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Inoculation history affects community composition in experimental freshwater bacterioplankton communities

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Summary

Priority effects occur when the arrival order of species or genotypes has a lasting effect on community or population structure. For freshwater bacteria, priority effects have been shown experimentally among individual species, but no experiments have been performed using complex natural communities. We investigated experimentally whether a foreign bacterioplankton community influences the community assembly trajectory when inoculated prior to the local community, whether inoculation time lag affects priority effects, and how the individual OTUs responded to time lag. Two bacterioplankton communities from dissimilar ponds were inoculated into one of the natural media with a time lag of 0, 12, 36 or 60 h, giving advantage in time to the foreign community. All three time lags resulted in priority effects, as the final community composition of these treatments differed significantly from that of the treatment with no time lag, but compositional shifts were not linear to inoculation time lag. The responses of individual OTUs to time lag were highly diverse and not predictable based on their immigration history or relative abundance in the inocula or control. The observed impact and complexity of priority effects in multispecies systems emphasize the importance of this process in structuring both natural and industrial bacterial communities.

Introduction

Field studies have shown that freshwater bacterioplankton communities are often strongly determined by local environmental conditions and to a much lower extent by spatial structure and dispersal limitation (Beisner *et al.*, 2006; Langenheder and Ragnarsson, 2007; Van der Gucht *et al.*, 2007; Souffreau *et al.*, 2015). However, an often even larger proportion of the variation in community structure cannot be explained by measured environmental or spatial variables (Beisner *et al.*, 2006; Langenheder and Ragnarsson, 2007; Van der Gucht *et al.*, 2007; Lindström and Langenheder, 2012; Souffreau *et al.*, 2015), and additional ecological processes and evolution need likely to be considered to better understand the drivers of local community assembly. The evolving metacommunity framework provides scope to dig into this unexplained variation in bacterioplankton community structure. In this framework local communities are linked by dispersal of multiple potentially interacting species (Leibold *et al.*, 2004; Holyoak *et al.*, 2005; Urban *et al.*, 2008). Local community assembly is influenced by local and regional ecological dynamics (Leibold *et al.*, 2004) while local populations can evolve in response to local (a)biotic conditions (Urban *et al.*, 2008). Under the species sorting perspective the metacommunity framework predicts a one-to-one match between local community composition and local (a)biotic variables, made possible by sufficient dispersal to ensure immigration of each species from the regional pool into each habitat (Leibold *et al.*, 2004; Holyoak *et al.*, 2005). Mechanisms that can explain divergences from this perfect match between community composition and environmental conditions include neutrality – where species occurrence within a habitat is the result of random immigration, speciation and extinction events (Hubbell, 2001; Leibold *et al.*, 2004; Holyoak *et al.*, 2005; Östman *et al.*, 2010), mass effects – where a maladapted species can remain present in a community by continuous or massive immigration from more suitable habitats (Leibold *et al.*, 2004; Holyoak *et al.*, 2005; Lindström and Östman, 2011; Souffreau *et al.*, 2014), and priority effects.

Priority effects occur when the arrival order and timing of species or genotypes have a lasting effect on the outcome of biotic interactions, and thus on the realized community and/or population structure (Alford and Wilbur, 1985; Almany, 2003; van Gremberghe *et al.*, 2009; Vannette and Fukami, 2014; Fukami, 2015). Variation in dispersal capacity of species, regional connectivity and chance can affect

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order and timing of species colonization from the regional species pool into an empty site during community assembly (Chase, 2003; van Gremberghe *et al*., 2009). In some cases early-arriving species can facilitate the establishment of late-arriving species by improving local conditions for these late immigrants, while in other cases early immigrants are expected to have a competitive advantage over late immigrants (Alford and Wilbur, 1985; van Gremberghe *et al*., 2009; Fukami, 2015). Different mutually non-exclusive mechanisms can result in favouring the establishment and growth of early over late immigrants. First, invasion success of late immigrants can be reduced when early immigrants decrease or deplete certain resources (known as niche preemption) or modify the habitat (known as niche modification), e.g., by producing toxic metabolites which increases their competitive advantage towards late immigrants (van Gremberghe *et al*., 2009; Vannette and Fukami, 2014; Fukami, 2015). Second, local genetic adaptation of early immigrants can facilitate rapid monopolization of resources by these early immigrants, a mechanism known as community monopolization (Loeuille and Leibold, 2008; Urban *et al*., 2008; Urban and De Meester, 2009, De Meester *et al*., 2016).

Evidence for priority effects has been found at population and community level in macro- and microscopic eukaryotes, both in laboratory experiments [Fukami, 2004 (protozoa); Fukami *et al*., 2010 (fungi); Peay *et al*., 2012 and Vannette and Fukami, 2014 (yeasts)] and in field experiments [Alford and Wilbur, 1985 and Wilbur and Alford, 1985 (amphibians); Facelli and Facelli, 1993 (plants); Almany, 2003 (fish); De Meester *et al*., 2007 and Mergeay *et al*., 2011 (zooplankton); Dickie *et al*., 2012 (fungi)]. For prokaryotes, priority effects have been studied and observed at population level using laboratory experiments with *Pseudomonas fluorescens* (Fukami *et al*., 2007; Knope *et al*., 2012) and *Microcystis aeruginosa* (van Gremberghe *et al*., 2009). At community level, only one laboratory experiment assessed priority effects using two bacterial and two yeast species (Tucker and Fukami, 2014). In all these studies, the investigated strains or species had an advantage when being introduced first compared to when being introduced after the establishment of other strain(s), species or communities, but the precise effects depended on the strain or species and on the level of environmental variability, with a higher environmental variability preventing extinction of late-arriving species (Tucker and Fukami, 2014). Moreover, also entire communities can colonize an empty habitat site at once following events such as floods, animal defecation, medical contaminations or birth. Despite the potential importance of this phenomenon both in nature as well as in human applications, data on the impact of such more extreme colonization events on the final community composition are not yet available for bacteria.

In this study, we determined the impact of whole-community priority effects in freshwater bacterioplankton, giving an entire bacterioplankton community an advantage in inoculation time over a second bacterioplankton community. To gain access to two dissimilar, natural communities, we selected bacterioplankton from two environmentally dissimilar ponds in Belgium, one being eutrophic, the other mesotrophic (Supporting Information Table S1). Because eutrophic ponds are the most common ones in Belgium, we used the associated eutrophic bacterioplankton community as the early-arriving pioneer community in our experiment and the mesotrophic community as the second-arriving colonist community. To mimic a colonization situation in which the early-arriving pioneers are not adapted to the empty habitat patch but the second-arriving colonists are, we used the mesotrophic medium as the medium of the empty habitat patch. This set-up assures that an observed priority effect is the result of one community arriving first and not of being *a priori* better adapted to the medium, although evolution-mediated priority effects might still play a role during the experiment itself. Throughout this article, we denote the first-arriving community, not *a priori* adapted to the medium, as 'naive pioneers' and the second-arriving community, originating from the medium, as 'native second colonists'. No reciprocal experiment was done as this was not the scope of our study.

