The article title: Cooperative interactions between invader and resident microbial community members weaken the negative diversity-invasion relationship

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Abstract

The negative diversity-invasion relationship observed in microbial invasion studies is commonly explained by competition between the invader and resident populations. However, whether this relationship is affected by invader-resident cooperative interactions is unknown. Using ecological and mathematical approaches, we examined the survival and functionality of *Aminobacter niigataensis* MSH1 to mineralize 2,6-dichlorobenzamide (BAM), a ground-water micropollutant affecting drinking water production, in sand microcosms when inoculated together with synthetic assemblies of resident bacteria. The assemblies varied in richness and in strains that interacted pairwise with MSH1, including cooperative and competitive interactions. While overall, the negative diversity-invasion relationship was retained, residents engaging in cooperative interactions with the invader had a positive impact on survival and functionality, highlighting the dependency of invasion success on community composition. No correlation existed between community richness and the delay in BAM mineralization by MSH1. The findings suggest that the presence of cooperative residents can alleviate the negative diversity-invasion relationship.

Introduction

Defined as the establishment of non-native microbe in a resident community, invasions constitute fundamental processes in microbial ecology (Kinnunen et al. 2016; Mallon et al. 2015a). The management of microbial invasion receives increasing interest to avoid invasion as in the case of pathogens but also to promote invasion as in the case of probiotics, biocontrol agents, biofertilizers, and bioremediation agents (Mawarda *et al.* 2020). An improved understanding of the factors that determine a strain's invasive potential and a community's resistance to invasion is required, encompassing not only abiotic factors and environmental filtering, but also biological factors and ecological mechanisms.

In analogy with ecological factors that govern invasion in macro-organism systems, the diversity of the resident community is proposed as a determining factor in microbial invasion (Van Elsas et al. 2012; Elton 1958; Hodgson et al. 2002; Tilman et al. 2004). The success of an invader in a microbial community often decreases with increasing resident community diversity, although this relationship is more nuanced with changes in nutrient conditions or other confounding factors (Ferreira *et al.* 2021; Mawarda *et al.* 2022a, b; De Roy *et al.* 2013). As in macro-species communities, the negative diversity-invasion relationship in microbial communities is commonly linked with the concept of resource competition (Mallon *et al.* 2015b; Wei *et al.* 2015; Yang *et al.* 2017). Each resident occupies a distinct and specialized niche, resulting in a diverse tapestry of individuals with unique resource utilization capabilities (Hardin 1960). Consequently, in diverse communities, resource availability is limited for invaders due to efficient resource utilization and exploitative competition by residents. This scarcity creates a stronger resistance barrier compared to less diverse communities (Davis *et al.* 2000; Mallon *et al.* 2015b; Tilman *et al.* 2004). Additionally, differences in resource usage, resource utilization patterns, and growth rates between

the invader and residents will influence resource competition strength at each diversity level (Mawarda *et al.* 2022b).

However, in microbial communities, in addition to exploitative competition, other direct social interactions exist including interactions beneficial and detrimental to a population's fitness. Direct interactions that hinder a population's fitness are termed interference competition and could arise from the production of antibiotic compounds, the disruption of signaling cascades, or predation (Mawarda *et al.* 2020, 2022c). Interactions that enhance a population's fitness are referred to as cooperative interactions, for instance through the exchange of metabolites or sharing of common goods (Little et al. 2008; Moons et al. 2009). Cooperative interactions can take the form of commensalism (where one partner benefits) or mutualism (where both partners benefit) (Moons *et al.* 2009; Morris *et al.* 2013; West & Cooper 2016). In invasion microbiology, beneficial interactions among resident community members were shown to improve the invaders' fitness and facilitate invasion (Li et al. 2019), while computationally modelling suggested that cooperation can support invasion in the context of community coalescence (the phenomenon when microbial communities invade one another as a whole) (Lechón-Alonso *et al.* 2021). However, even though Mawarda et al., (2020) has conceptualized that cooperation between residents and invaders could be a driving mechanism underlying the success of microbial invasion, experimental evidence is lacking.

Efforts studying how direct social interactions affect the diversity invasion relationship, particularly cooperative ones between an invader and residents, are crucial for understanding the ecology and management of microbial invasions, as they may assist in the establishment of pathogen infections or support beneficial microbial inoculants (Duan *et al.* 2003; Vandermaesen *et al.* 2022; Whiley *et al.* 2015). However, this can only be explored when the individual effect of

each resident on the invaders' behavior, is known. Yet, such studies are scarce, as most microbial invasion studies used a top-down approach, employing a natural resident microbial community to investigate the diversity-invasion relationship (Van Elsas *et al.* 2012; Mallon *et al.* 2018; Vivant *et al.* 2013), likely obscuring effects of cooperative and interference competition.

