



Invasion in microbial communities: Role of community composition and assembly processes

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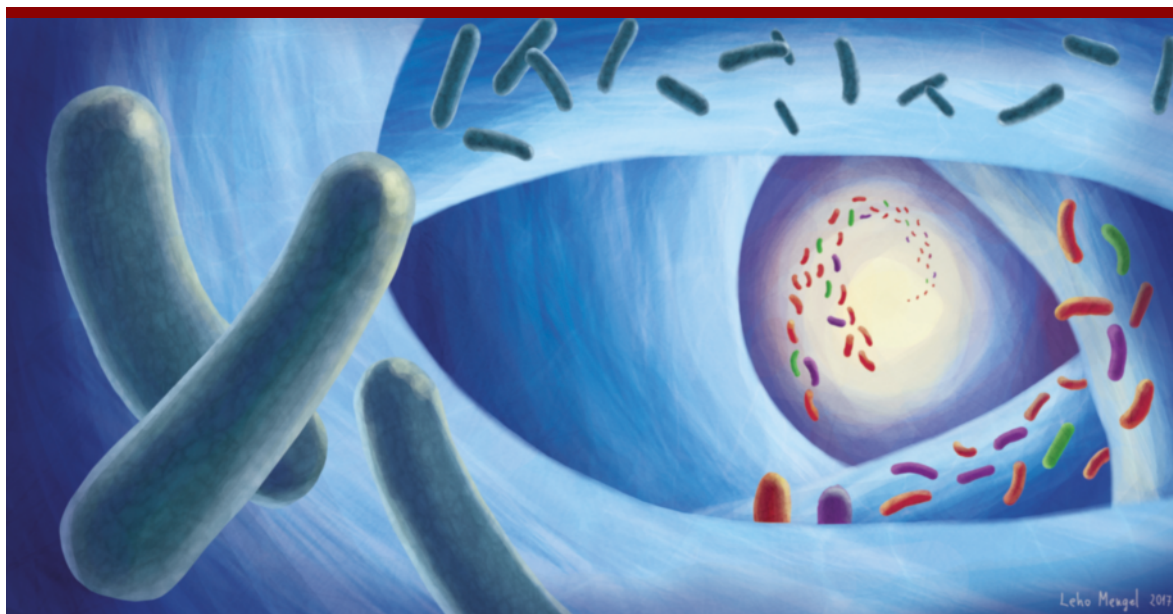
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Invasion in microbial communities: Role of community composition and assembly processes



Marta Kinnunen

PhD Thesis
October 2017

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DTU Environment
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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: <http://www.orbit.dtu.dk>.

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Preface

This thesis is based on research conducted at the Technical University of Denmark, Department of Environmental Engineering from June 2014 to August 2017. This thesis was conducted while participating in PhD training program Mermaid, ITN-EU-FP7 funded by the People Programme (Marie Skłodowska-Curie Actions). The research was performed under the main supervision of Professor Barth F. Smets and co-supervision of Senior Researcher Arnaud Dechesne and Professor Hans-Jørgen Albrechtsen.

The thesis is organized in two parts: the first part puts the overall approaches and findings of the PhD study in context and reviews the state-of-the-knowledge; the second part consists of the papers listed below that constitute the core scientific outcomes of the research. These will be referred to by their paper number written with the Roman numerals **I-IV**.

- I Kinnunen, M.**, Dechesne, A., Proctor, C., Hammes, F., Johnson, D., Quintela-Baluja, M., Graham, D., Daffonchio, D., Fodelianakis, S., Hahn, N., Boon, N., Smets, B.F (2016) A conceptual framework for invasion in microbial communities. *The ISME Journal*. **10**: 2773–2775.
- II Kinnunen, M.**, Gülay, A., Albrechtsen, H.-J., Dechesne, A., and Smets, B.F. (2017) Nitrotoga is selected over Nitrospira in newly assembled biofilm communities from a tap water source community at increased nitrite loading. *Environmental Microbiology* **19**: 2785–2793.
- III Kinnunen, M.**, Palomo, A., Fowler, J., Albrechtsen, H.-J., Dechesne, A., and Smets, B.F. Switching from ammonium to nitrite feeding induces a differential loss of comammox Nitrospira from a rapid sand filter community. *Submitted to FEMS Microbial Ecology*
- IV Kinnunen, M.**, Albrechtsen, H.-J., Dechesne, A., and Smets, B.F. Neutral processes govern invasion success in microbial communities when invader is phylogenetically similar to the resident community. *Manuscript in preparation*

In this online version of the thesis, paper **I-VI** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

Over the course of the PhD, the following contributions to international conferences were made with the following proceeding and conference papers:

Kinnunen, M., Dechesne, A., Albrechtsen, H-J., Smets, B.F. Invasion of nitrite oxidizer dominated communities: interactions between propagule pressure and community composition. Oral presentation at 2nd International symposium of Microbial Resource Management (MRM), 2017 in Ghent, Belgium

Kinnunen, M., Palomo, A., Fowler, J., Dechesne, A., Albrechtsen, H-J., Smets, B.F. Dramatic loss of comammox *Nitrospira* associated with long-term nitrite feeding. Poster presentation at 5th International Conference of Nitrification and Related Processes (iCoN), 2017 in Vienna, Austria

Kinnunen, M., Dechesne, A., Albrechtsen, H-J., Smets, B.F. De novo biofilm community assembly from tap water source communities favours *Nitrotoga* over *Nitrospira* under elevated nitrite surface loading. Poster presentation at 16th International Symposium on Microbial Ecology (ISME), 2016 in Montreal, Canada

Kinnunen, M., Dechesne, A., Albrechtsen, H-J., Smets, B.F. Establishing drinking water biofilms with varying alpha-diversity. Poster presentation at IWA joint specialist conference of Microbial Ecology in Water Engineering & Biofilms (MEWE), 2016 in Copenhagen, Denmark

Kinnunen, M., Dechesne, A., Albrechtsen, H-J., Smets, B.F. A novel high-throughput drip-flow system to grow autotrophic biofilms of contrasting diversities. Poster presentation at 13th Symposium on Bacterial Genetics and Ecology (BAGECO), 2015 in Milan, Italy

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Last but not least, I wouldn't have been able to do this without my mom and sisters! You have always supported me in whatever I have decided to do, and your unconditional love means the world to me!

Summary

Microbes contribute to all biogeochemical cycles on earth and are responsible for key biological processes that support the survival of plants and animals. There is increased interest in controlling and managing microbial communities in different ecosystems in order to make targeted microbiological processes more effective. In order to manage microbial communities, it is essential to understand the factors that shape and influence microbial community composition. In addition to abiotic factors, such as environmental conditions and resource availability, biotic factors also shape the dynamics of microbial community assembly. Biotic factors include interactions between different microbial groups as well as the community response to alien species – invaders.

Microbial invasions can have significant effects on the composition and functioning of resident communities. There is, however, lack of agreement on the core determinants of invasion in microbial communities. Current models and concepts for invasion in microbial ecology are largely based on the macro-ecology literature. Although attempts have been made to examine the applicability of these concepts to microbial communities, a general conceptual framework for microbial invasion applicable across ecosystems is missing. The overall aim of this PhD project was therefore to propose a conceptual framework to study microbial community invasion and to test this framework against experimental observations.

Based on a synthesis of earlier frameworks on invasion and community ecology, I defined invasion in a microbial community as the establishment of an *alien microbial type* in a *resident community* and have proposed simple criteria to define aliens, residents, and alien establishment, applicable across a wide variety of communities. I suggested the adoption of the community ecology framework advanced by Vellend (2010) to identify determinants of invasion. This framework lists the four fundamental processes that govern community assembly as: dispersal, selection, drift and diversification. We have suggested that it is important to determine which processes dominate the assembly of a resident community in order to understand what governs invasion in that community.