We used a time lag of 0, 12, 36 or 60 h (four treatments denoted as Δ0 h, Δ12 h, Δ36 h and Δ60 h respectively) between the inoculations of the naive pioneers' community and the native second colonists' community, and assessed the final community composition after eight days (Fig. 1). At the community level, we tested the following two specific hypotheses: (i) an inoculation time lag between two dissimilar bacterioplankton communities results in a change in final community composition, i.e., in a priority effect; (ii) the magnitude of the priority effect depends on the length of the inoculation time lag. We were also interested in how individual OTUs respond to a priority effect, and we, therefore, tested the following two hypotheses at the level of individual OTUs: (i) OTUs from the naive pioneers' community benefit from inoculation time lag, measured as an increase in their relative abundance in the treatments (Δ12 h, Δ36 h or Δ60 h) compared to that in the control condition (Δ0 h), whereas OTUs from the native second colonists' community are disadvantaged by inoculation time lag; (ii) OTUs with high relative abundances in the naive pioneers' community have a higher advantage of inoculation time lag compared to OTUs with lower relative abundances in this inoculum, whereas OTUs with high relative abundances in the native second colonists' community have a lower disadvantage of inoculation time lag compared to OTUs with lower relative abundances in this inoculum.

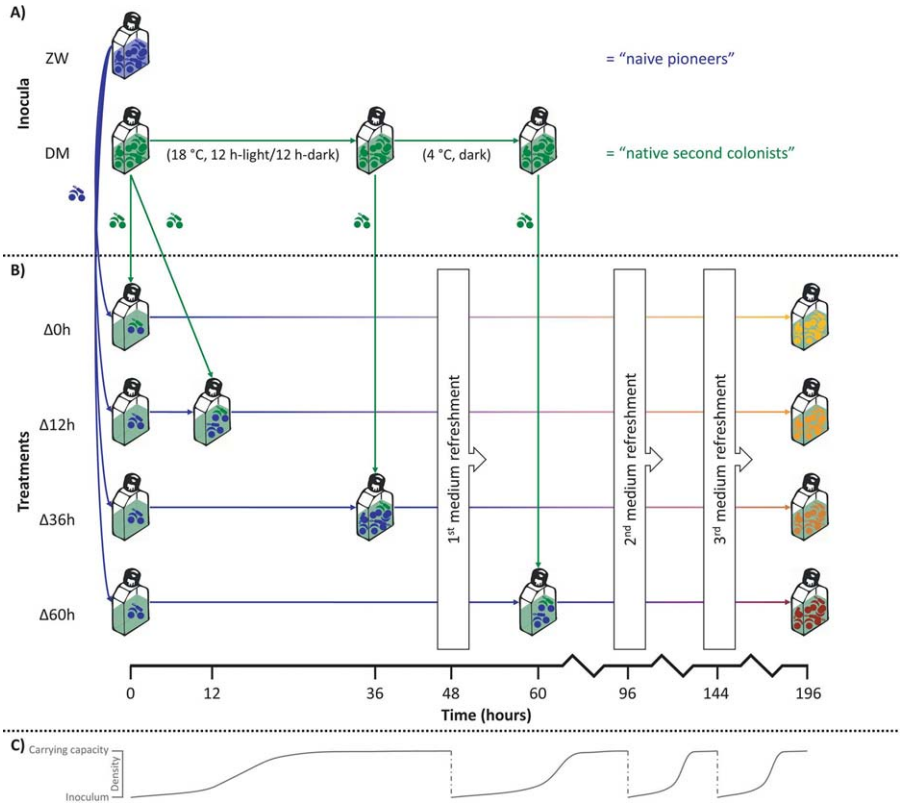

Fig. 1. Schematic representation of the experimental design. A. represents the bacterial community inocula. ZW designates the eutrophic Zoet Water community or the 'naive pioneers', DM designates the mesotrophic De Maten community or the 'native second colonists'. B. visualizes the experimental set-up and inoculation of the treatments. The naive pioneers were inoculated at the start of the experiment, while the native second colonists were inoculated with a time lag of 0, 12, 36 or 60 h (Δ0 h, Δ12 h, Δ36 h and Δ60 h). Medium was refreshed and communities re-inoculated each 48 h. Samples were taken after 196h (eight days). C. visualizes cell density evolution in the experimental culture flasks based on previously determined growth curves of naive pioneers and native second colonists in De Maten medium. Stationary phase was reached after ~ 24 h. [Colour figure can be viewed at wileyonlinelibrary.com]

Results

Effect of inoculation time lag on bacterial community composition

We assessed whether time lag between inoculation of naive pioneers and native second colonists (0, 12, 36 or 60 h) affected the final bacterial community composition. Principal Component Analysis (PCA) was used to visualize the variation in community composition among the different treatments and original inocula (Fig. 2): while PC1 (explaining 36.86% of the variation) separates the inoculum samples from the treatment samples, PC2 (13.70%;

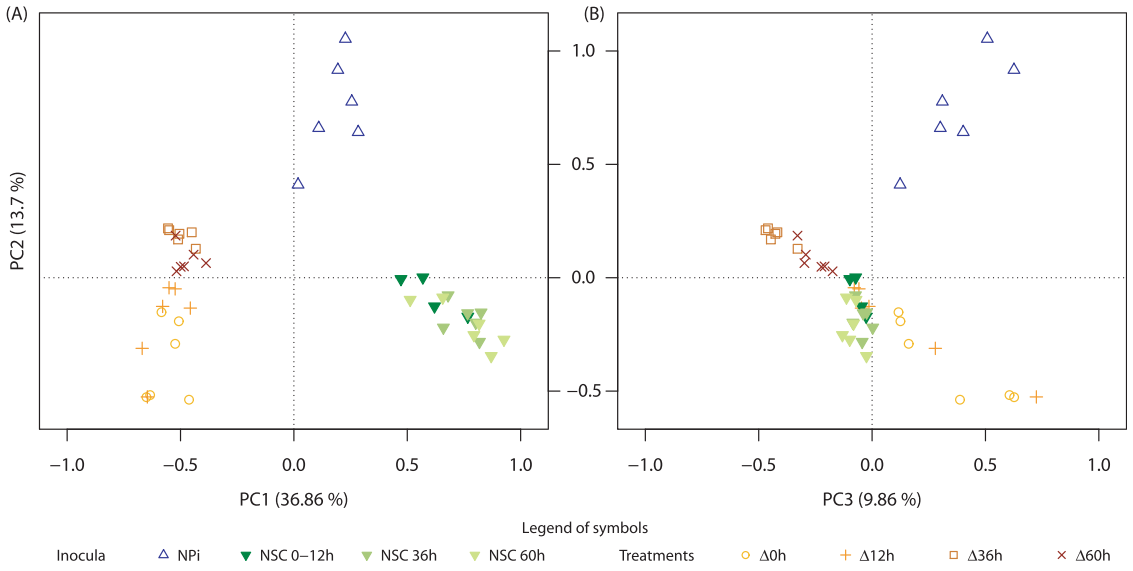

Fig. 2. PCA ordination diagrams showing (A) PC1 and PC2 and (B) PC3 and PC2, visualizing the largest variation in bacterial community composition within and among naive pioneers' (NPi) and native second colonists' (NSC) inocula, and the four treatments (Δ0 h, Δ12 h, Δ36 h and Δ60 h) eight days after initial inoculation. [Colour figure can be viewed at wileyonlinelibrary.com]