Through a combination of ecological and mathematical approaches, this study examines the net effect of direct social interactions on microbial invasion, using synthetic communities with known pairwise interactions between invader and residents, at different initial diversity levels. We hypothesized that cooperative interactions between the invader and the resident, in a form of mutualism and/or commensalism, affect the diversity invasion relationship. Recently, Vandermaesen et al., (2022), examined how pair-wise interactions between *Aminobacter niigataensis* MSH1, capable of mineralizing 2,6-dichlorobenzamide (BAM), and 13 heterotrophic bacterial strains isolated from sand filters exploited in drinking water treatment plants (DWTPs), affected the MSH1 BAM mineralizing functionality and MSH1 cell densities. BAM is a common groundwater micropollutant in Europe and a threat for drinking water production, while MSH1 is used as a bioremediation agent for bioaugmentation of DWTP sand filters to avert BAM pollution (Albers *et al.* 2015; Horemans *et al.* 2017). Hence, MSH1 can be considered as an invader and the sand filter isolates (SFI) as residents of the target environment of MSH1. Though resource competition accounted for 70% of the observed effects, there were also signs of interference competition. Moreover, two SFI enhanced the functionality of MSH1 to mineralize BAM while MSH1 increased the SFIs density, suggesting mutual cooperation. To test our hypothesis, we examined how the BAM mineralization functionality and survival of *A. niigataensis* MSH1, was affected when inoculated together with different synthetic combinations of the SFI strains at increasing initial richness (up to richness 13) in sand microcosms. Each SFI was previously shown

to either positively or negatively affect MSH1 functionality in pair-wise assemblies (Vandermaesen *et al*. 2022).

Materials and methods

Bacterial strains and culture conditions

Used SFI were *Rhodococcus* sp. K27, *Acidovorax* sp. K52, *Aeromonas* sp. K62, *Paucibacter* sp. K67, *Pelomonas* sp. K89, *Rhodoferax* sp. K112, *Rhodoferax* sp. K129, *Piscinibacter* sp. K169, *Acidovorax* sp. S9, *Undibacterium* sp. S22, *Brachybacterium* sp. S51, *Mesorhizobium* sp. S158 and *Acidovorax* sp. S164 originating from two DWTP sand filters (Vandermaesen *et al.* 2017). A GFP tagged variant of *A. niigataensis* MSH1 was used (Sekhar *et al.* 2016). All strains were cultured as described (Vandermaesen et al. 2017).

Microcosm set-up

Sand microcosm experiments were performed and synthetic communities were constructed at varying richness levels by combining SFI with MSH1 as described (Vandermaesen *et al*. 2022). Richness (R_{SFI}), meaning the number of SFI species inoculated, ranged from 1 to 13. Testing all possible combinations of SFI from the lowest (R_{SFI}=1) to the highest (R_{SFI}=13) richness would imply 8191 tests (without replicates), which is practically infeasible. To reduce the number of tested combinations, a combinatorial experimental design strategy was applied, based on covering arrays. This sampling method guarantees a certain coverage of the space of experimental conditions, requires a relatively small number of experiments (Shasha *et al.* 2001) and was used before in microbial studies (Kerr & Churchill 2001; Shasha *et al.* 2001). The rationale is testing all possible factor interactions in order to discover interesting or exceptional behavior. The results of

such an experiment may demonstrate strong correlations between factors in a more efficient way than random sampling. These correlations are not definitive, because of confounding factors, but rather suggestive about the factors that are most important for the system behavior (Colbourn & McClary 2008). In our case, the factors correspond to the individual SFI. Hence, there were 13 factors with two settings each (presence or absence). The interactions between the factors were the concurrent presence or absence of the SFI at varying richness or, in other words, the different combinations of factor settings.

The use of a covering array allowed testing all interactions between two, three and four SFI with 49 tests at $2 \leq R_{SFI} \leq 11$. Additionally, three series of tests were created from R_{SFI}=5 to R_{SFI}=12 by starting with a random combination of five SFI and successively adding individual SFI up to R_{SFI}=12, resulting in 24 additional tests. As controls, MSH1 without SFI (R_{SFI}=0), the 13 combinations at R_{SFI}=1 and the one combination at R_{SFI}=13 were included. Four remaining tests at $2 \leq R_{SFI} \leq 4$ were chosen randomly as internal controls. Table S1.1 shows the compositions of all tested synthetic communities at $1 \leq R_{SFI} \leq 13$. All tests were replicated four times.

MSH1 and SFI were combined in MMO medium (Dejonghe *et al.* 2003) containing 150 µg/L Na-acetate at final densities of 10^7 cells/mL of each community member. The resulting assemblages were inoculated (100 µL) into 150 mg sterilized sand microcosms inside 2 mL deep 96-well plates and incubated at 20°C for 7 days, allowing interactions between MSH1 and SFI to occur as previously shown (Vandermaesen *et al.* 2022). This time period (designated competition phase) coincided with the duration used by Vandermaesen *et al.* (2022) and was selected since the number and functionality (measured as specific BAM degradation rate) of MSH1 cells declined between 5 and 11 days after inoculation in laboratory and pilot sand filter systems (Albers *et al.* 2015b; Horemans *et al.* 2017a). MSH1 and the SFI were previously shown to colonize the microcosms

reaching cell densities of 10^6-5.10^8 cells/mL when individually inoculated (Vandermaesen *et al.* 2022).