To test invasion in microbial communities while controlling the processes driving community assembly, I developed a high-throughput flow-through experimental microcosm system that enabled me to manipulate the relative

importance of selection versus drift during initial community assembly. I used this new system to establish resident microbial biofilm communities dominated by nitrite-oxidizing bacteria, where the direction of selection as well as contribution of drift was manipulated through differential nitrite loading rates.

Subsequently, I experimentally characterized the community assembly processes in the biofilm communities, using replicate communities assembled under same conditions. Both total community and guild-level analyses provided evidence for contribution of neutral processes (drift) combined with selection. More precisely, I observed the deterministic enrichment of certain types of nitrite-oxidizing bacteria in the biofilms: elevated nitrite loading selected for a single *Nitrotoga* representative, while lower nitrite conditions selected for a number of *Nitrospira*.

I then repeated the assembly experiment and subjected the assembled biofilms to invasion by a *Nitrotoga* HW29 culture. I found no significant (negative) correlation between community diversity and invasion success, in contrast with the often cited diversity/invasibility relationship. Instead, I observed that at high phylogenetic similarity between invader and resident types, the effect of selection is surpassed by the effect of drift on invasion success. My results suggest that controlling invasion in communities that contain members that are phylogenetically similar to the invader is nearly impossible because stochastic processes determine the invasion outcome when selection towards invader and resident community is similar.

In conclusion, during this PhD project I proposed a simple conceptual framework to study and characterize microbial invasions. This conceptual framework allows comparison of experimental observations across ecosystems using a coherent ecological terminology and consideration of community assembly processes rather than indices to describe community composition. Furthermore, I experimentally identified the dominant processes in newly assembled biofilms enriched in nitrite-oxidizing guilds before subjecting the resident guilds to invasion by an alien nitrite-oxidizer. The results of my experiments indicate that neutral processes have significantly higher contribution to community assembly – including invasion – than previously suggested.

Dansk sammenfatning

Mikroorganismer bidrager til alle jordens biokemiske kredsløb og er ansvarlige for centrale biologiske processer for overlevelsen af planter og dyr. Der er øget interesse for at styre og håndtere mikrobielle samfund i forskellige økosystemer for at gøre specifikke mikrobielle processer mere effektive. For at styre disse mikrobielle samfund er det vigtigt at forstå de faktorer, der skaber og har indflydelse på sammensætningen af de mikrobielle samfund. Ud over abiotiske faktorer som miljøforhold og tilgængelighed af resurser, er de biologiske faktorer med til at skabe dynamikken i dannelsen af mikrobielle samfund. Dette omfatter blandt andet interaktionen mellem forskellige mikrobielle grupper og samfundets reaktion på fremmede arter – angribere.

Mikrobielle invasioner kan have betydelig effekt på sammensætning og funktion af det beboede samfund. Der er dog ikke enighed om, hvilke betydende faktorer, der ligger til grund for invasion af mikrobielle samfund. De nuværende modeller og koncepter om invasion i mikrobiel økologi er baseret på litteratur om makro-økologi. Selvom der er gjort forsøg på at undersøge anvendelsesmulighederne af disse koncepter i mikrobielle samfund, mangler der en grundlæggende konceptuel ramme for mikrobiel invasion, som er anvendelig på tværs af økosystemer. Det overordnede mål for dette PhD-projekt var derfor at foreslå en konceptuel ramme, indenfor hvilken man kan studere invasion i mikrobielle samfund, og at holde denne ramme op mod eksperimentelle observationer.

Baseret på en syntese af tidligere konceptuelle rammer for invasion og økologi af samfund, har jeg defineret invasion af et mikrobielt samfund som etablering af en *fremmed mikrobiel type* i et *beboet samfund*, og jeg har foreslået simple kriterier for at definere fremmede, beboere og etablering af fremmede, hvilket kan anvendes på tværs af en bred vifte af samfund. Jeg har foreslået, at man anvender den samfundsøkologiske ramme, som er udviklet af Vellend (2010) til at identificere betydende faktorer for invasion. Denne ramme indeholder fire grundlæggende processer, som er afgørende for samfundsdannelse: dispersion, selektion, drift og diversificering. Vi har foreslået, at det er vigtigt at afgøre, hvilke processer der dominerer dannelsen af et beboet samfund for at forstå, hvad der er afgørende for invasionen af et givent mikrobielt samfund.

For at undersøge invasion i mikrobielle samfund samtidigt med at kunne kontrollere de processer, der driver dannelsen af samfundet, har jeg udviklet et eksperimentelt *high-throughput* gennemstrømnings-mikrokosmos, som gjorde mig i stand til at regulere selektion i forhold til drift i den tidlige dannelse af samfundet. Jeg brugte dette nye system til at danne beboede mikrobielle biofilmsamfund, som var domineret af nitrit-oxiderende bakterier, hvor selektionsretningen samt indflydelsen af drift blev reguleret ved forskellige nitrittilførringsrater.

Dernæst karakteriserede jeg dannelsesprocesserne af biofilmsamfundet. Både analyser af hele samfundet og af funktionelle grupper gav bevis for bidrag af neutrale processer (drift) kombineret med selektion. Mere præcist så jeg en deterministisk berigelse af visse typer af nitritoxiderende bakterier i biofilmen: øget nitrittilførsel selekterede for en enkelt *Nitrotoga* art, imens forhold med lavere nitrittilførsel selekterede for en række af *Nitrospira* arter.

Dernæst gentog jeg dannelseseksperimentet og udsatte den nyligt dannede biofilm for invasion af en *Nitrotoga* HW29 kultur. Jeg fandt ingen signifikant (negativ) sammenhæng mellem diversitet i samfundet og invasionssucces, i modsætning til det ofte citerede diversitets/invaderbarheds-forhold. I stedet så vi, at ved høj fylogenetisk lighed mellem angriber- og beboertyperne blev effekten af selektion overgået af effekten af drift med hensyn til succes af invasionen. Mine resultater tyder på, at det næsten er umuligt at styre invasion i fylogenetisk ens samfund, da stokastiske processer bestemmer udfaldet af invasionen, ved tilsvarende selektion mod angriber og beboer.

Gennem dette PhD-projekt har jeg foreslået en simpel konceptuel ramme til at studere og karakterisere mikrobielle invasioner. Denne konceptuelle ramme tillader sammenligning af eksperimentelle observationer på tværs af økosystemer ved hjælp af en sammenhængende økologisk terminologi og ved at inddrage samfundsdannelsesprocesser i stedet for eksklusive indicier til at beskrive sammensætningen af samfund. Jeg har desuden studeret de mest dominerende processer i nyligt dannede biofilm beriget med nitritoxiderende grupper, før jeg udsatte dem for invasion af en fremmed nitritoxiderende bakterie. Resultatet af mine forsøg indikerer, at neutrale processer yder et signifikant højere bidrag til dannelsen af samfund - såvel som invasion – end førhen antaget.

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1 Introduction

1.1 Motivation

Microbes are some of the key players in all ecosystems on Earth, driving several major biogeochemical cycles. Most of these cycles are driven by microbes with different functional roles interacting within a community. Therefore, within microbial communities, the individual microbes can influence each other's behaviour and subsequently the overall functioning of the community. Consequently, understanding the processes that shape and influence these microbial communities is essential for controlling and managing biological systems. The need for conceptual principles complementing the observations and descriptions of microbial communities has led to the development of a new concept: Microbial Resource Management (MRM). This concept aims to solve practical problems associated with biological processes by answering the questions of who is there, who is doing what with whom and how can one adjust, control and/or steer these mixed cultures and communities (Verstraete *et al.*, 2007). However, in order to answer these questions, ecological frameworks are needed that are applicable to all biological systems and describe all possible processes and interactions happening in microbial communities.

