; Sigma-Aldrich) at 30 °C. Overnight cultures were concentrated into 200 μL, plated on LB agar (Sigma-Aldrich) supplemented with 50 μg mL^{-1} rifampicin (Sigma-Aldrich), and incubated overnight at 30 °C to select for spontaneous mutations. Colonies were then transferred two further times to ensure resistance, first on LB agar containing rifampicin at 100, and then 300 μg mL^{-1}. Three species were subsequently selected that were distinct from each other both phylogenetically (highest taxonomic separation at order level) and morphologically (Table S1). Using the 16S rRNA gene sequences species A, B & C were identified as members of the genera *Chryseobacterium, Pseudomonas,* and *Pantoea* respectively. Using molecular analysis of the 16S rRNA gene (methods below) it was confirmed that these invaders were absent from the communities (Fig. S1). Each invader was revived from frozen stocks on 100 μg mL^{-1} rifampicin-LB agar plates, incubated for 3 days at 22 °C, prior to the inoculation of the media with a single colony and incubation for 1 week at 22 °C.

Experiment design

In this experiment, the three isolates were used to invade two of the archived communities. We chose two compositionally distinct communities (Fig. S2) to better generalise the mechanisms behind invasion success. No rifampicin resistance colonies were detected in either community at the outset of the experiment or in the invader-free controls. Prior to the inoculation of the experimental microcosms the two communities were revived from frozen into 10 mL of beech leaf tea supplemented with 0.1 mM MgSO$_4$ (Sigma-Aldrich) and 0.1 mM CaCl$_2$ (Sigma-Aldrich) (beech leaf media), and incubated statically at 22 °C for one week. The communities were then diluted 1: 99 in fresh beech leaf media (final volume 1 mL) to give a starting inoculum of ~10^5 colony forming units per mL (CFU mL^{-1}) in 2 mL square-deep well plates. Microcosms were inoculated with either one of the two communities, or left uninoculated for the control, and all experiments were carried out simultaneously.

We investigated whether successional changes in community composition had an effect on the invaders by inoculating the invaders (species A, B & C) at three time points: at the same time as the communities (early), and five (mid) and twelve (late) days post-community inoculation. Each community was challenged with all possible combinations of 1, 2 or 3 invaders (7 combinations) and independently replicated three times. The total number of invader cells (regardless of invader richness) was 10^3 CFU mL^{-1} for all invader introductions. The invaders were also inoculated into sterile beech leaf media with no community to act as an invasion control and to assess how the invaders interacted without a community and incubated as above at 22 °C statically for 1 week. All communities were destructively sampled 1 week after the invaders were introduced.

Invasiveness assay

Invasions were regarded as 'successful' if the invaders were able to persist in the microcosms over 7 days. Seven days was approximately the amount of time it took for communities to achieve stable abundances, and so was a natural yardstick for gauging whether the invader had managed to become integrated into the resident community. The microcosms were serially diluted and plated (75 µL) onto two solid growth media to differentiate between the resident community and invading bacteria; the first medium was R2A agar (Sigma-Aldrich) to assess the total bacterial CFU in the microcosm, and the second was 100 µg mL^{-1} rifampicin-LB agar, to quantify the CFU of the invaders whilst inhibiting growth of the communities. Whilst plate counts are known to underestimate the true abundance of viable bacterial cells this method was used here in order to generate comparable plate counts, between invaders and community, required to assess invasions. Cultures were diluted and the number of CFU was counted after 3 days for both the community (10^{-7} to 10^{-4}) and invader (10^{-4} to 10^{-2}), with the mean value recorded across the dilutions. Counts of each of the distinct colony morphologies (Table S1) were recorded from the rifampicin-LB plates. If no invader was detected via plate counts but

sequencing data confirmed its presence ($n = 9$ microcosms) at these dilutions, a value lower than the limit of detection was assigned (10 CFU).