Determining MSH1 survival and functionality

After the competition phase, the survival and the BAM mineralization functionality of MSH1 were determined. The number of MSH1 cells surviving the 7-days period (S_{MSH1}), was examined by selective plate counting as described (Vandermaesen et al. 2022). BAM mineralization was determined by spiking the microcosms with 150 µg/L [Ring-U-^{14}C]-labeled BAM (Izotop, Budapest, Hungary) and monitoring of $^{14}CO_2$ production for 130 hours as described (Johnsen et al. 2009). Cumulative mineralization curves were generated by plotting the cumulative percentage of $^{14}CO_2$ in function of time and were described with the modified Gompertz model:

$$P\,(t) = A\ \exp\left(-\exp\left(\frac{\mathrm{e}}{A}(\mu\lambda - (\mu - c)t) + 1\right)\right) + \ c.t\ ,$$

where P (%) is the percentage mineralization at time t (h), A (%) the total extent of mineralization after the exponential mineralization phase, λ (h) the lag time, μ (%.h^{-1}) the maximum mineralization rate constant and c (%.h^{-1}) the endogenous mineralization rate constant. Kinetic parameters λ and μ were derived to determine the nature of interactions between the SFI and MSH1 as reported (Vandermaesen *et al.* 2017). Positive interactions were classified as those leading to a decrease in λ and/or increase in μ at a 95% significance level. Negative interactions were those that significantly increased λ and/or decreased μ. Interactions that did not affect λ and μ were categorized as neutral. A was not taken into account as the carbon of BAM can be both converted to CO_2 and assimilated into biomass, and it remains unclear how different conditions influence each fraction. Furthermore, in matrices with a continuous water flow as in sand filters, most crucial

is the invader's capacity to grow rapidly and establish itself, as indicated by μ and λ. Values of c were always close to zero and disregarded for further analysis.

Data analysis

To assess the effect of SFI diversity on invasion, the Pearson correlation coefficient (r) (Cohen *et al.* 2009) was used to examine correlations between R_{SFI} and λ, μ, and S_{MSH1}. Moreover, the average values of λ, μ, and S_{MSH1} per R_{SFI}-level (denoted as $\bar{\lambda}$, $\bar{\mu}$, and $\overline{S_{MSH1}}$, respectively) were compared to those of the control microcosm with R_{SFI}=0. To enable comparison between parameters with varying scales, the data for λ, μ, *and* the natural logarithm of S_{MSH1} were standardized by transforming them into z-scores. This was done by subtracting their respective means and dividing by the standard deviation of the parameter values determined for the microcosms containing only MSH1 (R_{SFI}=0, 16 replicates). Hence, the standardized parameter values, denoted as λ_s, μ_s, and S_s, are distributed with a mean of 0 (which is the value of λ_s, μ_s, and S_s at R_{SFI}=0) and a standard deviation of 1. Variability of the parameter values was then evaluated as the difference between the maximum and minimum value of λ_s, μ_s, and S_s, per R_{SFI}-level.

To investigate whether the dominant effects of individual SFI persisted at $R_{SFI} \geq 2$, we calculated the D-values for λ (D_λ), μ (D_μ) and S_{MSH1} (D_S) per R_{SFI}-level, defined as the difference between averages of standardized parameter values MSH1 survival (S_s), lag time (λ_s), and maximum mineralization rate (μ_s) obtained from communities in which a specific SFI was present and those where it was absent. The averages of standardized parameter values for a specific R_{SFI}-level and SFI were calculated by dividing the λ_s, μ_s, and S_s values for that R_{SFI}-level by the number of assemblies in which that specific SFI was present (denoted as $\lambda_{s,P}$, $\mu_{s,P}$, and $S_{s,P}$) or absent (denoted as $\lambda_{s,A}$, $\mu_{s,A}$, and $S_{s,A}$). Corresponding D_λ-values were then defined as $\lambda_{s,P}$ - $\lambda_{s,A}$, D_μ as μ_s - $\mu_{s,A}$ and D_S as $S_{s,P}$ - $S_{s,A}$. If the presence of a particular SFI strain in synthetic communities would have a

dominantly increasing or decreasing effect on parameter values, even at high R_{SFI}, D_λ, D_μ, and/or D_S would be consistently positive or negative for all R_{SFI}-levels, with high absolute values. The consistency of a SFI strain's effects was calculated as the number of R_{SFI}-levels for which D_λ, D_μ, or D_S was either positive or negative, respectively, relative to the total number of R_{SFI}-levels for which D_λ-, D_μ-, and D_S-values were available. Moreover, the strength with which a strain affected parameter values, was evaluated based on the average values of D_λ, D_μ, or D_S for R_{SFI}=2 to R_{SFI}=12 (denoted as $\overline{D_\lambda}$, $\overline{D_\mu}$, and $\overline{D_S}$).

To further identify which strain has the most significant impact on MSH1 fitness in case two SFI with contrasting effects were present, we compared communities containing particular pairs of two SFI - one positively affecting MSH1 and one negatively affecting MSH1 - with communities where only one of those two SFI was present, by evaluating the overruling effect. To this end, average standardized λ-, μ-, and S_{MSH1}-values were first calculated per R_{SFI}-level for communities in which the pair was present ($\lambda_{s,pair}$, $\mu_{s,pair}$, and $S_{s,pair}$). When the $\lambda_{s,pair}$, $\mu_{s,pair}$, and $S_{s,pair}$ were statistically different from the respective $\lambda_{s,P}$, $\mu_{s,P}$, and $S_{s,P}$ derived for systems in which only one of the two considered SFI was present, it was determined that the additional SFI present in the system containing the pair overruled the impact of the other SFI. All parameter values were compared between different R_{SFI} levels by ANOVA and post hoc Tukey tests, to examine significant differences (95% significance level) using Mathematica (version 10.0, Wolfram Research, Champaign, IL, USA).