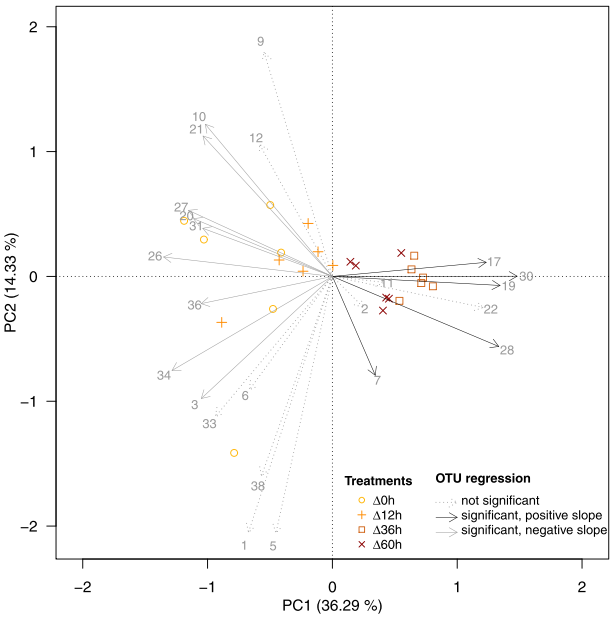

Fig. 3. PCA biplot, showing PC1 and PC2, visualizing the largest variation in bacterial community composition within the 24 treatment samples (symbols; Δ0 h, Δ12 h, Δ36 h and Δ60 h), and the relation to the 24 OTUs observed therein (arrows). OTU arrows are denoted with the OTU identifying number and shaded according to significance and slope sign of the regression between that OTU's relative abundance and inoculation time lag in treatments, as determined in Fig. S2. [Colour figure can be viewed at wileyonlinelibrary.com]

Fig. 2A) and especially PC3 (9.86%; Fig. 2B) separate the different treatment conditions according to time lag (Δ0 h, Δ12 h, Δ36 h and Δ60 h). This effect of time lag is even more visible on PC1 (36.29%) of Fig. 3 in which only the 24 treatment samples were plotted. When testing formally for effects of time lag on community composition using variation partitioning to control for effects of replicate, time lag (controlled for replicate effects) explained a significant proportion of the variation in community composition (46%–71% explained variance; $p < 0.001$), both when inserted as a categorical variable (assuming each time point is independent of the other time point) and when inserted as a numerical variable (assuming time points are related to each other) [partial redundancy analysis (pRDA), Table 1]. There was no interaction effect between time lag and replicate.

To test by which inoculation time lag a priority effect was induced, we assessed among which time lag treatments the final communities were significantly differentiated using pRDA to control for effects of replicate. By pairwise comparison of the community compositions among treatments, a significant effect of time lag was found for all pairs of treatments ($p < 0.05$; Supporting Information Table S2). To assess whether inoculation time lag affected the strength of the priority effect, we calculated Euclidean distances among each pair of time lag treatments based on PC1 of the treatment sub-data set (i.e., Fig. 3; PC1 separates treatment samples according to time lag and, therefore, reduces the variation in community composition to the variation due to the time lag treatments) and tested whether these distances differed significantly among the compared time lags. Figure 4 visualizes that pairs of treatments differed significantly in their Euclidean distances [one-way analysis of variance (ANOVA): $p = 0.00005$]. Samples of treatment Δ12 h showed significantly more resemblance to those of treatment Δ0 h than samples of treatment Δ36 h [Tukey's honest significant difference (HSD) test: $p = 0.0017668$], while samples of treatment Δ60 h had intermediate resemblance to those of treatment Δ0 h ($p > 0.05$). Additionally, samples of treatment Δ36 h bore significantly closer resemblance to those of treatment Δ60 h than to those of treatment Δ12 h ($p = 0.0196969$), with an intermediate resemblance between samples of treatments Δ12 h and Δ60 h ($p > 0.05$).

To examine the directionality of community changes (i.e., more/less resembling the naive pioneers' or native second colonists' inocula) under influence of inoculation time lag, we compared the Euclidean distances of each treatment with the naive pioneers' or native second colonists' inocula. Significant differences were observed among the inoculum-treatment pairs (Fig. 5; distances based on PC2 of the total data set, which separates communities according to culture medium and inoculation time lag while eliminating the variation induced by experimental conditions; one-way ANOVA: $p = 6.98 \times 10^{-12}$). The distance between the two inoculum types (naive pioneers and native second colonists) was equally large as the distances between the naive pioneers' inocula and each of the treatments (Tukey's HSD

Table 1. Percentage of variation (R^2_{adj}) in final bacterioplankton community composition of the treatments (eight days after initial inoculation) unexplained and explained by pure effect of inoculation time lag between naive pioneers and native second colonists (*I/R*; categorical or numerical), pure effect of replicate (*R/I*; categorical blocking factor) and their interaction effect (*I*∩*R*), based on variation partitioning.

Analysis components	Unexplained	Explained	*I/R*	*I*∩*R*	*R/I*
I (categorical) + R	37.63%	62.37% ***	71.13% ***	≤ 0%	7.70% **
I (numerical) + R	62.83%	37.17% ***	45.94% ***	≤ 0%	1.28%

Significance levels: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.
Significances of these factors' effects were tested by permutation tests on partial RDAs.

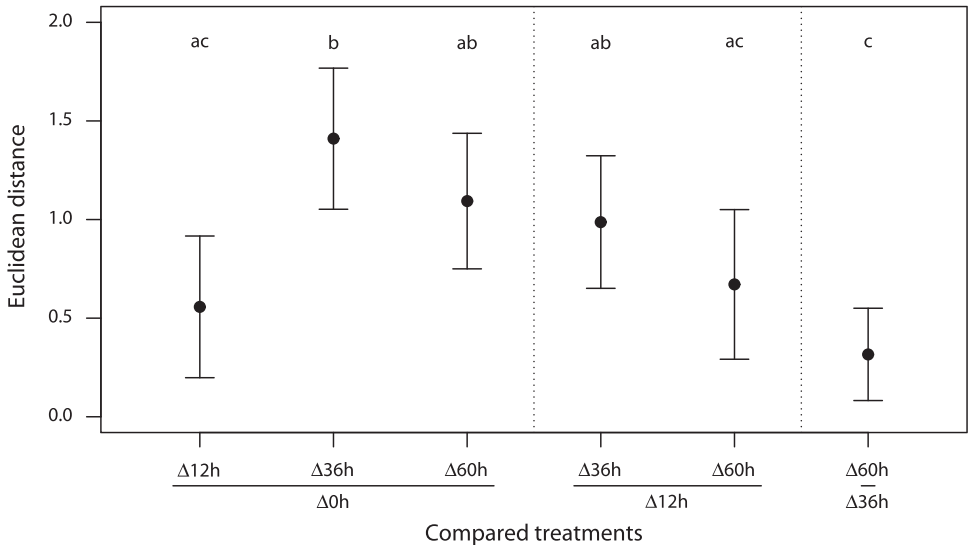

Fig. 4. Euclidean distances among each pair of treatments (Δ0 h, Δ12 h, Δ36 h and Δ60 h) based on the first axis of PCA diagram of the treatment sub-data set (see Fig. 3). Points represent mean Euclidean distance of six replicates, and whiskers one standard deviation from the mean. Different letters represent significant differences.

test: $p > 0.05$), while the distances between the native second colonists' inocula and each of the treatments were all significantly smaller compared to this inter-inoculum distance (Tukey's HSD test: $p < 0.001$) and close to zero. The four treatments (Δ0 h, Δ12 h, Δ36 h and Δ60 h) did not differ significantly in Euclidean distance with the native second colonists' inocula (Tukey's HSD test: $p \geq 0.646$), but there was an increasing resemblance of the treatment communities to the naive pioneers' inocula with increasing inoculation time lag: the distance to naive pioneers' inocula was significantly smaller for treatment Δ36 h in comparison to treatments Δ0 h and Δ12 h (Tukey's HSD test: $p = 0.0006223$ and $p = 0.0450308$ respectively), and for treatment Δ60 h in comparison to treatment Δ0 h (Tukey's HSD test: $p = 0.0100779$).