Molecular analysis of community structure

Experimental microcosms were briefly (10 s) homogenised by vortexing and frozen 7 days after invasion and DNA was later extracted from ~25% of the microcosm (200 µL) using the ZR-96 Fungal/Bacterial DNA extraction kit (Zymo Research) as described above. The V4 region of the 16S rRNA gene was amplified, using universal primers containing sequencing indexes (Kozich *et al.* 2013). Sequencing was performed on the MiSeq platform with version 3 chemistry (Illumina Inc., San Diego, CA, USA). Sequences were processed in MacQIIME and VSEARCH. Default settings were used to clean sequences in MacQIIME with the addition of a maximum unacceptable phred quality threshold of Q20. Cleaned sequences were checked for chimeras in VSEARCH, using the RDP Gold database and clustering and OTU identification were carried out in MacQIIME using the Silva database (SSU Ref NR 99 128) at a resolution of 97% sequence identity.

Statistical analysis

All data were log (base 10) transformed prior to analysis to conform to the assumptions of parametric statistics. If the assumptions were not met, non-parametric analyses were employed as stated in the text. Analyses of community composition were conducted using absolute abundances, where numbers of sequences were equilibrated by randomly sampling 10 000 sequences per community. The relative abundances were multiplied by the CFU mL^{-1} of the community to produce an estimate of absolute abundance. Analysis of variance was used to assess the effects of invader richness and community establishment on the persistence and abundance of the invaders in the microcosms. *Post hoc* Tukey's HSD tests were performed to assess the significant differences (p_{adj}) between the levels of the factors entered into the analysis of variance. For each invader species, we constructed a linear model where the response variable was the log$_{10}$-transformed invader abundance, and the independent variables scored the binary presence/absence of the other two invader species and their interaction, using R syntax $\alpha_i \sim x_j * x_k$, where α_i represents the model estimate for the abundance (CFU mL^{-1}) of invader i and x is the binary presence/absence of invaders j and k.

We estimated the importance of complementarity and selection effects in determining the total number of viable invader cells. We used the additive partitioning equation (Loreau & Hector 2001): $\Delta Y = N\overline{\Delta RY}\,\overline{N} + Ncov(\Delta RY, M)$, where ΔY is the difference between the observed total number of invader cells in mixtures and the expected number in the absence of selection or complementarity effects, N is the number of species in the mixture, ΔRY is the difference between the invasion success of the starting inoculum of a particular isolate in monoculture and the invasion success of the same isolate while in the mixture and M is the monoculture invasion success. In the equation above, $N\overline{\Delta RY}\,\overline{N}$ ('complementarity

effect') reflects the degree to which invasion success depends on interactions between the invaders, and $Ncov(\Delta RY, M)$ ('selection effects') reflects the degree to which inclusion of a particular species in mixture influences invasion success.

Community data were visualised using non-metric multidimensional scaling (NMDS) ordination. Prior to the analysis of the sequencing data, sequences that matched the invaders were removed to avoid artificial inflation of the dissimilarities between the communities. NMDS ordinations used 99 permutations, and were repeated if the stress was greater than 0.16. Analysis of similarity (ANOSIM) was calculated using 999 permutations. Multivariate analysis of variance was conducted to assess whether the treatments impacted the distance between communities in the ordination space (NMDS axis 1 & 2). Statistical significance was assigned using $\alpha \leq 0.05$. Visualisation and analysis were performed using R v.3.0.1 (https://www.r-project.org). Multivariate statistics were performed using the vegan (v2.3-4) package (Oksanen *et al.* 2015).

RESULTS

Impact of invasion on community composition

We investigated whether the invaders altered the final community composition of the resident communities across time and invasion treatments. Using Bray–Curtis dissimilarity, significant differences were observed between the two community identities (ANOSIM R = 0.54, $P < 0.001$), whether the communities were invaded (ANOSIM R = 0.45, $P < 0.001$), and the time after inoculation that invasion occurred (ANOSIM R = 0.25, $P < 0.001$) (Fig. 1). Due to the communities being initially chosen to have different compositions (Fig. S2), community identity was treated as a categorical variable in the analysis. Multivariate analysis of variance found that the starting community composition was the largest source of variation in final community composition (Table 1) and was therefore entered first into the model. The next most significant variables was the time of invasion followed by the number of invader species. Significant interactions were observed between the different communities and increasing the number of invaders, and the time of the invader introduction. Overall, communities destructively sampled at later time points were more similar than communities destructively sampled at earlier time points.