One of these influential processes, with the potential to alter community composition and functioning, is invasion by new microbial types. There is growing interest in controlling microbial invasions, whether promoting invasions to increase community functioning by adding microbial types with different metabolic capabilities, or avoiding invasion by potentially harmful or pathogenic microbial types. Yet, the mechanisms facilitating microbial invasions are largely unknown. It is not clear, who is interacting with whom during the invasion and how can we steer the microbial resources to facilitate desired outcome of invasion. Microbial invasions are important in different natural (aquatic and terrestrial) systems. Here, we focus on drinking water microbial communities as model systems.

Drinking water has been shown to have a diverse microbiome, consisting of microorganisms competing for limited nutrient availability. A stable microbial community is required to provide safe drinking water for human consumption. Fluctuations in drinking water microbial community can result in changes of water quality with subsequent negative effects on distribution

systems and final consumers, which can be linked to health hazards caused by invasion of unwanted microorganisms (pathogens).

Therefore, a better understanding of microbial invasion in drinking water systems could have significant pay-off in providing biologically safe drinking water. More generally, it is surmised that the conclusion made within this model system are be applicable across many domains including management of human and animal diseases, development of plant growth promoting inocula and bioaugmentation efforts in food and environmental industries.

1.2 Aim of the PhD thesis

In this PhD thesis, the processes governing invasion in microbial communities are addressed using drinking water microbial communities as model systems. More specifically, the objectives of this PhD were to:

- Synthesize the state-of-the-knowledge to provide a comprehensive conceptual framework to study invasion in microbial communities based on community assembly processes
- Develop and test a novel experimental system for studying invasion in microbial communities with controlled manipulation of community assembly and composition
- Using nitrite oxidizing bacteria as an example, investigate the extent of deterministic and stochastic community assembly processes in microbial guild assembly
- Quantify and discern the interactions between community assembly processes and invasion success in a model microbial guild of nitrite oxidizing bacteria

2 Background and state-of-the-art

2.1 Community assembly processes

It has been long suggested that diversity plays an important role in ecosystem functioning (Elton, 1958). Most observations regarding community assembly, its resilience, resistance and other relevant properties for stable functioning are made based on community diversity (see Table 2.1 for terminology) (Langenheder *et al.*, 2012; Philippot *et al.*, 2013; Steiner, 2014; Gibbons *et al.*, 2016). Therefore, it is important to understand what shapes community diversity. In order to explain the diversity in any given community, we need to reflect on processes governing community assembly and maintaining the diversity. While, the governing processes of microbial community assembly have shown to be largely dependent on the environment (May and MacArthur, 1972; Langenheder and Ragnarsson, 2007), the stage of community assembly (Langenheder and Szekely, 2011; Hao *et al.*, 2016) and the identity of the community members (Kraft *et al.*, 2007), several attempts to make universal assumption of community assembly processes have been made.

Table 2.1 Terminology and definitions used in community ecology

Term	Definition	Adapted from
Community assembly	The sum of all processes that shape the composition of a microbial community, including dispersal, selection, drift and speciation	Vellend, 2010
Microbial community	Group of potentially interacting microbial types that co-occur in space and time	Nemergut <i>et al.</i> , 2013
Resident community	A specific community considered in the context of possible invasion	Paper I
Metacommunity	A set of local communities that are linked by dispersal of multiple interacting species	Leibold <i>et al.</i> , 2004
Local community	The individuals of all species that potentially interact within a single patch or local area of habitat	Leibold <i>et al.</i> , 2004
Alien type or invader	A microbial type that was not part of the resident community prior to point of observation	Davis <i>et al.</i> , 2005
Invasion	Entry and establishment of an alien type in a resident community	Blackburn <i>et al.</i> , 2011
Establishment	The maintenance of an active population of an alien type in a resident community	Paper I
Community resistance	The ability of a community to withstand change in the face of a potentially disturbing event (invader)	Levine and D'antonio, 1999
Community resilience	The rate of recovery to original state after the disturbance	Shade <i>et al.</i> , 2012

Two ecological theories of community assembly have received most focus and are often referred to when interpreting the composition of microbial communities; the niche theory (Box 1) and the unified neutral theory of community assembly (Box 2). Later, theories combining both have been proposed (Tilman, 2004; Leibold and McPeck, 2006; Latombe *et al.*, 2015), based on the knowledge that ecological communities are rarely governed by single dominating process (Stegen *et al.*, 2016).

Box 1: Niche theory

A niche is an n-dimensional volume, where dimensions (parameters) including environmental conditions, resources and interactions between community members, define the requirements of an individual to live, more particularly, for its population to persist (Hutchinson, 1957). According to niche theory, the community forms as a result of parameters available for each individual. When one parameter the individual requires for growth is limited, the only individual that can maintain this specific parameter at the lowest level, while still being able to reproduce, will outcompete all others in that given niche. When two or more parameters are limited, two or more different individuals can coexist when they are able to trade off in their ability to utilize those resources. However, if the resources fluctuate over space and time, two or more different individuals can coexist, using the same resource, when they are able to trade off in their abilities to utilize the resource. (Vandermeer, 1972)

It is challenging to experimentally quantify the niche of an individual due to the abstract definition of *niche*. As an obvious approach when defining the niche of any individual, most experimental studies of community assembly focus on resource availability (Waldrop *et al.*, 2006; Litchman *et al.*, 2015; Ma *et al.*, 2015; Larson *et al.*, 2016), substrate affinity of different community members (Maixner *et al.*, 2006), or known environmental conditions required by these individuals (e.g. optimum temperature, pH, availability of oxygen) (Princic *et al.*, 1998; Downing and Nerenberg, 2008; Alawi *et al.*, 2009).

While niche theory considers differences between the niches of different individuals to be essential for explaining which species coexist, neutral theory considers niche differences to be irrelevant (Box 2). Hubbell (2001) states that “niche differences are unnecessarily complex and that the coexistence of species is a result of ‘chance’, along with historically-contingent initial population size”. In other words, the community assembly is a result of continuous cycles of immigration, births and death (Sloan *et al.*, 2006). If the neutral theory is true, then any free space could be filled

randomly by any individual from within or from outside the community, but most probably it is filled by the one that is most abundant (Burke *et al.*, 2011). These assumptions can all be tested, because if a community is neutrally assembled then the abundance of each community member in the metacommunity can be used to predict the frequency and abundance with which a given community member is observed in the local community (Sloan *et al.*, 2006).

Box 2: Neutral theory

Neutral theory is based on the hypothesis of *functional equivalence*. According to neutral theory all individuals in a community are equivalent regarding their prospects of reproduction and death. The community assembly is a result of species extinction and immigration or speciation of new species. (Hubbell, 2005)

Neutral community models (NCM) can successfully reproduce the observed species abundance distribution in communities of macro-organisms (Hubbell, 2001) as well as micro-organisms (Sloan *et al.*, 2006; Woodcock *et al.*, 2007). In this PhD project, we focus on microbial communities, therefore we follow the *near-neutral model for prokaryotes* proposed by Sloan *et al.* (2006). Although, core concepts of the earlier neutral model by Hubbell (2001) are retained, two main differences are noted. First, Sloan *et al.* model allows individuals to have competitive advantage or disadvantage by introducing a new advantage parameter allowing relaxing the assumption of equivalent specific growth rate in all individuals. Although, when given parameter is set to zero, as it was done in simulations part of this PhD project, the original assumption of functional equality is followed. Second, the model was translated into a continuous form so that it could be applied to large population sizes typical for microorganisms. The model uses the relative abundance of microbes in metacommunity to predict their establishment frequency in replicate local communities. Briefly, the model assumes that the community is saturated with a total of N_T individuals and that an individual must die or leave the system for the composition to change. To characterize this in the model, a species-independent rate δ is introduced. The dead individual is then immediately replaced by an immigrant from a source community, with probability m , or by reproduction of a member of the local community with probability $1 - m$ (Sloan *et al.*, 2006).