OTU patterns in treatment communities and relation with inocula OTUs

The total data set contained 40 OTUs, of which 34 were observed in the inocula and 24 in the treatments. Two of the 24 OTUs observed in the treatments could unambiguously be linked to naive pioneers' inocula (OTU 27, 30), 6 to native second colonists' inocula (OTU 2, 3, 5, 10, 22, 31), 10 to both inoculum types (OTU 1, 6, 7, 9, 11, 17, 19, 21, 26, 28) and 6 OTUs could not be linked to either inoculum type (OTU 12, 20, 33, 34, 36, 38) (Supporting Information Fig. S1A). To assess how individual OTUs responded to the different inoculation time lags and whether OTUs from the naive pioneers' inocula had an increasing advantage (higher relative abundance) while OTUs from the native second colonists' inocula had an increasing disadvantage (lower relative abundance) with

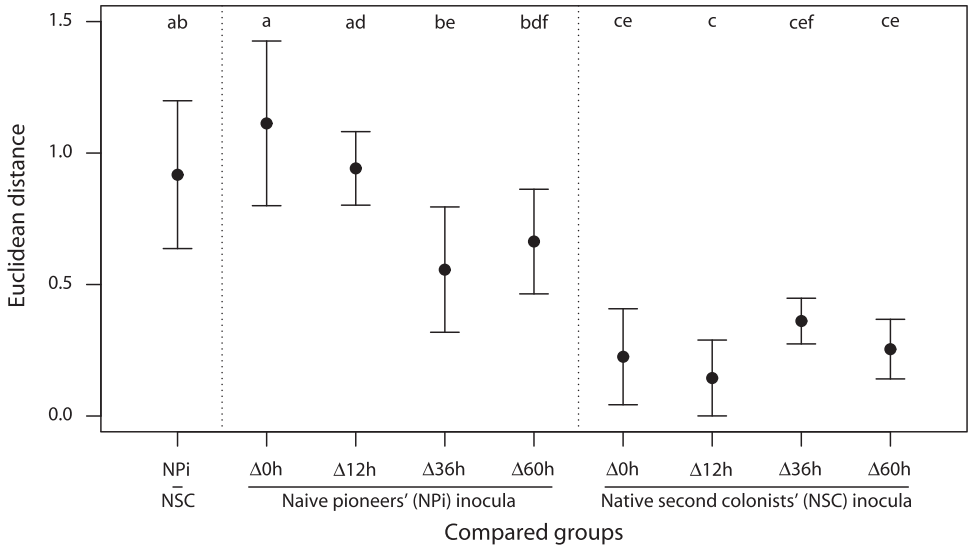

Fig. 5. Euclidean distances between the naive pioneers' (NPi) or native second colonists' (NSC) inocula and each of the four treatment (Δ0 h, Δ12 h, Δ36 h and Δ60 h) based on the second axis of the PCA diagram of the entire data set (see Fig. 2). Points represent mean Euclidean distance of six replicates, and whiskers one standard deviation from the mean. Different letters represent significant differences.

Table 2. Allocation of the 40 OTUs observed in this experiment (denoted by their identifying number) to one of four response types in relation to inoculation time lag (not-detected, increase in relative abundance, decrease in relative abundance or none/unclear) and categorized according to their inoculum origin.

	Response type			
OTU's inoculum origin	OTUs present in inocula that are not detected in any of the four treatments.	OTUs that have an (significant) increase in mean relative abundance with non-zero inoculation time lags compared to control treatment Δ0h.	OTUs that have a (significant) decrease in mean relative abundance with non-zero inoculation time lags compared to control treatment Δ0h.	OTUs that have a mean relative abundance not significantly in- or decreasing with non-zero inoculation time lags compared to control treatment Δ0h.
Naive pioneer	13, 32, 39, 40 (66.67%)	**30** (16.67%)	27 (16.67%)	/ (0.00%)
Naive pioneer and native second colonist	4, 8, 14, 18, 35, 37 (37.50%)	**19**, **28**, 7, **17** (25.00%)	**26**, 21, 6 (18.75%)	11, 9, 1 (18.75%)
Native second colonist	15, 16, 23, 24, 25, 29 (50.00%)	22 (8.33%)	**10**, 31, 3 (25.00%)	5, 2 (16.67%)
Unknown	/ (0.00%)	/ (0.00%)	20, **34**, **36** (50.00%)	12, 33, 38 (50.00%)

OTUs were allocated to a response type when the differences between treatments in deviations from treatment-mean relative abundance were significant (Supporting Information Fig. S1) and/or when the linear regression between relative abundances and time lags was significant (Supporting Information Fig. S2). OTUs whose responses were significant in both figures are denoted in bold, and OTU identifying numbers are written down in the order of their occurrence on these figures. Percentages between brackets are proportions of OTUs displaying that response type within that inoculum origin.

increasing time lag, we compared the relative abundances of the OTUs over the time lag treatments, taking into account their inoculum origin. We observed no consistent relation between the response to inoculation time lag and inoculum origin of an OTU (Supporting Information Figs. S1 and S2; Table 2). The correlation between relative abundance of an OTU and inoculation time lag was significantly positive – suggesting facilitation – for OTUs 30, 19, 28, 7 and 17 (one-way ANOVA: $p < 0.05$), while it was significantly negative – suggesting inhibition – for OTUs 27, 26, 21, 10, 31, 3, 20, 34 and 36 ($p < 0.05$) (Supporting Information Figs. S1 and S2; Table 2). However, there was no clear relation between the response to inoculation time lag (increase, decrease or none/unclear) and origin of OTUs (naive pioneers, native second colonists, both inocula, or unknown).

Additionally, we tested whether there was a relationship between the relative abundance of the OTUs in the inocula or control treatment and their subsequent response in the time lag treatments. We, therefore, plotted the OTUs regression slopes of relative abundance with time lag (cf. Supporting Information Fig. S2) against the OTU's relative abundance in inocula or control treatment, but no significant relationships were detected (Supporting Information Fig. S3). Nevertheless, when we colour the OTU's arrows on the PCA biplot of the treatment samples (Fig. 3) based on the sign of their regression slope, we see that OTUs with a significantly negative regression slope are negatively correlated to those with a significantly positive slope, and together these OTUs divide treatment communities along the first PC axis according to inoculation time lag. OTUs with a non-significant regression have directions more perpendicular to this first PC axis (except for OTU 22 whose regression is borderline non-significant; $p = 0.05595$), indicating they do not distinguish treatment samples according to inoculation time lag.

Discussion

We inoculated in the laboratory two bacterioplankton communities from two dissimilar freshwater environments into the environment of one of the communities with a time lag of 0, 12, 36 or 60 h between inoculations, giving advantage in time to the community originating from the opposite environment, and observed that time lag affected community composition as measured after eight days (Table 1). This effect of time lag on community composition was not only observed compared to the treatment in which the two communities were inoculated simultaneously (Δ0 h), but also among all other pairwise combinations of time lag treatments (Δ12 h, Δ36 h or Δ60 h) (Supporting Information Table S2, Fig. 4), proving that not only order of inoculation but also magnitude of time lag between inoculation events impacts community assembly of complex bacterial communities. After eliminating the variation induced by experimental conditions, the bacterial communities of all four treatments exhibited a high resemblance to the late-arriving community native to the used environment (native second colonists) irrespective of the time delay of its inoculation, while resemblance towards the early-inoculated, foreign community (naive pioneers) significantly increased with increasing time lag (Fig. 5). These results demonstrate that while species sorting played an important role in bacterial community assembly in all four treatments,

priority effects were able to shift the final community composition in favour of the early-inoculated, foreign community when it was given a time advantage.