Further multivariate analysis of variance found that the abundance of invader cells persisting in the microcosm had a significant effect on the community dissimilarity of microcosms ($F_{2,121} = 23.9$, $P < 0.001$). This relationship (mean $\pm$ 1 standard deviation throughout) was found to be positive (an increase in dissimilarity of 0.17 ± 0.03 per $\log_{10}$ invader) with greater numbers of invaders having a greater effect on community compositional shifts. Despite the strong impact of multiple invasions on overall community structure, we detected significant changes for only 3 out of 56 OTUs (Fig. S3) the genus *Sphingomonas* (mean increase 5.1×10^5; $P = 0.044$) increased abundance with invasion whereas the genera *Rheinheimera* (mean increase 2.4×10^5; $P = 0.037$) and *Rhodoplanes* (mean decrease 6.7×10^7; $P = 0.010$) changed abundance at the mid-time invader introduction. The result
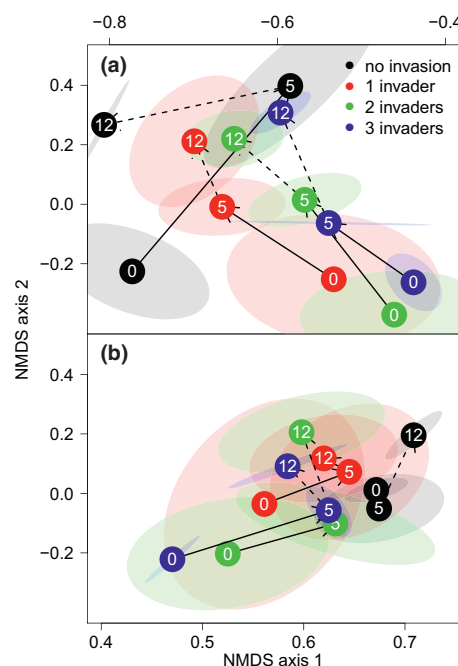

Figure 1 Despite invasion-led differences in composition, communities became more similar over time. Changes in mean NMDS score for community A (a) and B (b) across time and with different numbers of invaders. Shaded ellipses denote the standard deviation of the means, with the numbers representing the days the communities aged prior to invasion. Black lines between the points represent the movement of the mean dissimilarities from 0 to 5 days (solid lines) and 5–12 days (dashed).

Table 1 Multivariate analysis of variance showed highly significant factors in determining the dissimilarity between microcosms

Effect	Pillai's trace	d.f.	F-value	P-value
Community	0.984	2119	3657.6	< 0.001
Time before invasion	0.570	4240	23.9	< 0.001
Invader richness	0.114	6240	2.4	0.028
Community × richness	0.302	6240	7.1	< 0.001
Community × time	0.376	4240	13.9	< 0.001
Richness × time	0.100	12 240	1.1	0.398
Community × richness × time	0.165	12 240	1.8	0.048

implies that invasions consistently impacted the communities, but that the impacts on the underlying OTUs were relatively more variable and therefore more difficult to detect using the sample sizes available.

Invasion success

There was no significant difference in invasion success between the two resident communities (paired-$t_{62} = 0.53$, $P = 0.599$) despite the substantial differences in community composition at the conclusion of the experiment. The timing of invader introduction was found to have a significant effect on the success of the multispecies invasion (Table 2). Invasion success was highest when the invaders were co-inoculated with the community at the start of the growth period (5.61 ± 0.97 $\log_{10}$ invader CFU mL^{-1}). This was significantly higher than

Table 2 Analysis of variance showed the significant impact of time pre-invasion, and richness of invaders, on the total persistence of the three invaders

Factor	Df	*F*-value	*P*-value
Community	1	0.14	0.706
Time before invasion	2	64.96	< 0.001
Invader richness	2	11.04	< 0.001
Time × richness	4	3.25	0.015
Residuals	116		

if invaders were introduced at a mid (4.79 ± 0.31 log$_{10}$ invader CFU mL^{-1}; $p_{adj} < 0.001$) or late (4.98 ± 0.49 log$_{10}$ invader CFU mL^{-1}; $p_{adj} < 0.001$) stage of resident community growth. There was also a significant effect of the richness of the invaders on invasion success (Table 2). These data showed that when a single invader was present the invasion success (5.11 ± 0.89 log$_{10}$ invader CFU mL^{-1}) was lower than if two (5.60 ± 0.83 log$_{10}$ invader CFU mL^{-1}; $p_{adj} < 0.001$) or three (5.62 ± 0.77 log$_{10}$ invader CFU mL^{-1}; $p_{adj} = 0.005$) invaders were present.