Within microbial community ecology, evidence of community assembly following the niche theory exists. Moroenyane *et al.* (2016), for example, observed strong clustering of biological replicates, indicating strong

deterministic assembly. Other studies provide support for neutral theory. For example, Burns *et al.* (2015) showed that the intestine microbial community of zebrafish mostly follows the NCM model described above. Most commonly; however, a combination of both is suggested (for example Dumbrell *et al.* (2010) and Lee *et al.* (2013) all observe significant fit with the NCM model, but also see strong clustering of replicate samples as a result of environmental conditions). These different observations indicate that it is important to acknowledge the relative importance of niche-based and neutral/stochastic processes, rather than trying to determine which theory alone provides better explanation for community structure and diversity.

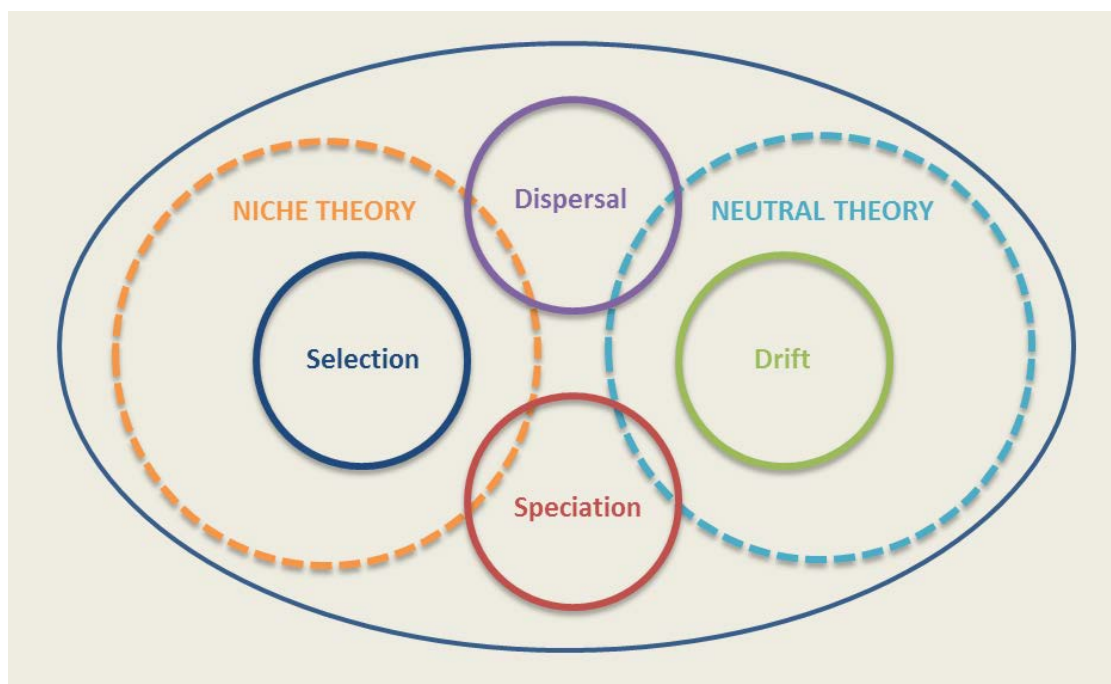


Figure 2.1. Conceptual affiliation of Vellend's community ecology processes within the niche and neutral theories

Recently, Vellend (2010) proposed a conceptual framework for community assembly considering the relative importance of different community assembly processes. Instead of grouping governing forces explicitly into niche or neutral theory frameworks, Vellend emphasizes that all community ecology processes are ultimately made of four processes – classified as selection, dispersal, drift and speciation – therefore, these processes have features of both, niche and/or neutral theory (**Figure 2.1**)

Selection is a process whereby changes in community composition are caused by deterministic fitness differences between types. Selection is the result of biotic and abiotic pressures causing variation in reproductive success across

individuals and species. Selection process is probably the closest one to the niche theory assumption, used to describe the deterministic fitness differences among individuals of different species. Just like in the niche theory, selection occurs when individuals in a community vary in some respect.

Dispersal is a process representing the movement of organisms across space, considering the immigration in and emigration out of the community. Dispersal process has features of both niche and neutral theory. For example, if selection favours specific individuals in a community, the overall diversity is usually decreased. Dispersal can, however, increase this diversity by continuous addition of individuals that might otherwise have fitness disadvantage in that community, but are able to persist due to stochastic component of the assembly supported by strong immigration.

Drift is a process whereby changes in the relative abundance of individuals in a community are caused by stochastic processes. Drift is probably closest to neutral theory assumptions that all changes in community composition are stochastic. If drift dominates the community assembly, then eventually all but one species will drift to extinction. In drift dominated communities, the probability of each species achieving dominance is related to their initial abundance in the metacommunity.

Speciation is a process where increase in diversity is caused by the generation of new genetic variants.

Table 2.2 Overview of the main concepts used to interpret the processes of community assembly

Concept	Explanation	Reference
Drift inferred from within-group dissimilarities	Low within-group dissimilarities indicate strong deterministic influence on community assembly, suggested from high similarity of replicate communities	Evans <i>et al.</i> , 2017
Selection inferred from between-group dissimilarities	If the centroids of communities assembled from the same source community are dissimilar then this indicates strong effect of determinism in the community assembly.	Evans <i>et al.</i> , 2017

2.2 Theoretical frameworks of invasion ecology

Biological invasions can have significant effect on altering the community composition and functioning. Therefore, understanding biological invasions is central to successful control and management of biological communities. The determinants of invasion have been tested using different taxa and varying environments, and different research communities have therefore adopted different theoretical frameworks. This results in a range of concepts, terms and definitions being used to interpret the experimental observations. Synonymous terms are used to describe the same processes and at the same time different definitions are proposed for the same terms. This affects the definition of everything from invader and resident/native species to what is considered as successful invasion. In this chapter I will briefly discuss the frameworks in invasion ecology of macro organisms and the attempts that have been made to explain invasion in microbial communities.

Most animal ecologists follow the theoretical framework proposed by Williamson and Fitter (1996). This framework is based on ‘the tens rule’ that states: “for a variety of British groups of animals and plants, the statistical rule holds that 1 in 10 of those imported appear in the wild, 1 in 10 of those introduced become established, and 1 in 10 of those established become a pest”. This framework focuses on invasion of entire natural areas (even countries) and does not consider specific resident communities. From the perspective of the invaded area (country), they introduce four different terms to distinguish among non-native species (invaders): ‘imported’, ‘introduced’, ‘established’ and ‘pest’ (see Table 2.3). The framework then elaborates on four exceptions to the ‘tens rule’ primarily linked to island geography and dispersal limitations. Williamson’s framework provides three sets of factors that are suggested to be important in explaining invasion success. The first is propagule pressure, the rate at which propagules, seeds, breeding individuals, and so on, are released. The second is the set of factors that allow species to survive, and multiply, from low densities. The third is the set of factors that determine local abundance. Williamson and Fitter acknowledge that their framework is only the first step to developing a more precise and general framework. The authors agree that propagule pressure and population dynamics are both involved, but also point out that both deserve better quantification and the involvement of these processes is unclear and not elaborated on in this framework.

Table 2.3 Different terminology with the same definition that has been used to define invader type in two different frameworks suggested for macro-organisms, and the added category representing the given invader in different stage (see Figure 2.2 for description of stages) in the unified framework

Term ¹	Term ²	Category ³	Adapted general definition
Imported	Alien	B1, B2 and B3	Invader type brought into the country (due to human-mediated transport)
Introduced	Casual alien	C0	Invader type found in the wild and able to occasionally flourish but eventually dies out
-	-	C1, C2	Invader type surviving in wild without reproduction or self-sustaining population
Established	Naturalized	C3	Invader type is able to maintain self-sustaining population (for at least 10 years in Richardson's framework)
-	-	D1, D2	Self-sustaining population of invader type with individuals surviving (and reproducing) at a significant distance from point of introduction
Pest	Invasive	E	Invader-type is fully established with wide dispersal range and reproduction. In Williamson this type also needs to have negative economic effect

¹ Williamson et al. ² Richardson et al. ³ Blackburn et al.