Results

Relationship between initial resident community richness and MSH1 performance

The correlation analysis showed that increasing species richness of the sand filter community R_{SFI}, significantly declined both MSH1 population density S_{MSH1} (R^2=-0.46, p<0.001) and maximum BAM mineralization rate μ (R^2=-0.46, p<0.001) (Figure 1). In the case of S_{MSH1} this negative relationship holds true only for R_{SFI}=0 to 3, since the trend seems to weaken when more than 3 SFI were present. Moreover, the average values of both parameters per R_{SFI}-level ($\bar{\mu}$ and $\overline{S_{MSH1}}$) determined for assemblies with R_{SFI}-level ≥1, were lower compared to the control microcosm with R_{SFI}=0 (Tukey's post hoc, p<0.05). In contrast to S_{MSH1} and μ, there was no significant correlation between R_{SFI} and lag time λ (Figure 1a, R^2<0.1, p>0.05). Although the average λ values per R_{SFI}-level ($\bar{\lambda}$) in the assemblies with R_{SFI}>0 were higher than the control (Tukey's post hoc, p<0.05), $\bar{\lambda}$ values did not differ for assemblies with R_{SFI} ≥1 (Tukey's post hoc, p>0.05). These findings suggest a negative biodiversity-invasion relationship, particularly concerning MSH1 survival and maximum BAM mineralization rate. Yet this effect was less pronounced for the BAM mineralization lag time.

If the survival (S_{MSH1}) and BAM mineralization functionality (λ, μ) of MSH1 were influenced by the specific type of SFI present, - meaning by the community composition, - it would lead to deviations from the overall trends observed between R_{SFI} and λ, μ and S_{MSH1}. Consequently, there would be a high variability of the S_{MSH1}, λ and μ parameters for each R_{SFI}-level. The results showed that aside from diversity, the community composition indeed affected the functionality of MSH1, especially regarding λ (Figure 2). Between R_{SFI}-values of 2 and 4, the variability of all three parameters decreased compared to R_{SFI}=1, mainly due to the limited number of tested synthetic

communities (Table S1.1). As R_{SFI} increased from 5 to 8, differences in variability were observed among the parameters. In that particular R_{SFI}-range, the variability for μ and S_{MSH1} was similar while the variability for λ was generally higher. When R_{SFI} exceeded 9, the variability became similar for all three parameters.

Identification of SFI that dominantly affect MSH1 performance

To identify SFI strains that dominantly affect MSH1 performance, we calculated the *D*-values (i.e., the difference between standardized MSH1 survival, lag time, and maximum mineralization rate obtained from communities where a specific SFI was present and those where it was absent) for each SFI, spanning from R_{SFI}=2 to R_{SFI}=12 and examined whether the *D*-values consistently showed positive or negative values for the considered R_{SFI}-levels. Moreover, the strength with which a strain affected the three parameter values, was evaluated based on the average values of D_λ, D_μ, or D_S for R_{SFI}=2 to R_{SFI}=12, i.e., $\overline{D_\lambda}$, $\overline{D_\mu}$, and $\overline{D_S}$. Effects of each individual resident SFI on invader MSH1 for R_{SFI}=1 were highly similar to those observed by Vandermaesen *et al.* (2022) and hence highly reproducible between experiments (Document S2; Figures S2.1 and S2.2), for example, strains S9 and S158 displaying negative interactions with MSH1, whereas strains S51 and K169 positively impacted MSH1 performance.

Based on the *D*-values for R_{SFI}=2 to R_{SFI}=12, for most SFI, D_λ, D_μ and D_S-values were either small or not consistently positive or negative across the entire R_{SFI} range suggesting that the presence of those SFI generally did not exert a dominant influence on the survival and functionality of MSH1. Interestingly, the dominant effect of several SFI at R_{SFI}=2 did not persist in higher richness systems (i.e., at R_{SFI}>2). For example, even though strains S164 and K52 exerted strong negative effects on BAM mineralization at $R_{SF}\leq$2 (Vandermaesen et al 2022), these strains did not maintain the same effect at higher richness levels as their $\overline{D_\lambda}$-, $\overline{D_\mu}$- and $\overline{D_S}$-values indicated weak negative effect to no

effect (Table 1). Otherwise, the negative effects on MSH1 fitness by strains K67 and K112 surprisingly persisted when R_{SFI} increased despite their weak competitiveness at R_{SFI}=2 (Vandermaesen et al 2022) and the two strains showed relative low $\overline{D_\lambda}$-values and high $\overline{D_\mu}$- and $\overline{D_S}$-values (Figures S3.1, S3.2 and S3.3, Table 1).