We also observed a significant interaction between the successional stage during invasion and invader richness affecting invasion success (Fig. 2; Table 2). When an early invader introduction occurred, single invaders were less successful (5.62 ± 1.22 log$_{10}$ invader CFU mL^{-1}) than two invaders (6.67 ± 0.32 log$_{10}$ invader CFU mL^{-1}; $p_{adj} < 0.001$). However, invasion success was reduced when three-invaders were simultaneously introduced (6.59 ± 0.34 log$_{10}$ invader CFU mL^{-1}) compared with the two-invader treatment. When invaders were introduced at a mid-stage of community growth, the introduction of a single invader (4.72 ± 0.38 log$_{10}$ invader CFU mL^{-1}) was less successful than the introduction of two invaders (4.88 ± 0.23 log$_{10}$ invader CFU mL^{-1}), and the

Figure 2 Persistence of the invaders a week post-invasion at different times and richness levels. Significant differences in the number of invaders counted were observed with increased time prior to invasion ($P < 0.001$). When invasions occurred after the community acclimatised to the environment, richness increased the persistence of the invaders, however, this was at a much lower value than if an early invader introduction occurred. Shaded area represents ± 1 standard deviation.

introduction of three invaders was more successful yet (4.90 ± 0.20 log$_{10}$ invader CFU mL^{-1}). This continual increase was also found with late invader introductions; single invaders (4.98 ± 0.62 log$_{10}$ invader CFU mL^{-1}) had a lower success than pairs of invaders (5.25 ± 0.35 log$_{10}$ invader CFU mL^{-1}), which in turn was lower than invader trios (5.37 ± 0.12 log$_{10}$ invader CFU mL^{-1}).

We assessed the changing effects of selection and complementarity using the Additive Partitions Equation (Loreau & Hector 2001). When the three species were grown in sterile beech leaf media (i.e. when the background community was not present), complementarity between the species increased the overall abundance of the invader cells, whereas the influence of selection effects were negative (Fig. 3a). When the species were invaded into a community, however, a negative complementarity was shown alongside positive selection effects (Fig. 3b–d). This change in the direction of complementarity effects was dependent on the presence of the communities ($F_{1,21} = 50.34$, $p < 0.001$), and increased with the number of invading species from two to three ($F_{1,21} = 16.85$, $P = 0.006$). Successional stage only influenced complementarity significantly in the presence of a community and in microcosms with three invading species ($F_{2,21} = 9.17$, $P = 0.002$). Successional stage was significantly ($F_{1,21} = 9.77$, $P < 0.001$) associated with positive contributions of the selection effects in the presence of the community ($F_{1,21} = 16.14$, $P < 0.001$). Decreasing selection effects were associated with longer times pre-invasion and in three species invasions in the presence of the community ($F_{2,21} = 4.27$, $P = 0.028$).

To better understand the complementarity effects, we characterised the pairwise interactions among the invader species. Pairwise interactions were calculated as the difference between the mean abundance of an invader in monoculture and their mean abundance when invaded with the other invader species. A positive value would indicate that co-invasion was beneficial. We calculated pairwise interactions between each of the three invaders species in the presence of a community and over time. For assemblages grown without the presence of a background community (Fig. 3e), we found that the presence of another species, on average, increased the growth of the invaders (0.67 ± 1.32 log$_{10}$ invader CFU mL^{-1}). This, however, changed when a community was present (Fig. 3f–h), starting negative with a simultaneous invasion (-0.09 ± 0.89 log$_{10}$ invader CFU mL^{-1}) and becoming more negative during early (-0.11 ± 0.25 log$_{10}$ invader CFU mL^{-1}) and late invasions (-0.15 ± 0.30 log$_{10}$ invader CFU mL^{-1}). The change in the mean interaction at each time (Fig. S4), when compared to the mean abundances without a community, indicated that the later the invasion time, the more inhibitive the invaders became towards each other (Kruskal–Wallis rank sum test: $\chi_2^2 = 9.53$, $p < 0.009$).