Our observations of priority effects at already short time lags of inoculation (12 h) are in agreement with previous research on priority effects and historical contingency in bacteria. Time lags used in previous studies on priority effects in bacteria varied from 6 h, 1 day and 2 days in work on *Pseudomonas fluorescence* populations (Fukami *et al*., 2007; Knope *et al*., 2012), 2 days in work on two bacterial and two yeast species (Tucker and Fukami, 2014), to one week in work on two-strain *Microcystis* populations (van Gremberghe *et al*., 2009), and all these evaluated time lags resulted in significant effects on final population or community structure. Fukami *et al*. (2007) also studied and observed an effect of time lag on the amplitude of priority effects. While these previous studies used individual species as early and late immigrants to assess priority effects (Fukami *et al*., 2007; van Gremberghe *et al*., 2009; Knope *et al*., 2012; Tucker and Fukami, 2014), we worked with complex bacterial communities as early and late immigrants. To our knowledge, no other data on whole-community priority effects in bacterial communities are yet available. Dickie and colleagues (2012) focused on forest-soil fungal communities of 30 species in a natural setting and observed lasting priority effects on the final community structure after manipulating the inoculation history of 10 lab-cultured species. Our results are in line with their observations. Both these experimental studies thus indicate that priority effects can also be of significant importance in complex microbial communities. However, our experimental set-up does not allow us to determine whether the observed priority effect is primarily a consequence of niche preemption and/or niche modification, or whether also evolution drove historical contingency.

At the level of individual OTUs, we expected that a priority effect would correspond to an advantage for several early-inoculated species, measured as an increase in their relative abundance in the treatments (Δ12 h, Δ36 h or Δ60 h) compared to that in the control condition (Δ0 h), and to a disadvantage for species inoculated later on, measured as a decrease in their relative abundance compared to that in the control condition. We also expected that OTUs with high relative abundances in the naive pioneers' inocula would have a higher advantage of inoculation time lag compared to OTUs with lower relative abundances in these inocula, whereas OTUs with high relative abundances in the native second colonists' inocula would have a lower disadvantage of inoculation time lag compared to OTUs with lower relative abundances in these inocula. Though both an increase and decrease in OTUs' relative abundances with non-zero time lags compared to control treatment Δ0 h of OTUs were clearly observed (Table 2, Supporting Information Fig. S1), our analyses showed that responses of individual OTUs to inoculation time lag were highly diverse and could not be predicted by their immigration history (Table 2; Supporting Information Figs. S1 and S2) nor by their relative abundance in inocula or in the control treatment Δ0 h (Supporting Information Fig. S3). In line with our expectations, three OTUs that were more dominant in the naive pioneers' community than in the native second colonists' community showed a clear increase in relative abundance with increased time lag (namely OTUs 30, 19 and 28), a pattern that might be the result of facilitation due to priority effects. Surprisingly, two such OTUs demonstrated a decrease in relative abundance (namely OTUs 27 and 26). Likewise, for the OTUs that were more dominant in the native second colonists' community than in the naive pioneers' community three exhibited – as expected – a decrease in relative abundance when inoculated after a longer time lag (namely OTUs 10, 31 and 3), potentially attributable to inhibition due to the priority effect, while in contrast two OTUs increased their relative abundances under longer time lags (namely OTUs 17 and 22). It was not possible to categorize the significant response of OTUs 7, 21, 6, 20, 34 and 36 to time lag as (un)expected based on inoculum origin, due to the absent or small difference in relative abundance among inocula (Table 2). Although these patterns suggest the presence of inhibition or facilitation due to priority effects, we have no information on the exact mechanisms underlying the observed increase or decrease in abundance of OTUs. Altogether, our results suggest a trend where an OTU might be facilitated or inhibited with increasing time lag as the advantage for early-arriving over late-arriving OTUs increases respectively decreases. Nevertheless, there was no clear-cut pattern in the changes in relative abundance of OTUs based on their immigration history or according to their relative abundance in the inocula or in control treatment Δ0 h. These observations show that although priority effects can play an important role in the total assembly of complex bacterial communities, responses of individual species to immigration time lags are very complex in communities consisting of multiple tens of species. Also in studies using a limited number (one to two) of early-arriving microbial strains or species (Fukami *et al*., 2007; van Gremberghe *et al*., 2009; Knope *et al*., 2012; Tucker and Fukami, 2014), the strength of priority effects was often strain- or species-specific and responses could strongly depend on specific genotype characteristics. This unpredictability of species' responses to priority effects could have severe implications in areas where priority effects might play a role and predictability or standardization is important, think of technologies that are dependent on highly controlled microbial communities such as food fermentation processes, bioremediation or probiotics. The high variation in response of species and

strains to priority effects observed in previous and this study is most likely the result of trait differences among species and strains. For the present experiment we have, however, no data on species' identities or traits to set up more specific hypotheses based on our results. One way to enhance the predictability of species' responses is by increasing our knowledge on species' traits and the link between traits and responses to priority effects. The use of high-throughput sequencing methods, by which specific genes (associated to specific functional traits) or even full genomes can be sequenced, offers ways to get more insight into the mechanisms that shape these divergent responses.

Facilitation and inhibition of early- or late-arriving species as suggested in our experiment based on their change in relative abundance with increasing inoculation time lag, have been reported before in the context of priority effects and can be explained by different mechanisms. An increase in relative abundance (facilitation) of early-arriving species and a decrease in relative abundance (inhibition) of late-arriving species are two responses that have been mostly observed in previous experimental work on priority effects (e.g., van Gremberghe *et al.*, 2009; Dickie *et al.*, 2012; Peay *et al.*, 2012; Vannette and Fukami, 2014), and can be explained by niche preemption (usage and/or monopolization of available resources by early-arriving species) or niche modification by early-arriving species, negatively affecting late-arriving species (Fukami, 2015). Facilitation of late-arriving species has also been observed in previous work (e.g., van Gremberghe *et al.*, 2009; Fukami *et al.*, 2010; Ottosson *et al.*, 2014), and can be explained by niche modification where early-arriving species create new or change available niches to some that can be occupied by late-arriving species. A negative effect on early-arriving species by the absence of the late-arriving community (i.e., decreasing relative abundance of early-arriving species with longer time lags; here OTUs 27 and 26) has been observed previously for a non-toxic non-colony-forming *Microcystis* strain which could only survive under grazing pressure of *Daphnia magna* when inoculated simultaneously or secondly to another toxic or colony-forming strain (van Gremberghe *et al.* 2009). This phenomenon can thus be explained by niche modification by late-arriving species which positively affects early-arriving species: the shorter time lag between immigration events is, the earlier late-arriving species can start modifying available niches or environment (e.g., the production of toxins), positively ensuring survival and growth of early-arriving species, and the less time this early-arriving species has to survive under unfavourable conditions (e.g., grazing by *Daphnia*).