However, several strains showed rather consistently positive effects. D_λ was consistently negative and D_μ was consistently positive for strains K27, K169 or S51 over the entire R_{SFI}-range. The $\overline{D_\lambda}$- and $\overline{D_\mu}$-values calculated for those strains were strongly negative and strongly positive respectively, when compared to other SFI, suggesting positive effects when one of these strains was present (Table 1; Figure S3.1 and S3.2). Moreover, highly positive D_S-values were observed in the presence of K27 at all considered R_{SFI}-levels leading to a high $\overline{D_S}$ for K27 (Table 1; Figure S3.3). D_μ was also consistently positive among all R_{SFI}-levels when strain K62 was present leading to a relatively high $\overline{D_\mu}$ for K62 (Table 1; Figure S3.2).

Other strains showed consistently negative effects on MSH1 fitness over the R_{SFI}-levels. For instance, S158 showed consistently a positive D_λ (longer lag time) and negative D_μ (lower maximum mineralization rate) at almost all R_{SFI}-levels, leading to a relatively high $\overline{D_\lambda}$ and low $\overline{D_\mu}$ (Figures S3.1 and S3.2, Table 1). S9 showed consistently negative D_s-values leading to the highest $\overline{D_S}$ of all SFI (Table 1, Figure S3.3). Moreover, despite more variable D_λ and D_μ among the R_{SFI}-levels, S9 showed a relatively high $\overline{D_\lambda}$ (Tukey's posthoc, $p < 0.05$) (Figures S3.1 and S3.2, Table 1). Our findings conclude that several SFI and especially K27 and K169 retained their dominant effects on MSH1 when species richness of the resident community increased (

Table 1).

Cooperative resident strains overrule strains with strong negative effects on MSH1 performance

To further identify SFI strains that had the most significant impact on MSH1 fitness in case two SFI with contrasting effects were present, we compared communities containing one SFI that strongly positively affected MSH1 and one SFI that strongly negatively affected MSH1, with communities where only one of those two SFI was present. This was done by evaluating their overruling effects, comparing $\lambda_{s,pair}$, $\mu_{s,pair}$, and $S_{s,pair}$ with respectively $\lambda_{s,P}$, $\mu_{s,P}$, and $S_{s,P}$ at the different S_{SFI} levels. Selected SFI with strong positive effects were K27 and K169, while S9 and S158 were selected as SFI with strong negative effects on MSH1. Hence, reported results concern assemblies containing the pairs K27/S158, K169/S158, K27/S9 and K169/S9 (

Figure 2). Overruling effects of individual SFI decreased as diversity increased, given similar values for $\lambda_{s,P}$, $\mu_{s,P}$, and $S_{s,P}$ among strains regardless their effect, and hence no significant differences with $\lambda_{s,pair}$, $\mu_{s,pair}$, and $S_{s,pair}$ (ANOVA, $p>0.05$). However, when $R_{SFI}<10$, positive effects of K27 and K169, particularly on lag time λ, overruled the negative effects of S158 or S9, as $\lambda_{s,pair}$-values were lower than $\lambda_{s,P}$-values derived from communities containing either S158 or S9 (

Figure 2). This aligns with the lower $\overline{D_\lambda}$-values calculated for K27 and K169 compared to S9 and S158 (Tukey's post hoc, $p<0.05$) (

Table 1). The lower $\overline{D_\lambda}$, the stronger the effect of the corresponding strain on MSH1, the more likely this effect will overrule that of other strains. In terms of maximum rate of BAM mineralization, the positive effect of strain K27 overruled the negative effect of S158 when R_{SFI} <9, showing $\mu_{s,pair}$-values that were higher than the $\mu_{s,P}$-values derived from communities containing S158 (Tukey's post hoc, $p<0.05$) (Figure 3). Strain K27 also showed the highest $\overline{D_\mu}$ among all SFI, reflecting its overruling positive effect (Table 1). For the MSH1 survival S_{MSH1}, the positive effect of K27 and K169 overruled the negative effect of S158 when R_{SFI} <9, as their $S_{s,pair}$-values were higher than $S_{s,P}$-values of communities containing S158 (Tukey's post hoc, $p<0.05$) (Figure 3). This is also in line with $\overline{D_S}$ being highest for K27 and K169 (Tukey's post hoc, $p<0.05$) (Table 1). Finally, the positive effects of K27 and K169 were stronger than the negative effects of S9 and S158 on MSH1's survival/functionality.

Discussion

In the framework of microbial invasion, the well-established negative relationship between diversity and invasibility has centered on resource competition. We postulated that this dynamic could shift when, additional to competitive interactions, cooperative interactions exist between invader and residents (Mawarda et al. 2020). Therefore, by using ecological and mathematical approaches, we evaluated the survival and functionality of the bioremediation agent *A. niigataensis* MSH1 when inoculated together with bacterial isolates native to its target environment - i.e., sand filters exploited in DWTPs - at increasing initial richness (R_{SFI}). The isolates exhibited known pair-wise effects on MSH1 including cooperative interactions (Vandermaesen *et al.* 2022).