DISCUSSION

Like global hotspots of plant and animal invasions, microbial communities in natural environments experience a continual, diverse rain of foreign cells, which have the potential to displace native populations and disrupt existing interaction networks (Dawson *et al.* 2017). Multiple, simultaneous invasions
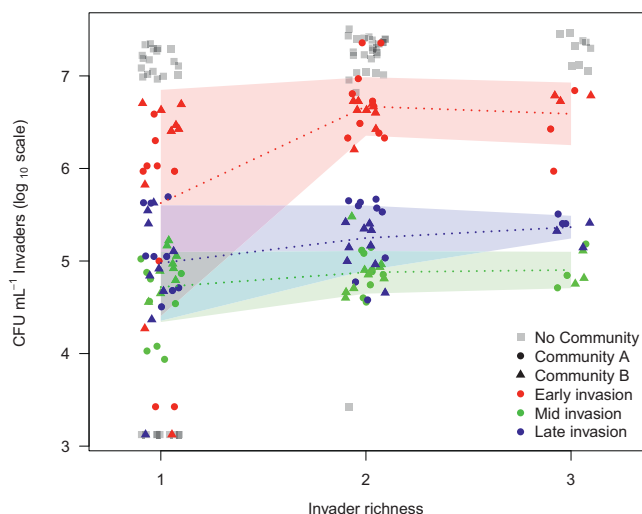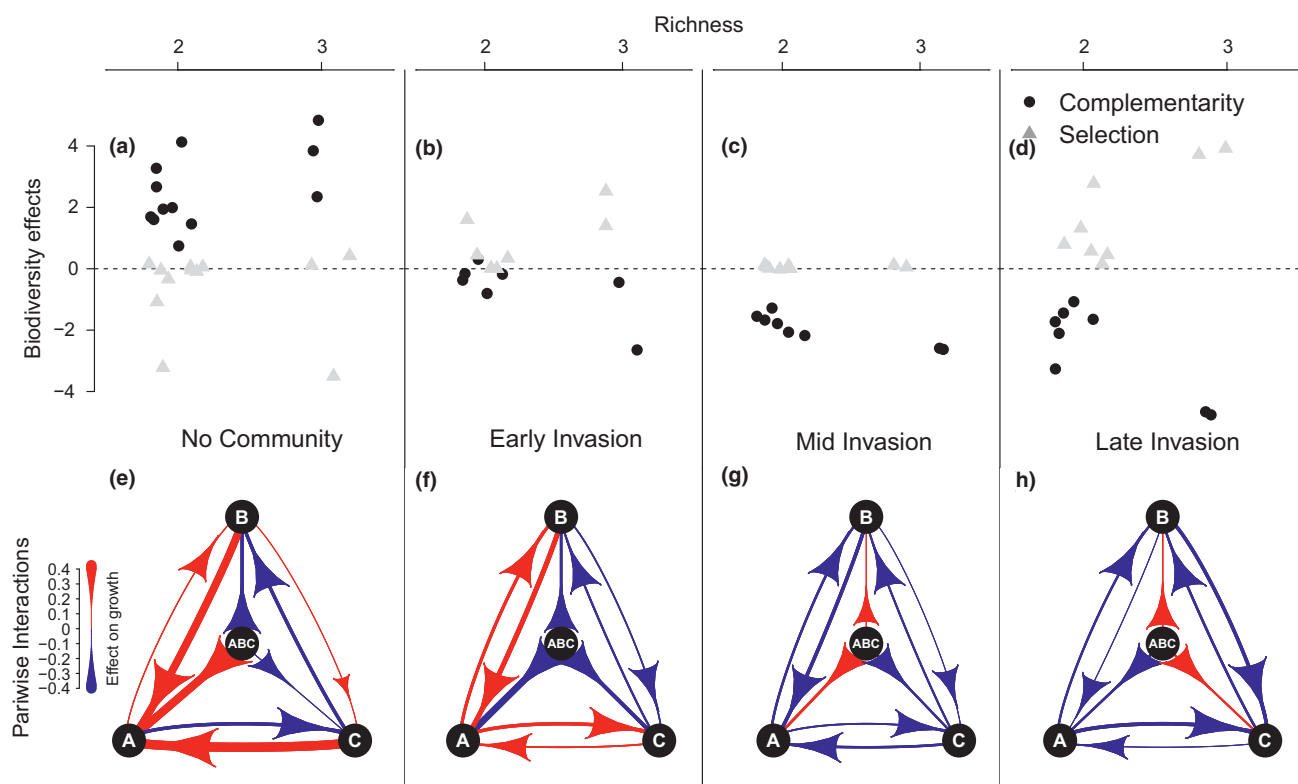
Figure 3 Interactions between the invading species are dependent on the age of the resident community. We used the Additive Partitions Equation to estimate the role of selection effects (grey triangles) and complementarity effects (black circles) on invasion success averaged across the replicate microcosms (a) with no community to invade, or (b) early (c) mid (d) late invasions. We plotted the pairwise interactions among the 3 invasions in the lower panels, showing interactions when there is (e) no community, or (f) early (g) mid (h) late invasions. The thickness of the arrows represents the degree of positive (red) or negative (blue) change in1 growth (log$_{10}$ CFU mL^{-1}) of each species when co-invaded with every other species. The outer arrows are the pairwise invasions, whereas the arrows radiating from the central 'ABC' show the impact of removing any of the 3 species from the 3 species mixture.