We further observed a non-linear change in community composition with increasing time lag of inoculation in our study. There was a discrepancy in the community variance explained by time lag as numerical (46%) or categorical (71%) variable (Table 1), while PCA (Figs. 2 and 3) and analyses on Euclidean distances (Figs. 4 and 5) showed that the community structure of treatment Δ36 h was more different from that of treatment(s) Δ0 h (and Δ12 h) than was the case for treatment Δ60 h. Additionally, the mean relative abundance of specific OTUs in treatment Δ60 h was often intermediate to that of treatments Δ12 h and Δ36 h (Supporting Information Fig. S1). We see two mutually non-exclusive explanations for this observed smaller change in community composition from treatment Δ0 h to treatment Δ60 h than to treatment Δ36 h. First, this could be an effect of the growth phase and its associated cell density of the naive pioneers' community at time of inoculation of the native second colonists' community (Fig. 1C). In treatments Δ0 h, Δ12 h and Δ60 h the native second colonists' community was inoculated during lag or exponential growth phase of the naive pioneers' community (with respective cell densities ca. 1.7×10^4 cells ml;$^{-1}$ for Δ0 h and ca. 3.5×10^5 cells ml^{-1} for Δ12 h and Δ60 h), whereas in treatment Δ36 h this was done during stationary growth phase of this community (cell densities ca. 7.4×10^6 cells ml^{-1}). In treatment Δ36 h, the naive pioneers' community had thus already reached its carrying capacity – resulting in a high cell density ratio of naive pioneers over native second colonists, which could have given this community a higher advantage over the native second colonists' community compared to treatments Δ12 h and Δ60 h by having depleted or monopolized all available resources and/or produced high amounts of toxic metabolites (Fukami, 2004; 2015; van Gremberghe *et al.*, 2009). In treatments Δ12 h and Δ60 h carrying capacity was not yet reached by the naive pioneers' community – resulting in a lower cell density ratio of naive pioneers over native second colonists, and thus some niche space might still have been available for the native second colonists' community. The importance of the growth curve phase of the resident community for priority effects has been suggested previously in the literature (Fukami, 2004; 2015; van Gremberghe *et al.*, 2009). In protozoan and rotifer species larger priority effects have been observed in smaller microcosms than in larger ones when inoculated under identical initial population sizes, which were attributed to the relatively larger initial population densities in smaller microcosms (Fukami, 2004). For priority effects to occur, the rate of local population dynamics must be high enough in comparison to immigration rates for early-arriving species to preempt or modify niches substantially before immigration of late-arriving species (Fukami, 2015). In this study, the early-arriving community of treatment Δ36 h had reached carrying capacity of the environment at the moment the late-arriving community was inoculated, and local population dynamics had thus been fast enough to preempt or modify niches. By refreshing the medium after 48 h in

treatment Δ60 h, we reset this preemption or modification of niches, potentially diminishing the strength of priority effects that could be exerted by the naive pioneers' community and providing species in the native second colonists' community with a higher chance of survival.

Second, it is possible that the strength of priority effects does not increase linearly with time lag between immigration events, but reaches a threshold at a certain time lag after which the effect of time lag on final community composition stabilizes. When a first-arriving community colonizes a new environment, its species acclimatize, undergo species sorting and/or become genetically adapted to the new environment over time. After a certain time frame, this might result in a stable community. If the time lags of inoculation between two communities are longer than the time frame needed for the early-arriving species to reach a stable community, these inoculation time lags would result in a similar end community and thus a similar priority effect. In this case, the effect of inoculation time lag on final community composition might have stabilized at a time lag between 12 and 60 h. If the effect of inoculation time lag stabilized after the threshold, the differences observed between treatments Δ36 h and Δ60 h could also be explained as natural variation. Of course, it is possible that these two proposed mechanisms acted simultaneously on the communities, resulting in the observed non-linear response of the strength of priority effects to time lag. Even additional factors could have played a role, for example the number of disturbances (such as medium refreshments) that potentially lead to a period of increased growth rates (Chase, 2003).

In summary, our results clearly demonstrate the potential importance of priority effects and historical contingency – alongside species sorting by environmental conditions – on the assembly of complex bacterial communities, and the complexity of their effects at the individual OTU level. Nevertheless, this study does not allow to determine the exact mechanisms resulting in this priority effect (niche preemption, niche modification or evolution-mediated) nor their relative contribution to the effect. Also the mechanisms behind the observed differences in individual OTU responses remain unknown. Although natural freshwater bacterioplankton communities experience little dispersal limitation at more local scale (Van der Gucht *et al.*, 2007; Lindström and Langenheder, 2012; Souffreau *et al.*, 2015), colonization events of individual species are probably spread in time. Therefore, further studies should focus on the circumstances under which priority effects can overcome pure species sorting and influence composition of natural communities, and which effect they have on occurrence and relative abundance of particular species (in relation to their traits) and on higher level community- and ecosystem functions.

Experimental procedures

Sampling of natural bacterial communities and pond water

In October 2012, the bacterioplankton community and pond water of two dissimilar ponds were sampled: one eutrophic pond situated in the public area 'Zoet Water' in Oud-Heverlee (Belgium), and one mesotrophic pond situated in the nature reserve 'De Maten' in Genk (Belgium). Just before sampling we measured water depth, pH and conductivity following De Bie *et al.* (2012). Of each pond, water was collected for characterization and experimental use with a submersible pump at a single sampling point (distance to the shoreline 1–3 m, depth 10–20 cm and min. 10 cm above the sediment), immediately sieving the water over a sequence of 1000 μm to 50 μm sieves. A mixed 50 μm-filtered sample was used to measure Sneller depth and Chlorophyll a concentration, and to take water samples for the determination of total nitrogen, total phosphorus and dissolved organic carbon concentration following De Bie *et al.* (2012). Environmental characteristics of the two ponds are given in Supporting Information Table S1. Mixed 50 μm-filtered water from each pond was filter-sterilized (final filter pore-size 0.2 μm; Sartobran® P MidiCaps®, Sartorius Stedim Biotech S.A., France) in the laboratory and stored at −20°C. Defrosted pond waters were used as natural growth media to perform the experiment after a second step of filter-sterilization (0.22 μm GSWP Nitrocellulose Membrane Filter, Merck Millipore, Germany), and ensured constant media composition during the course of the experiment. To sample the natural bacterioplankton community, mixed 50 μm-filtered pond water was subsequently filtered (11 μm, Whatman® Grade 1 Qualitative Filter Paper, GE Healthcare) in the laboratory. A subsample of the filtrate was stored at 4°C for abundance measurement (fixated with 4% formaldehyde), and living bacterioplankton was isolated from 11 μm-filtered pond water by centrifugation (7000 g, 15 min, 4°C). The pellet was dissolved in a cryoprotectant solution (final concentrations of 7% skim milk and 30% glycerol, and bacterial abundance eight times their natural abundance) and stored in aliquots of 1 ml at −80°C.

Re-growth of bacterioplankton communities from −80°C stock

To re-grow the bacterial communities of Zoet Water and De Maten from the cryopreserved stocks, six replicate aliquots of each pond community were defrosted and the entire volume of 1 ml inoculated into individual culture flasks (Tissue culture flask 75 cm^2 with filter screw cap, TPP Techno Plastic Products AG, Switzerland) filled with 150 ml of growth medium (i.e., filter-sterilized defrosted pond water) of the respective original ponds, and incubated at 18°C with a 12 h-light/12 h-dark regime. After 48 hours, 5 ml of each bacterial culture was transferred to 145 ml of fresh, sterile growth medium from the respective original pond. After a second incubation period of 48 h, the bacterioplankton communities were used as inocula for the experiment. Per water type (Zoet Water and De Maten), bacterioplankton abundances were determined using microscopic counts on a pooled sample of the six replicates. Briefly, 4%-formaldehyde-fixated samples were stained with

4',6-diamidino-2-phenylindole (final concentration 100 μg ml^{-1}), filtered onto a 0.2 μm filter (0.2 μm GTBP Isopore™ Membrane Filter, Merck Millipore, Germany), and cells were counted using epifluorescence microscopy (magnification × 1000, min. 200 bacterial cells in min. 10 random fields counted; BX51 System Microscopy, Olympus Corporation, Japan) (Porter and Feig, 1980; Rejas *et al.*, 2005).