Richardson and Pyšek (2006), on the other hand, suggested a framework explicitly focusing on invasions in plant ecology through human-mediated introductions. The primary difference from Williamson's framework is that it focuses explicitly on plant invasions. Similarly to Williamson, they focus on invaded areas and not the invaded communities. Richardson's framework introduces a sequence of (geographical) barriers that a plant species need to overcome to establish as an invader. They argue that a plant is invasive only when it overcomes geographic, environmental, reproductive and dispersal barriers. They also define the invader differently depending on the barrier it is facing: 'aliens', 'casual aliens', 'naturalized' and 'invasive' plants. Although, in general, the stages follow the same distinguishing approach as proposed by Williamson (see Table 2.3). Richardson also refers to 'the tens rule' and agrees that stochastic factors, like propagule pressure contribute to the invasion success. For an introduced plant to invade a new region, two options are suggested in this framework: the plant must either possess

sufficiently high levels of physiological tolerance and plasticity or it must undergo genetic differentiation to achieve required levels of fitness.

As presented above, the two first frameworks view the process of invasion similarly, but have adopted various definitions for some processes (Table 2.3). Therefore, Blackburn *et al.* (2011) suggested merging the two above-mentioned frameworks, claiming that similarities within the two frameworks outweigh the differences. After some clarification and alteration Blackburn *et al.* designed a new framework with the aim of being applicable to all invasions, regardless of taxa or location. The framework is suggested to be an improvement because it now explicitly separates the stages of invasion by barriers introduced by both Richardson and Williamson (Figure 2.2). Furthermore, the unified framework introduces an additional barrier to invasion: human-imposed barrier originating from growing plant and animal species in captivity and/or cultivation. By revising the stages and barriers, Blackburn also elaborates on the definitions by adding additional categories to describe invader type between barriers with even more detail depending on its survival, and (self-sustaining) reproduction, and spread.

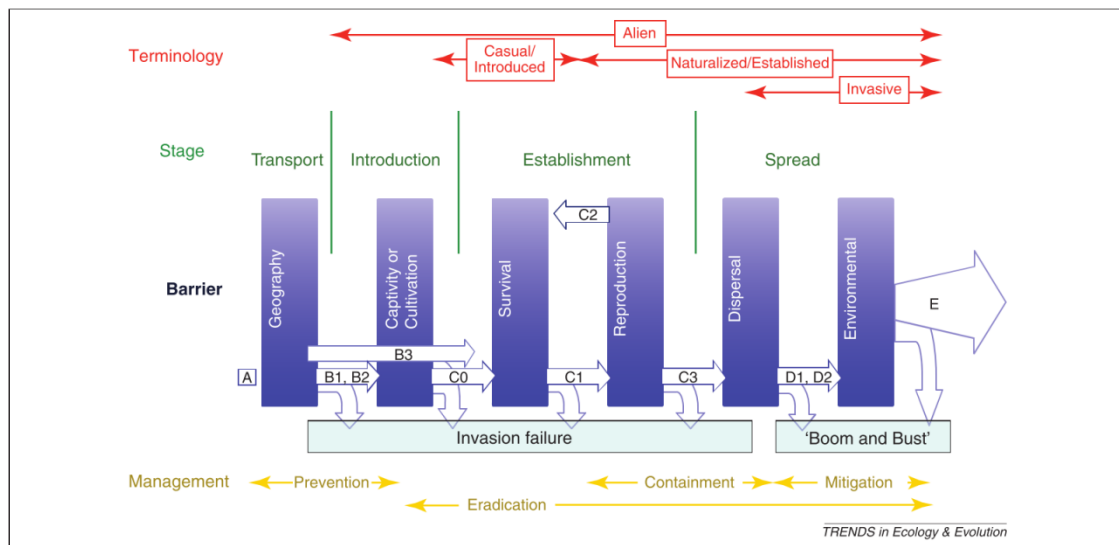


Figure 2.2 The proposed unified framework for biological invasions by Blackburn *et al.* (2011) The proposed framework recognizes that the invasion process can be divided into a series of stages, that in each stage there are barriers that need to be overcome for a species or population to pass on to the next stage, that species are referred to by different terms in the terminology depending on where in the invasion process they have reached, and that different management interventions apply at different stages. Different parts of this framework emphasize views of invasions that focus on individual, population, process, or species. The unfilled block arrows describe the movement of species along the invasion framework with respect to the barriers, and the alphanumeric codes associated with the arrows relate to the categorization of species with respect to the invasion pathway (see synthesis of the terminology in Table 2.3)

Indeed, Blackburn’s attempt for a unified framework is a major advancement; however, even though they claim that it could be applied to all invasions, regardless of taxon or environment, it is challenging to use this to determine invasion in microbial communities, primarily, because none of the described frameworks is community based. There are multiple factors suggested that determine the invasion probability in microbial communities (Figure 2.3). For example, microbes, due to their small sizes, fast growth and large populations, are thought to disperse faster and over longer distances than most macro-organisms. Hence, oftentimes the stages proposed in the above-mentioned frameworks are not explicitly detected in microbial invasion. Because methods are used only to test the establishment, and conclude only whether or not the invader type is present. Of course, with well-designed time series sampling, self-sustaining reproduction as well as spread could be confirmed if surrounding areas are sampled. However, it is difficult to determine if a microbial type is an ‘alien’ or a ‘casual alien’, because it is suggested that “everything is everywhere and the environment selects”. Although, if dispersal barriers exist for microbes, and we now know that there is some biogeographical limitation to microbial dispersal (Martiny *et al.*, 2006), then microbial invasions may be qualitatively, if not quantitatively, interpreted similarly to macro-organisms in their dispersal patterns (Litchman, 2010).

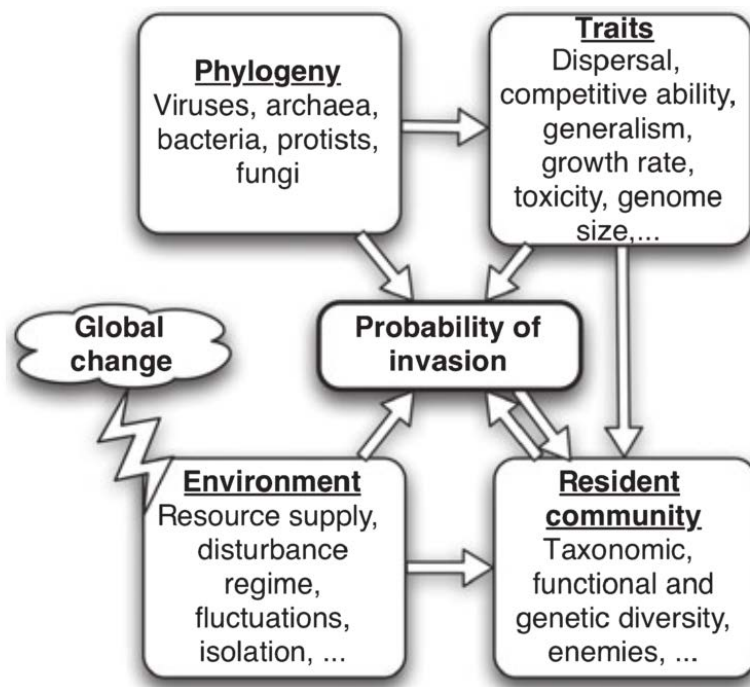


Figure 2.3. Conceptual diagram showing the influence of multiple factors determining invasion probability for microbes according to Litchman (2010).

The first conceptual framework specifically addressing microbial invasion in microbial communities was proposed by Mallon *et al.* (2015a). This framework defines the process of microbial invasion and acknowledges the difficulty of tracking microbial invasions from start to finish. Therefore, they simplify the stages and barriers approach described for macro-organisms to become more applicable in microbial ecology (Figure 2.4). They attempt to answer key questions posed by Litchman (2010), like what are the key traits associated with high invasive potential and what are the community and ecosystem characteristics that facilitate microbial invasion (Figure 2.4).