are therefore expected to be the rule rather than the exception, yet most studies have conducted invasion experiments using individual invaders. We found that co-invasions of bacteria significantly increased the persistence of the invading species and altered the resident community composition. The results imply that future studies of invasion ecology need to account for interactions among invading cells in order to understand invasion dynamics in natural systems.

We found that invaders had significant impacts on community composition, and that this impact was greater at the earliest invasion time (co-inoculation with the community), in which the persistence of the invaders was highest. Later invasions were less successful and had a lower impact on community composition. A similar finding was reported in previous studies in which compositional shifts only occurred when communities were invaded at early stages or when supplied with additional resources (Symons & Arnott 2014; Mallon et al. 2015). The result is consistent with a role for priority effects, in both bacterial (Tan et al. 2012; Gómez et al. 2016) and studies of larger organisms (Fukami 2015; Pantel et al. 2015); where early invaders are able to utilise existing resources to either monopolise nutrients or to create new niches (e.g. by releasing secondary metabolites), but that such options become closed to them at later stages of succession. Invasion can thus change species abundances early in

succession, and thus the trajectory of succession. Later invaders are required to utilise less favourable resources which could impact less on the species in the community likely because more abundant or more labile resources have already been monopolised (Sanders et al. 2003; Rivett et al. 2016; Taillefumier et al. 2017).

Invader species richness had a significant impact on invader success. Previous studies of invasions of organisms have demonstrated that consecutive invader introductions affect the success of 'secondary invasions,' through interactions between invaders (Preston et al. 2012; O'Loughlin & Green 2015), similar to those observed here. We were able to monitor these interactions among invaders over a time course, by utilising the short-generation times of bacteria, to show that the duration of community development reduced the impact of multiple invaders. When inoculated in a new environment, a community undergoes a period of ecological sorting in which bacterial populations stabilise their niche space through the response to abiotic factors, resource uptake, and the establishment of new biotic interactions (Nemergut et al. 2013). Empirical work with bacteria has shown that, as a community stabilises during succession, strong negative interactions become more neutral (Rivett et al. 2016), and positive interactions are given the opportunity to develop (Lawrence et al. 2012; Coyte et al. 2015). Previous work has shown that

invasion resistance in low diversity microbial communities is determined by community composition (Jousset *et al.* 2011; Jones *et al.* 2017), potentially because of differences in inter-specific interaction networks that develop in different communities (Coyte *et al.* 2015) as predicted from theory (HilleRisLambers *et al.* 2012). Increasing positive interactions within communities during succession have also been observed in plants (Tilman *et al.* 2001), animals (Vermeij 1991), and in theoretical work (Gilpin 1994; Tikhonov 2016). Community development therefore potentially reduces the number of available niches, which is reflected in the lower invasion success over time.