Community composition influences the biodiversity-invasion relationship

This study confirms previous findings of microbial invasibility negatively correlating with increasing species richness in soil (Van Elsas *et al.* 2012; Jousset *et al.* 2011; Mallon *et al.* 2015b), in surface water (Van Nevel *et al.* 2013) and in wastewater (Cook *et al.* 2006) as the survival of MSH1 (S_{MSH1}) and the maximum rate (μ) of BAM mineralization negatively correlated with SFI richness (R_{SFI}). However, the lag time of BAM mineralization (λ) was highly variable but did not correlate with R_{SFI}. The high variability of λ and μ values per R_{SFI}-level indicates that the BAM mineralizing functionality of MSH1, largely depends on the composition of the community, rather than species richness. Thus, the biodiversity-invasion relationship is besides species richness, influenced by resident community composition. This is in line with previous findings showing that different community structure affects invasion differently (Mawarda et al. 2022a,c,b). The prevailing theory of microbial invasion builds upon the presence of an available ecological niche for invaders to exploit and the competition for that niche with resident community members (Ives & Carpenter 2007; Shade *et al.* 2012). The negative diversity-invasion relationship is explained through the concepts of niche complementarity and the sampling effect (Eisenhauer *et al.* 2013; Hodgson *et al.* 2002; Mallon *et al.* 2015a; Mawarda *et al.* 2022a; Shade *et al.* 2012). Niche complementarity proposes that increased community diversity involves more species occupying more niches, increasing the likelihood of niche overlap with invaders (Elton 1958; Stachowicz JJ & Tilman D 2005). The sampling effect theory suggests that a more diverse community has a greater probability of containing residents dominantly impacting the invader (Fargione & Tilman 2005). Our observation that invasion is predominantly influenced by community composition, aligns with the concept of the sampling effect. Related to the negative biodiversity-invasion relationship, dominant SFI species might exert negative effects on invasion, occupying the same niche as the invader and exhibiting higher growth rates resulting in exploitative competition. Alternatively, this might be SFI strains that exert interference competition towards MSH1, for

instance by producing antagonistic compounds (Ghoul & Mitri 2016; Jensen 1987). Vandermaesen *et al.* (2022) previously showed that the survival and functionality of MSH1 in pair-wise systems is largely explained by the intrinsic exploitative competitiveness of its SFI opponents with MSH1, while SFI *Acidovorax* sp. S9 was suggested to exert interference competition towards MSH1.

Interactions between the SFI affect MSH1 performance in higher richness assemblies.

Interestingly, several SFI that showed dominant effects at R_{SFI}=2 did not continue to show that effect in higher richness systems (R_{SFI}>2), as observed for *Acidovorax* sp. strains S164 and K52, two strains that give strong negative effects on BAM mineralization at $R_{SFI}\leq 2$. Additionally, negative effects of *Paucibacter* sp. K67 and *Rhodoferax* sp. K112 on MSH1 fitness surprisingly remained as R_{SFI} increased, despite their weak competitiveness and weak dominant behavior at R_{SFI} =2. These observations are indicative of the occurrence of non-transitive interaction networks such as rock-paper-scissors interactions (Allesina & Levine 2011; Hibbing *et al.* 2010; Kerr *et al.* 2002) or higher-order interactions (HOI) in which a third species modifies the interactions between a species pair (Mayfield & Stouffer 2017) within the higher richness assemblies. Also Li et al. (2019) showed that interactions among residents affect invasion. Further information on the pairwise interactions among the SFI and the use of pairwise models to predict co-existence in the higher richness systems, eventually supported by high throughput community sequencing to recover relative densities of each member, can further substantiate this notion and distinguish non-transitive interaction networks and HOI (Friedman et al. 2017; Guo & Boedicker 2016; Venturelli et al. 2018; Li et al. 2019). Moreover, information concerning the molecular mechanisms underlying cooperative and antagonistic interactions among SFI as well as among SFI and MSH1, can be incorporated in mechanistic models to capture and predict community dynamics. Although such information is still challenging to identify for multi-species systems, progress in multi-omics

and single-cell omics provide opportunities (Liu *et al.* 2021; Mauger *et al.* 2022; Momeni *et al.* 2017).

Cooperative interactions alleviate the biodiversity-invasion relationship

Remarkably, although effects diminished at higher R_{SFI}, the presence of strains like K27 and K169, dominantly influenced invasion. Moreover, both K27 and K169 overruled the strong negative effects of S9 and S158 across species richness levels. The overall dominant effect of K27 and K169 over other SFI explains the high dependence of λ and μ on community composition, and likely the lack of correlation between R_{SFI} and λ, i.e., their presence counteracted the negative effect of increasing richness. K169 was previously shown to cooperatively interact with MSH1 (Vandermaesen *et al.* 2022). Our findings suggest that the presence of suitable ecological niches can be influenced by cooperation with residents. For instance, when residents turn a substance that the invader cannot directly use into accessible compounds, new ecological niches for the invader arise. The significance of synergism and cooperative interactions was highlighted before. Li et al. (2019) showed that pairwise interactions facilitated the invasion potential of the plant-pathogen *Ralstonia solanacearum*. However, their study limited to interactions between residents, without encompassing interactions between residents and invader, and did not dwell into the biodiversity-invasion relationship. Moreover, previous studies studying the biodiversity-invasion relationship (Eisenhauer *et al.* 2013; Van Elsas *et al.* 2012; Jousset *et al.* 2011; Mallon *et al.* 2015b, 2018; Mawarda *et al.* 2022a) focused on the invader survivability rather than its functionality. The current study fills this knowledge gap and reveals that pairwise cooperative interactions between invader and residents might alleviate the negative biodiversity-invasion relationship, affecting both the invader's densities and functionality. How K27 exerts its positive dominant effect in higher-richness systems is less clear as K27 showed mild negative effects on MSH1 in pairwise systems

(Vandermaesen *et al.* 2022) but is likely explained by strong negative interactions towards other SFI.