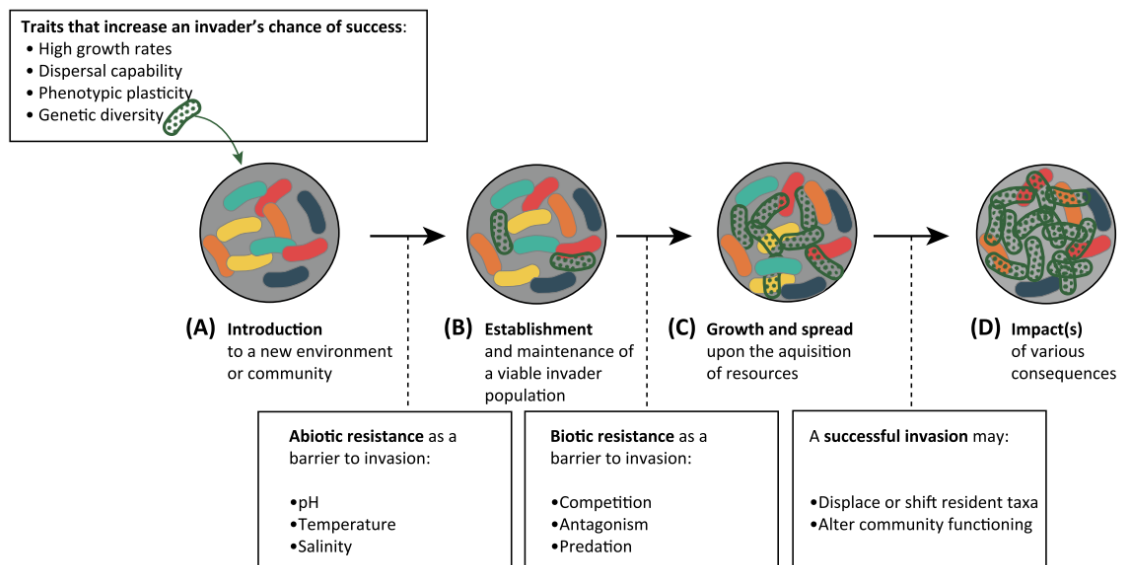


Figure 2.4 A successful invasion is depicted as a four-step process, envisioned through both the invader and resident community perspectives. The green, polka-dotted microbe represents an invader that is introduced into a community with various microbial taxa depicted in different colours. The same community is represented as it transitions through each phase of the invasion process. Each phase is a key step with a particular 'barrier value', meaning that there may be large (insurmountable) or small (surmountable) barriers on the path to a successful invasion. From an invader's perspective, several of the listed factors may aid in bypassing the barriers of invasion resistance. (A) Should an invader be able to withstand the abiotic pressures of a new environment it is then considered to be introduced. (B) Biotic resistance will keep the invader in the establishment phase as it maintains a viable population. (C) If an invader gains access to resources that support its growth, it will pass to the growth and spread phase. (D) Growth and spread of the invader may cause impacts to the resident community (from Mallon *et al.*, 2015a).

Mallon's framework can be used to follow invasion of microorganisms and determine the stages of invasion. However, it still lacks depth in describing the determinants that control the suggested processes. They see diversity-invasion effect (explained in detail in the next paragraph) as the primary mechanism that drives invasion success in most natural microbial

communities. It is; however, important to recognise, that diversity is maintained through active community assembly processes and that similar diversity can be because of different processes (Shade, 2017). Hence, the behaviour (as well as resistance to invasion) of two microbial communities with identical diversity can be completely different. Furthermore, it is unlikely that all microbial communities are inherently stable in their resource utilization and diversity, and the resistance to invasion therefore is a process rather than a single event.

Therefore, we suggest that a consistent set of definitions and a rigorous conceptual framework are still needed and we proposed new conceptual framework for invasion in microbial communities based on the community assembly processes (**Paper I**).

Within this framework, we proposed to follow a simple and inclusive definition of an invader and resident community, which is commonly used in ecology (Table 2.1). These definitions are very general, as they make no assumptions regarding community properties. Furthermore, we emphasise that invasion does not necessarily have to have negative consequences.

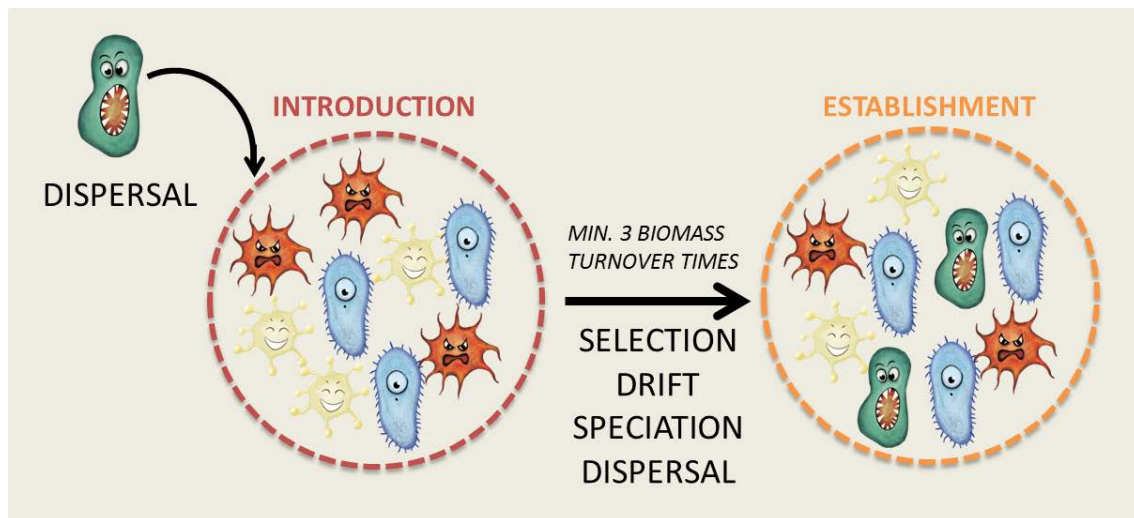


Figure 2.5 A successful invasion is considered to be a two-step process in our new conceptual framework, where introduction of an alien type is facilitated by dispersal processes and the establishment is determined by selection, drift and speciation processes

We relax the constraints of following subsequent stages of invasion, monitoring only introduction and establishment (Figure 2.5) should be sufficient (i.e. no spread is needed for an alien type to be called invader). This is because we focus on invasion of microbial communities, and if after establishment, the invader spreads to microbial community, where it is not present, it can be considered as a new (separate) invasion event.

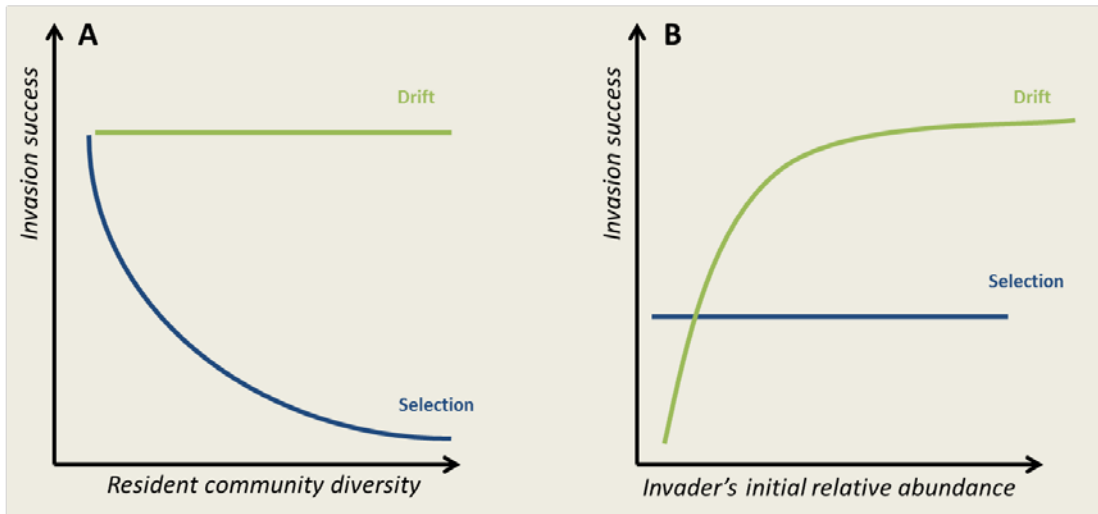


Figure 2.6 Hypothetical relation between average invasion success and (a) resident community diversity, and (b) initial relative abundance of the invader, in communities where either competition (selection) or drift govern the community assembly. In *a*, the exponential curve is illustrative; the curve can be of any monotonic form, with the rate of decline depending on the distribution of the competitive abilities of the resident microbes relative to that of the alien. In *b*, the curve associated with drift highlights the fact that, in a drift-only situation, invasion success increases rapidly with the size of the invading population (Paper I).