Experimental set-up and bacterioplankton inoculation

To investigate the occurrence of priority effects over four time lags (0, 12, 36 and 60 h) of inoculation between bacterioplankton communities of Zoet Water and De Maten, four treatments were set up, performed in six replicate units, following a randomized block design (Fig. 1). The bacterial community from Zoet Water was inoculated into 150 ml sterile growth medium from De Maten simultaneously for all treatments, while the original bacterial community from De Maten was inoculated 0, 12, 36 or 60 h later (treatments denoted as Δ0 h, Δ12 h, Δ36 h and Δ60 h respectively). Replicate was introduced as a blocking factor by pairing the same replicates of Zoet Water and De Maten inocula in all four treatments. Corresponding with their inoculation timing and their relation to the growth medium, the Zoet Water community, not *a priori* adapted to the medium, will from here on out be denoted as 'naive pioneers' and the De Maten community, originating from the medium, as 'native second colonists'. The three inoculation time lags were chosen based on the growth curve of both bacterioplankton communities in sterile growth medium from De Maten (Fig. 1C). For treatments Δ12 h and Δ60 h, the native second colonists were inoculated at the middle of the exponential phase of the naive pioneers (after ca. 3.4 and 11.1 generations respectively). In treatment Δ36 h, the native second colonists were inoculated at the stationary phase of the naive pioneers (after ca. 7.8 generations).

Each inoculum consisted of in total ca. 5×10^6 bacterial cells (ca. 3.3×10^4 cells ml^{-1} medium), except for treatment Δ0 h where both bacterial communities were inoculated at equal abundance of ca. 2.5×10^6 cells. The inocula were prepared from the re-grown bacterial communities (six replicates per treatment, see above) by diluting growth medium in physiological water (0.9% NaCl) so that 100–1000 μl of the dilution contained ca. 5×10^6 bacterial cells. Because of the time lag between inoculations of the native second colonists into different treatments, inocula of this community were prepared from re-grown cultures in the following way (Fig. 1A). Per replicate, native second colonists' inocula used for treatments Δ0 h and Δ12 h originated from the same dilutions of re-grown De Maten cultures, for which the dilution factor was based on the initial DAPI-count of bacterial abundances of re-grown cultures, and which were preserved at 4°C during 12 h. The De Maten re-growth cultures were incubated as described above between inoculation time points 0 and 36 h, and were then preserved at 4°C until time point 60 h. The native second colonists' inocula used for treatments Δ36 h and Δ60 h were – for each replicate separately – freshly prepared at these time points from re-grown De Maten cultures, with the dilution factor based on a new DAPI-count of bacterial abundances at time point 36 h. To avoid volume differences between treatments after inoculations, the same volume of physiological water was added at every inoculation moment to each treatment that did not receive an inoculum. A sample for DNA analysis was taken from the 0 to 12 h, 36 h and 60 h inoculum dilutions by centrifuging (7000 g, 20 min, 4°C) 2–4 ml of bacterial suspension, dissolving the pellet in 1 ml of 10 mM Tris-buffer (pH 9.0) and storing at −20°C. Statistical analyses on the inocula described in Supplementary Information S3 show that the inocula were suited to carry out the designed experiment because (i) there was a large, significant difference in community composition between naive pioneers' and native second colonists' inocula, (ii) the variation in community composition of native second colonists' inocula explained by replicate was larger than that explained by inoculation time point, making our design conservative and (iii) community composition of native second colonists' inocula did not differ significantly among inoculation time points.

Bacterioplankton growth and sampling during the experiment

During the experiment bacterial communities were incubated under standard culture conditions (18°C, 12 h-light/12 h-dark regime). To avoid nutrient depletion of the medium and declining bacterial abundances during the growth curve's death phase, 885 μl of each experimental replicate was transferred every 48 hours into 150 ml of fresh De Maten medium (Fig. 1B). According to growth curves determined for both bacterial communities in De Maten medium, 885 μl of medium contained ca. 5.17×10^6 cells, and after every transfer at 48 h the bacterial community went through an exponential growth phase followed by ca. 24 h of stationary phase (Fig. 1C). Eight days after initial inoculation of the naive pioneers (after ca. $4 \times 7.5 = 30.0$ generations), a sample for DNA analysis was taken from each replicate unit by centrifuging (7000 g, 20 min, 4°C) 4 ml of the medium, dissolving the pellet in 1 ml of 10 mM Tris-buffer (pH 9.0) and storing at −20°C.

DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis

Bacterioplankton community structure of the diluted inoculum samples and samples taken at the end of the experiment was analysed by T-RFLP fingerprinting analysis of 16S rDNA, following the protocol as described by Souffreau *et al.* (2014). DNA was extracted using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, California) following the 'Experienced User Protocol' of the manufacturer's manual with one exception: defrosted DNA samples were first centrifuged (12 000 rpm, 15 min, 20°C), had 900 μl of supernatant removed, after which the content of a PowerBead Tube provided with the isolation kit was transferred to each of the DNA sample tubes. Extracted DNA was amplified by polymerase chain reaction (PCR) using labelled universal primers 8–27f 6-FAM (5-AGAGTTTGATCCTGGCTCAG-3) and 907–926r (5-CCGTCAATTCCTTTTAGTTT-3) (Smalla *et al.*, 2007). The PCR mix (total volume 25 μl) contained 2 mM $MgCl_2$, 2.5 μl 10× PCR buffer, 200 μM dNTPs, 0.2 μM of each primer, 0.25 μl Taq-polymerase and 1 μl template-DNA. Thermal cycling conditions included a denaturation of 2 min at 94°C, 25 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, and a final extension of 5 min at 72°C. PCR products were UV-visualized

on a 1.5%-agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany), and subsequently purified using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, USA). DNA concentrations were measured using a Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 260 nm. Digestion was performed in a total reaction volume of 20 μl with ca. 200 ng PCR product and 20 units of the *Hha*I enzyme (Fermentas, Thermo Fisher Scientific) (Smalla *et al.*, 2007), incubated for 4 h at 37°C, and stopped by freezing at −20°C. Digestion products were UV-visualized, purified, and their DNA concentrations measured as described above. Restriction fragments were separated by capillary electrophoresis on an ABI Prism 3130-Avant Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific) using POP 7 polymer. In a total reaction volume of 10 μl, 30 ng of the DNA restriction product was mixed with 0.5 μl of GeneScan™ 1200 LIZ® Size Standard (Applied Biosystems, Thermo Fisher Scientific) and formamide. T-RFLP electropherograms were analysed using GeneMapper® software v.4.0 (Applied Biosystems, Thermo Fisher Scientific) with an analysis range of 30–1200 bp, a variation range of 1 bp and a minimal fluorescence threshold of 30 fluorescence units. To assign each T-RF to the corresponding operational taxonomic unit (OTU), electropherograms from different capillary electrophoresis runs were manually aligned in MS Excel using technical replicates of DNA restriction products included in these runs. Thereafter, no correction of OTU assignment was needed based on the mean size of each T-RF: only two pairs of T-RFs overlapped in mean size ± 1 bp, but in both cases the two T-RFs were simultaneously present in at least one sample. Two replicates of native second colonists' inocula of inoculation time point 0–12 h were removed due to low T-RFLP electropherogram quality. Relative peak height of T-RFs within an electropherogram was used as a proxy for relative abundance of OTUs in the bacterial community.

Statistical analyses

The bacterial community composition data set consisted of 46 samples in total: naive pioneers' inocula (six replicates), native second colonists' inocula 0–12 h (four replicates), 36 h (six replicates), 60 h (six replicates) and the four treatments (each six replicates). Before statistical analyses, OTUs occurring only once in the total data set were removed, where after the data set comprised 40 OTUs. From the total data set three sub-data sets were constructed for specific analyses: one including all 22 inocula, one including the 16 native second colonists' inocula, and a third including the 24 treatment samples (denoted as 'treatment sub-dataset'). All subsequent analyses were performed on Hellinger-transformed relative abundance data. Hellinger transformation is particularly suited to species abundance data as this transformation gives low weights to variables with low counts and many zeros (Legendre and Gallagher, 2001).