However, our study takes into account the initial presence or absence of the SFI without considering their abundance dynamics during and after the competition phase, nor does it perform invasion of an established community. While the same approach was used by others to study invasion-community diversity relationships (De Roy *et al.* 2013), it comes with some remarks. Although all SFI were inoculated at the same density and reach densities at 10^7-10^8 cells/mL when inoculated alone (Vandermaesen *et al.* 2022), the proportions may change after the competition period, while interactions might be density-dependent (Katsuyama *et al.* 2009). Moreover, final densities of each strain might depend on the initial richness and on among-SFI fitness differences (Katsuyama *et al.* 2009). We also did not invade an established community. Not only might the proportions of the SFI change upon assembly but the assembly would have been independent from MSH1, possibly resulting in different interactive forces within the assemblage compared to when MSH1 was present. Additionally, the sand in the microcosms can act as a substratum for biofilm formation and result into spatial segregation. Although MSH1 was shown to invade established biofilms of native sand filter communities (Horemans *et al.* 2017b), biofilm formation and spatial segregation can affect invasion and microbial interactions (Nadell et al. 2015; Wu et al. 2023).

Consequences for applying MSH1 to remove BAM in DWTP sand filters

While this study examines an ecological-driven hypothesis, it also provides new insights into the management of invasion for biotechnology and specifically for using MSH1 as a bioremediation agent to treat BAM pollution in DWTPs. Many bioremediation agents fail to maintain high population densities and functionality upon introduction in the target environment and biotic

interactions with resident bacteria are a possible explanation (Albright *et al.* 2022; Kaminsky *et al.* 2019). Microbial invasion studies examining biotic interactions, rarely used true candidate bioremediation agents and/or communities native to the target environment. This study not only examined the cooperative interactions that potentially foster the inoculant density and functionality but also used a bioremediation agent and residents of its target environment as a model. Our results show that the presence of SFI strains like K27 and K169, might facilitate establishment of MSH1 in DWTP sand filters as suggested by the reduction in lag time when they were present, even at higher richness. In addition, previous research showed that the BAM degrading activity of MSH1 in DWTP sand filters decline over time (Albers *et al.* 2015b; Horemans *et al.* 2017a). Our study shows that the presence of K169 and K27 supports the preservation of MSH1's fitness even at high R_{SFI} levels. This reinforces the idea to use specific residents of the target environment as co-inocula for prolonging MSH1's viability and functionality as previously proposed for K169 based on its cooperative pairwise interactions with MSH1 (Vandermaesen *et al.* 2022). The support of growth of a lower fitness micro-organism by commensals was reported before (Morris *et al.* 2013).

Otherwise, we realize that the experimental environment in this study differs from a real DWTP sand filter. Operational sand filters contain an established biofilm community which comes with its challenges for invasion as discussed above. Furthermore, its microbial diversity is broader than anticipated in this study, including non-culturable bacteria or particular bacterial guilds like chemolithotrophs that have relative high abundances in sand filters (Albers *et al.* 2015a; Gülay *et al.* 2016). Moreover, DWTP sand filters contain fungi and protozoa including bacterivores that might affect inoculum dynamics as shown for MSH1 (Ellegaard-Jensen *et al.* 2016; Harder *et al.* 2019). Performing bottom-up experiments as in this study but using established resident assemblies and including alternative natives like chemolithotrophs and protozoa, is one approach to proceed.

Top-down experiments using native sand filter communities, accompanied by inventive meta-omics to reveal community composition and functionality, can further unravel how cooperative community members affect MSH1 invasion in DWTP sand filters (Xu *et al.* 2019).

Author Contributions

JV, AJD, and PCM contributed equally to this paper and are first authors. JV, AJD, PCM, JMB, BDB, NC, and DS designed research; JV and AJD performed research. JV, AJD, PCM analyzed data; JV, AJD, PCM, JMB, BDB, NC, and DS wrote the manuscript; all authors contributed to the final draft.

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Conflict of interest statement

The authors confirm that there are no competing interests.

Data availability statement:

The combined data set and code used in this study are provided in a public repository: https://doi.org/10.5281/zenodo.10945604

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Figure captions and table.

Figure 1. Lag time (λ), maximum rate (μ) of BAM mineralization by *A. niigataensis* MSH1, and the number of MSH1-cells surviving the competition phase (S_{MSH1}) as a function of initial SFI richness (R_{SFI}). Average parameter values are shown (empty circles), based on four replicate microcosms. Black diamonds represent average parameter values per R_{SFI}-level. Dotted and dashed lines represent average parameter values for R_{SFI} = 0 and R_{SFI} = 1, respectively. The Pearson correlation coefficient (r) and corresponding p-value are given for the correlations between R_{SFI} and either λ, μ, or S_{MSH1}.