Furthermore, we proposed that adopting the framework of community ecology developed by Vellend (2010) can further clarify the determinants of invader success. The invader is introduced to the resident community through dispersal processes. Invader type(s) will have more opportunities to immigrate into a community when dispersal to the resident community is strong. Dispersal often ignored in experimental systems, because studies are conducted in batch environments, but has been shown to play an important role in open environmental systems (Lindström and Langenheder, 2012; Comte *et al.*, 2016). We then suggested that selection and drift are the main processes determining invader establishment after its entry to the resident community. Selection pressure can be positive or negative depending on the resident community composition and environmental conditions. But when selection process in resident community dominates, then invasion success is expected to decrease with increasing resident community diversity (Figure 2.5a). This is based on assumption that the presence of a community member capable for outcompeting the invader is more probable in more diverse resident communities. Contrarily, when drift dominates then resident community diversity would be irrelevant to invader success. The establishment of the invader would be random, but can be highly influenced by propagule pressure, defined as the relative abundance of the invader at the

time of immigration (Figure 2.6b). It is challenging to determine the effect of speciation (diversification) in microbial communities because most experiments are not conducted within sufficiently long timeframe. However, when the diversification rate of the invader is high, it can increase the chances for variants of the invader type to establish if some mutations are adaptive (Tayeh *et al.*, 2015). Conversely, strong speciation in the resident community might increase resident community resistance to invasion.

Our proposed framework follows ecological research terminology and is easily and coherently applicable to any microbial community. It allows comparisons between seemingly different communities based on the ecological similarities. With this framework, we suggest that invasion should be considered as a part of ‘normal’ community assembly and observations should be interpreted with that in mind.

2.3 State-of-the-art of determinants of invasion in microbial communities

Invasion experiments can be categorized broadly into two classes, depending on whether the focus is on the invader or on the invaded community (resident community). Invader-centric experiments focus on traits of the invader and most often include known invasive microbial types as model organisms. This does not allow us to make general observations as to what mechanisms underlie microbial invasions. Instead, these observations mostly link to particular microbial types (Buse *et al.*, 2014; Yao *et al.*, 2014; Acosta *et al.*, 2015; Hambright *et al.*, 2015). Invader-centric experiments have been performed to shed light on invasion success of *Prymnesium parvum*, a marine algae which is considered to be a globally invasive species in many freshwater systems (Acosta *et al.*, 2015; Hambright *et al.*, 2015), *Escherichia coli* O157:H7, a significant pathogen carried by animals (Yao *et al.*, 2014) and *Legionella pneumophila*, a frequent health concern in engineered water systems associated with several disease outbreaks (Buse *et al.*, 2014). These invader-centric studies primarily aim at explaining the spread of a particular (often pathogenic) invasive type in order to learn mechanisms for preventing invasion and the negative consequences associated with invasion. While community assembly processes could also be used to interpret the experimental observations from invader perspective, this has received little attention in these studies. Below, I highlight two invader-centric studies that reach diverse conclusions as to what governs invasion, in order to provide

examples of invader-centric experiments that are generally not applicable to any invader *per se*.

The first study focuses on the invasion of *P. parvum* in natural environment. Although some microbes have been suggested to be dispersal limited, *P. parvum* is evidently not, as extremely low-density populations have been detected in habitats that do not experience *P. parvum* blooms (Zamor *et al.*, 2012). While common assumptions about invasions in natural ecosystems would suggest that environmental selection determines invasion outcome in highly dispersive communities, experimental evidence have failed to support that. Instead, propagule pressure was shown to determine invasion success over resource availability (Acosta *et al.*, 2015). Not interpreted as such by the Acosta *et al.*, these two studies suggest that invasion by harmful algae is governed by drift and dispersal rather than selection.

The second study aims to unravel the invasion success and growth of *L. pneumophila* in drinking water biofilms (Buse *et al.*, 2014). They concluded that invasion success was governed solely by nutrient availability. However, the effect of propagule pressure was not tested in this study, as the invader was introduced at one concentration during a single immigration event. While this study provides valuable information regarding the selective conditions towards *L. pneumophila*, it cannot be compared to *P. parvum* study conducted in completely different environment.

The largest share of the observations are, however, ‘resident community-centric’ and evaluate community’s susceptibility to invasion. These experiments usually test invasion in situations when selection is the strongest governing process. Most often community diversity-invasibility relationships are explored. This theory suggests that “species-rich communities are more resistant to invasion than species-poor communities” (Robinson and Valentine, 1979). See Table 2.4 for overview of main assumptions these ecological concepts. These experiments are designed to explain the success of any invader under defined environmental conditions. These studies often assemble communities with variation in a diversity attribute (richness, evenness or both) by manipulating natural communities (dilution-to-extinction method) or using synthetically assembled communities. This approach allows evaluating the importance of that particular attribute on invasion success. However, it comes short in recognizing the processes behind community assembly. Oftentimes invader is introduced together with the ‘resident’ community or the time for ‘resident’ community to reach

steady state has not been sufficient. In these cases, invader is not required to displace any community members and can be just considered as part of the inoculum during initial community assembly. Furthermore, synthetic communities of limited number of pure strains, usually without any history of co-evolution, are used because they provide great control on experimental design (De Roy *et al.*, 2014). The ecological interactions between community members in synthetic communities; however, are rarely as complex as in natural communities. Thus, it remains unknown to what extent, if at all, the evidence from the experiments made with synthetic communities applies to natural ecosystems.

Table 2.4 Overview of main concepts referred to in invasion ecology

Concept	Explanation	Reference
Diversity-invasibility relationship	Main assumption is that more diverse communities are also likely to have more diverse resource consumers, and this may limit the number of possible invaders due to lack of available resources.	Elton, 1958
Phylogenetic similarity as an indicator for competition and selection	This hypothesis originates from a related hypothesis suggesting that closely related species tend to possess similar niches and hence perform similarly under the same environmental conditions. This can be translated into strong competition imposed by resident species on closely related invaders that reduces their success.	Darwin, 1859

To highlight controversy of synthetic vs. natural communities, most diversity-invasibility studies in synthetic communities conclude that biodiversity is the central factor determining invasion success. This is often the only feature tested experimentally when resident community diversity is manipulated by mixing pure strains at varying abundance (Hodgson *et al.*, 2002; Jiang and Morin, 2004; Dillon *et al.*, 2005; Jousset *et al.*, 2011; van Elsas *et al.*, 2012; De Roy *et al.*, 2013; Eisenhauer *et al.*, 2013; Mallon, *et al.*, 2015b; Tan *et al.*, 2015). These studies almost always agree that diverse communities exploit resources more efficiently, leaving less free niche space available for invaders. Hence, negative correlation between resident community diversity and invasion success is commonly observed. This is not surprising; invasion experiments with synthetic communities are mostly performed in closed batch systems where dispersal is never considered. In natural communities; however, dispersal processes have been shown to determine invasion success in multiple occasions (Acosta *et al.*, 2015; Bellucci *et al.*, 2015). Dispersal is suggested to increase community diversity

(Zha *et al.*, 2016) as well as theoretically facilitate successful invasion (**Paper I**). Therefore, linking diversity without context of other possible community assembly processes to invasion success can be misleading.