To visualize the largest variation in bacterial community composition in the total data set and in the treatment sub-data set, principal component analyses (PCAs) were performed using function *rda* of R-package vegan (Oksanen *et al.*, 2014) in R (version 3.1.2) (R Core Team, 2014). Using function *varpart* of the same R-package we calculated the fractions of variation in bacterial community composition that could uniquely and by interaction terms be explained by inoculum origin (naive pioneers or native second colonists) and replicate (1–6; categorical blocking factor) in the sub-data set containing all 22 inocula, by inoculation time point (0–12, 36 or 60 h; categorical; representing different inocula) and replicate in the sub-data set containing the 16 native second colonists' inocula, and by inoculation time lag (0, 12, 36 or 60 h; categorical or numerical; representing different experimental treatments) and replicate in the treatment sub-data set. Significant effects of these factors in the respective sub-data sets were tested for using partial redundancy analyses (pRDAs). pRDAs – controlling for effect of replicate, inoculum origin, inoculation time point or inoculation time lag – were performed followed by permutation tests (10 000 permutations) using functions *rda* and *anova.cca* of R-package vegan. Inoculation time lag was entered as a categorical or as a numerical factor to test for categorical differences in bacterial community composition of treatments and for an effect of the magnitude of time lag on these communities respectively. Pairwise comparisons of the community composition of native second colonists' inocula from different inoculation time points (0–12, 36 and 60 h; categorical), and of treatments with different inoculation time lags (0, 12, 36 or 60 h; categorical) were performed using pRDA to test for the effects of these categorical grouping variables and the blocking factor replicate between pairs of groups, using functions *rda* and *anova.cca* as above. To correct for multiple testing, p-values of these analyses were adjusted according to Benjamini and Hochberg's method using function *p.adjust* of R-package stats (R Core Team, 2014).

To examine the directionality of community changes (i.e., more/less resembling naive pioneers'/native second colonists' inocula) under influence of inoculation time lag, Euclidean distances within each pair of naive pioneers' or native second colonists' inocula and one of the four treatments were calculated between site scores of paired replicates on the second principal component of the PCA diagram based on the Hellinger-transformed total data set. Whether inoculum-treatment pairs differed in their mean Euclidean distance was formally tested by performing one-way analysis of variance (ANOVA) followed by a *post hoc* test (Tukey's honest significant difference (HSD) test) using functions *aov* and *TukeyHSD* of R-package stats. In the same way, Euclidean distances among each pair of two treatments were calculated using site scores on the first principal component of the PCA diagram based on the Hellinger-transformed treatment sub-data set to explore the magnitude of differences in community composition between treatments. Whether pairs of treatments differed in their mean Euclidean distance was formally tested by performing one-way ANOVA and *post hoc* test (Tukey's HSD test) as described above.

For each OTU occurring in the treatments, effect of inoculation time lag on the OTU's relative abundance was visualized as the deviation from that OTU's mean relative abundance over all four treatments. The significance of each of these deviations was formally tested by a two-sided t-test (expected value equal to 0) using function *t.test* of R-package stats. After

testing homogeneity of variances using function *leveneTest* of R-package car (Fox and Weisberg, 2011), significant differences between treatments in deviations from treatment-mean relative abundance were formally tested for each OTU using one-way ANOVA followed by a *post hoc* test (Tukey's HSD test) as above when variances were equal or by performing non-parametric Kruskal-Wallis rank sum test and multiple comparison test using functions *kruskal.test* and *kruskalmc* of R-packages stats and pgirmess (Giraudoux, 2014) when variances were unequal. In the same way we tested for each OTU whether relative abundances in naive pioneers' and native second colonists' inocula and treatment-mean relative abundance differed from each other. Furthermore, to test for significant changes in an OTU's relative abundance with inoculation time lag regression analyses were performed between these variables by fitting a linear model using function *lm* of R-package stats. The slopes of these regressions for each OTU were subsequently plotted in scatterplots as response variable to the OTU's relative abundance in naive pioneers' and native second colonists' inocula, to the ratio thereof, and to its relative abundance in the control treatment $\Delta 0$ h. Subsequent regression analyses were performed as above to investigate the relationship of these OTU regression slopes with their relative abundance in the inocula and control treatment. Due to the low number of OTUs that could be unambiguously assigned uniquely to one of the two inocula (two OTUs assigned uniquely to the naive pioneers' inoculum; six OTUs assigned uniquely to the native second colonists' inoculum), we could not test formally for an effect of OTU inoculum origin on the change in relative abundance with increasing time lag.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Bar plots visualizing (A) relative abundances and (B) changes in relative abundance for each of the 24 OTUs occurring in the treatments. OTUs are ordered according to their inoculum origin and with decreasing ratios of relative abundance in naive pioneers' (NPi) over native second colonists' (NSC) inocula. In top plot (A), the mean relative abundance of naive pioneers' inocula, native second colonists' inocula and the four treatments are plotted. In bottom plot (B) the deviation in relative abundance from the mean relative abundance of all treatments is visualized for each treatment. Bars represent mean (deviation in) relative abundance of six replicates, and whiskers one standard deviation from this mean. Asterisks above/below individual bars in (B) denote significances of these deviations (i.e., significantly different from zero). Asterisks above groups of 3–4 bars in A and B denote significant differences in (deviation in) relative abundance within these group, while different letters represent significant differences among bars of these groups (significance levels: $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$). White bars in (B) represent OTUs that were not observed in any replicate of that treatment and, therefore, had a constant deviation from treatment-mean relative abundance.

Fig. S2. Scatterplots of relative abundance of OTUs in relation to inoculation time lag between naive pioneers and native second colonists, for all 24 OTUs observed in the treatments. The OTU identifying number is denoted in the upper left corner of each scatterplot, and significance of regression between relative abundance and inoculation time lag is denoted with asterisks in the upper right corner (significance levels: $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$). The regression line and mean relative abundance of the OTU in naive pioneers' and/or native second colonists' inocula are shown in each scatterplot

Fig. S3. Scatterplots of the slopes of the regressions between the OTUs' relative abundances and inoculation time lag (as determined in Supporting Information Fig. S2) in relation to the OTUs' mean relative abundances in naive pioneers' inocula (A), in native second colonists' inocula (B), to the ratio of relative abundance in naive pioneers' over native second colonists' inocula (C), or to the mean relative abundance in the control treatment Δ0h (D). Regressions between these slopes and the (ratio of) relative abundances in the inocula are drawn for data points from all origins, for those from 'both' origin and those from 'both' and naive pioneers ('NPi') origin (i.e., those that should have advantage). *p* values of these regressions are shown to the right of each plot.

Table S1. Environmental characteristics of the two ponds – 'Zoet Water' and 'De Maten' – from which bacterioplankton and water was used during this experiment.

Table S2. Explained variation (R^2) by pRDA and Benjamini and Hochberg-corrected *p* values of permutation tests for effects of inoculation time lag and replicate on final bacterial community composition within each pairwise comparison of treatments (Δ0 h, Δ12 h, Δ36 h and Δ60 h).

Table S3. Bacterial community composition of naive pioneers' and native second colonists' inocula

Table A. Explained fraction of the total variation (R^2) by pRDA and Benjamini and Hochberg-corrected *p* values of permutation tests for effects of inoculation time point and replicate on bacterial community composition within each pairwise combination of native second colonists' inocula (time points 0–12, 36 and 60 h).