Figure 1. Variability of the lag time (λ, full black line), maximum rate (μ, dashed black line) of BAM mineralization by *A. niigataensis* MSH1 and of the number of MSH1-cells surviving the competition phase (S_{MSH1}, dashed grey line) as a function of initial SFI richness (R_{SFI}) in sand microcosms. Variability was calculated as the difference between the maximum and minimum standardized parameter value (λ_s, μ_s, or S_s) per R_{SFI}-level and is expressed in units of standard deviations (SD).

Figure 2. Standardized lag time (λ_s, row 1), maximum rate (μ_s, row 2) of BAM mineralization by *A. niigataensis* MSH1 and number of MSH1-cells surviving the competition phase (S_S, row 3) as a function of initial SFI richness (R_{SFI}). Average values are shown (circles), based on four replicate microcosms. Blue, green, red and orange circles represent average parameter values recorded for synthetic communities in which either *Rhodococcus* sp. K27, *Piscinibacter* sp. K169, *Mesorhizobium* sp. S158 or *Acidovorax* sp. S9 was present, respectively. Diamonds represent average parameter values per R_{SFI}-level ($\lambda_{s,P}$, $\mu_{s,P}$, and $S_{s,P}$,) for all communities in which strains K27 (blue), K169 (green), S158 (red) or S9 (orange) were present. Black circles represent average parameter values for synthetic communities in which K27 and S158 (column 1), K169 and S158 (column 2), K27 and S9 (column 3) or K169 and S9 (column 4) were simultaneously present. Black diamonds represent average parameter values per R_{SFI}-level ($\lambda_{s,pair}$, $\mu_{s,pair}$, and $S_{s,pair}$) for all communities in which the corresponding pair (per column) was present. Empty circles indicate average values for communities in which neither of the two corresponding SFI was present.

Table 1. Summary of the effects of SFI and consistency thereof, on the lag time (λ), maximum rate (μ) of BAM mineralization by *A. niigataensis* MSH1, and on the number of MSH1-cells surviving the competition phase (S_{MSH1}), based on comparison of the parameter values recorded for communities in which a specific SFI strain (indicated in the first column) was either present or absent. Effects are divided into categories as not affecting (0) or resulting in slightly lower/higher (-/+) or strongly lower/higher (--/++) values of the corresponding parameter, compared to communities in which that specific SFI strain was absent.

		λ				μ					S_{MSH1}				Conclusion[6]
		$\overline{D_\lambda}$	Effect[1]	Cons.[5]		$\overline{D_\mu}$	Effect[2]	Cons.[5]			$\overline{D_S}$	Effect[4]	Cons.[5]		Effect on MSH1
Acidovorax sp. S9		1.57	+	86%		-0.06	0	n.a.			-1.56	- -	100%		Intermediate negative effect
Undibacterium sp. S22		0.95	0	n.a.		-0.21	0	n.a.			-0.04	0	n.a.		No effect
Brachybacterium sp. S51		-2.07	- -	89%		1.18	++	100%			0.49	0	n.a.		Intermediate positive effect
Mesorhizobium sp. S158		2.31	++	100%		-0.93	- -	80%			-0.55	0	n.a.		Intermediate negative effect
Acidovorax sp. S164		1.64	+	71%		-0.13	0	n.a.			-0.75	-	71%		Weak negative effect
Rhodococcus sp. K27		-3.23	- -	100%		0.57	+	100%			2.05	++	100%		Dominant positive effect
Acidovorax sp. K52		-0.19	0	n.a.		-0.34	0	n.a.			0.27	0	n.a.		No effect
Aeromonas sp. K62		0.09	0	n.a.		0.41	+	86%			-0.21	0	n.a.		No effect
Paucibacter sp. K67		2.06	++	88%		-0.91	- -	75%			-0.76	-	88%		Intermediate negative effect
Pelomonas sp. K89		-0.15	0	n.a.		0.26	0	n.a.			0.64	0	n.a.		No effect
Rhodoferax sp. K112		1.75	+	86%		-0.72	-	71%			-1.14	-	71%		Intermediate negative effect
Rhodoferax sp. K129		-1.19	-	71%		0.30	0	n.a.			0.58	0	n.a.		No effect
Piscinibacter sp. K169		-2.45	- -	90%		0.42	+	60%			0.94	+	70%		Dominant positive effect

[1] "+" = $\overline{D_\lambda} > 1$; "++" = $\overline{D_\lambda} > 2$; "-" = $\overline{D_\lambda} < -1$; "- -" = $\overline{D_\lambda} < -2$

[2] "+" = $\overline{D_\mu} > 0.4$; "++" = $\overline{D_\mu} > 0.8$; "-" = $\overline{D_\mu} < -0.4$; "- -" = $\overline{D_\mu} < -0.8$

[4] "+" = $\overline{D_S} > 0.7$; "++" = $\overline{D_S} > 1.4$; "-" = $\overline{D_S} < -0.7$; "- -" = $\overline{D_S} < -1.4$

[5] Consistency of the effect, calculated as the number of R_{SFI}-levels for which D > 0 or D < 0 for + or - effects, respectively, relative to the total number of R_{SFI}-levels tested, n.a. = not applicable

[6] Lower λ, higher μ or lower A are regarded as positive effects on MSH1. Higher λ, lower μ or higher A are regarded as negative effects on MSH1.