Disturbances to succession that alter species frequencies can have widespread repercussions on the community. These are potentially due to downstream destabilisations of species interaction networks (Sanders *et al.* 2003), which might result in an increase in invasiveness for less competitive invasive partners (Ricciardi *et al.* 2013), or the invasive species becoming part of the resident community after a period of sorting (Donlan & Wilcox 2008). There has been substantial work on interaction networks in microbial communities (Coyte *et al.* 2015; Wei *et al.* 2015), and future work could quantify whether inferred interactions are disrupted for example through changes in abundance of keystone or hub species. Further empirical research should test whether more diverse invader assemblies can break the interaction networks established within resident communities, including under scenarios when there is the simultaneous invasion by whole communities of invaders during community coalescence (Rillig *et al.* 2015, 2016). Whilst it is possible that during invader introductions, that take place at a single time point, the community could be disturbed, the microorganisms may re-stabilise their networks by incorporating the invaders. Successful invasions of larger organisms typically occur once the invasive species has become stably incorporated into the new community. Here, we have simply measured the persistence of the invader, but it would be useful to also understand the extent of incorporation of invaders into bacterial communities. This might involve, for example, measuring whether interactions between the invader and the surrounding community attenuate over time, as has been shown during succession of similar bacterial communities (Rivett *et al.* 2016). Understanding the mechanisms by which invaders are incorporated into a community, thus potentially allowing the long-term persistence that we observe, would aid in gaining a more fundamental understanding of what factors facilitate or hinder invasions, and would also serve to bridge the gap between the understanding of the invasion ecology of micro- and macro-organisms (Balser *et al.* 2006).

Whilst we originally postulated that a successful invader would affect the interactive landscape of the community (Simberloff & Von Holle 1999), resulting in observable complementarity between multiple invaders, our results suggest invasion success is primarily due to selection effects. In addition, invaders are apparently increasingly forced into a narrower range of strategies, resulting in the increasingly negative interactions among invader species over time, and the increasing importance of selection effects rather than complementarity effects in later stages of succession. The result implies that

the negative interactions among multiple invaders, frequently observed in communities of larger organisms, could be due to those studies focusing on late-successional communities. Our work leads to the prediction that studies of early successional communities or perturbed communities might yield a greater number of positive interactions among multiple invasions. It also raises the possibility for successive invaders to benefit from positive feedbacks as the community becomes increasingly perturbed.

In conclusion, multi-species invasions increase overall invasion success relative to single species invasions due to selection effects. Furthermore, the inclusion of successively more invaders causes the final composition to shift more away from the 'normal' (no invasion) trajectory, although limited exclusion of existing members was observed. We also demonstrate that early invader introductions have a greater impact upon the composition of the community and allow greater persistence of invasive species in species-rich bacterial communities. These results present an interesting dichotomy from studies looking at larger organisms; bacterial communities appear to be more resilient to multiple invaders, retaining the same successional trajectories, compared to communities of larger organism that can become substantially altered (Johnson *et al.* 2009). This disconnect may be due to the different numbers of generations, and levels of diversity, achievable by micro- and larger organisms observed during an experiment. However, in agreement with previous ecological studies (Buřič *et al.* 2009; Johnson *et al.* 2009; Hudina *et al.* 2011; Preston *et al.* 2012) we demonstrate the importance of considering the resilience of the community composition, and the interactions between the invaders, as a result of invasion. This aspect of invasion ecology is crucial in both micro- and macro-communities and should be accounted for before being able to predict the outcome of multiple invasion events.

AUTHORSHIP

DWR, JR and MLJ conceived the experiment. DWR designed and executed the experiment with help from SBM. JR conducted extensive pilot experiments. SBM and ER generated the community composition data. DWR analysed the data and wrote the first draft of the manuscript which was completed by all authors.

DATA ACCESSIBILITY STATEMENT

Data available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.m5r87

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