The second widely investigated concept is based on phylogenetic relatedness (Table 2.4). It has been suggested that if invader is phylogenetically related to the resident community, the chances for successful establishment are reduced. This is better known as Darwin's naturalization hypothesis (Procheş *et al.*, 2008; Jiang *et al.*, 2010; Thuiller *et al.*, 2010). Studies using assembled communities where phylogenetic similarity was manipulated have found that resident-invader phylogenetic relatedness is indeed negatively correlated with invasion success (Tan *et al.*, 2012, 2015), suggesting again that selection is predominantly responsible for invader establishment. Interestingly, when similar experiments were done with plants, then the support for negative correlation seen with synthetic communities was lost when natural communities were used (Price and Pärtel, 2013).

Last, while speciation can be considered irrelevant in most of these experiments due to small timeframe, there are excellent conditions for drift to act in batch systems. Strong drift has been suggested to govern the assembly of beech tree holes – batch systems – determined by NCM (Sloan *et al.*, 2006). Drift is, however, rarely discussed when interpreting the results from batch experiments.

In conclusion, I have shown that invasion experiments performed using state-of-the-art do not adhere to a systematic framework (**Paper I**). Although, there are many applied studies that may be relevant for unravelling the determinants of invasion in microbial communities, these studies do not use explicit definitions or measurement criteria. For example, the concept of a resident community is often approached loosely, particularly when synthetic assembled communities are added together with the 'invader'. From an ecological perspective, in these cases nothing distinguishes the 'resident' community members from the 'invader' because none of them have had a prior common existence.

2.4 Experimental approaches taken in this study

Most microbial invasion experiments are performed in closed batch systems. Batch systems are practical and allow high replicability. However, there are several limitations to using batch systems to test invasion in microbial communities. First, in these systems dispersal in the community is completely

removed, except from the single addition of the invader type (De Roy *et al.*, 2013; Eisenhauer *et al.*, 2013; Bonanomi *et al.*, 2014; Acosta *et al.*, 2015; Chapelle *et al.*, 2015). As discussed above, dispersal can have a large effect in maintaining resident community diversity and therefore govern invasion success. Second, it needs to be noted that the absence of continuous supply of nutrients to the community during an invasion experiment might not simulate the native conditions of the community correctly. In fact, when nutrients are supplied in a dynamic manner, the outcome of invasion has been shown to change compared to conditions of continuous nutrient supply (Liu *et al.*, 2012; Mallon *et al.*, 2015b). More precisely, increased resource pulses showed to decrease the competition with the invader and increase invasion success. Subsequently, during these dynamic resource pulses, the higher resident community diversity did not decrease invasion success. Therefore, I suggest that testing invasion in any ecosystem requires an experimental setup mimicking the natural conditions of given system as closely as possible.

Furthermore, different approaches to assemble resident communities subjected to invasion are used. While mixed synthetic communities provide increased control over the experimental design by allowing to create precise gradients of community diversity or richness, they are often less complex than natural systems. On the other hand, enriched communities from natural environment, for example obtained from soil (Liu *et al.*, 2012; van Elsas *et al.*, 2012; Chi *et al.*, 2013; Yao *et al.*, 2014; Chapelle *et al.*, 2015; Ma *et al.*, 2015; Mallon *et al.*, 2015b), fresh water (Hambright *et al.*, 2015) or drinking water (Van Nevel *et al.*, 2013; Buse *et al.*, 2014) provide higher similarity to real-world ecosystems. While these enrichment communities are more difficult to control, the interactions between community members are occurring in nature and therefore experimental observations are more likely to be applicable in real ecosystems.

During this PhD, the focus was on invasion in microbial communities inhabiting drinking water systems. Drinking water systems are known to experience continuous nutrient loading, hence, similar conditions should be applied when testing determinants of invasion in model communities inhabiting drinking water systems. For the purpose of this PhD project, I enriched nitrite-oxidizing bacteria from naturally occurring drinking water community as a model guild in a new lab-scale system for growing replicate biofilms under continuous flow-through conditions.

2.4.1 Nitrite oxidizing bacteria as model organisms

I enriched nitrite-oxidizing bacteria (NOB) from naturally occurring community in drinking water. This community is not a synthetic assemblage of pure strains or a completely natural community. Using this enrichment approach allows me to maintain some control over experimental design and community composition while still favouring naturally co-occurring microbes.

Canonical NOB carry out the second step of nitrification where they convert nitrite (NO_2^-) to nitrate (NO_3^-) (eq. 1.1). Due to their relatively well-known metabolism as well as established detection and quantification methods, NOB are excellent model organisms for studying community assembly processes in drinking water related environments.



The diversity of NOB is notable, with genera across several bacterial phyla (Nitrospinae, Nitrospirae, Proteobacteria, Chloroflexi) and lineages (*Nitrobacter*, *Nitrococcus*, *Nitrospina*, *Nitrospira*, *Nitrotoga*, *Nitrolancetus*) (Ward *et al.*, 2011; Sorokin *et al.*, 2012). *Nitrospira* is often detected as dominant NOB in natural environments where nitrite concentrations are low (drinking water systems) (Gülay *et al.*, 2016; Palomo *et al.*, 2016; **Paper II**; **Paper III**). *Nitrotoga*, however, has been detected in high nitrite environments (wastewater treatment) (Lücker *et al.*, 2014; Saunders *et al.*, 2015) as well as medium (to low) nitrite environments (Hüpeden *et al.*, 2016; Ishii *et al.*, 2017). During this PhD we observed primarily *Nitrospira* and *Nitrotoga* genera when determining the total NOB abundance in our model communities.

In order to be able to interpret the community assembly processes in the model communities, we need to elaborate on the niche differences of known NOB. When focusing on canonical NOB, it is safe to assume that nitrite concentration is the primary determinant of niche differentiation (Nowka *et al.*, 2015; Daims *et al.*, 2016). For example, it has been experimentally verified that nitrite concentration selects for different sublineages of *Nitrospira*. Lineage I *Nitrospira* is suggested to resemble r-strategists more than the organisms in sublineage II. Thus sublineage I *Nitrospira* has been shown to outcompete sublineage II *Nitrospira* as soon as the nitrite concentration is high enough to support the required growth rate (Maixner *et al.*, 2006). Furthermore, there is a large difference in nitrite oxidation kinetics of *Nitrospira* and *Nitrobacter*. *Nitrobacter* has highest maximum activity, but

also generally lowest substrate affinities (Blackburne *et al.*, 2007; Nowka *et al.*, 2015). This has been suggested to be the primary reason why *Nitrobacter* is found more in wastewater treatments and rarely detected in drinking water where *Nitrospira* dominates thanks to its, by far, the lowest affinities to nitrite of currently known NOB (Table 2.5).

Table 2.5. Kinetic constants of selected nitrite-oxidizers adapted from Nowka *et al.* (2015)

Organism	Temp. (°C)	V _{max} *	K _m **
<i>Nitrobacter vulgaris</i>	28	164 ± 9	49 ± 11
<i>Nitrobacter hamburgensis</i>	28	64 ± 1	544 ± 55
<i>Nitrobacter winogradskyi</i>	28	78 ± 5	309 ± 92
Ca. <i>Nitrospira defluvii</i>	28	48 ± 2	9 ± 3
<i>Nitrospira moscovensis</i>	37	18 ± 1	9 ± 3
<i>Nitrospira lenta</i>	28	20 ± 2	27 ± 11
Ca. <i>Nitrotoga arctica</i>	17	26 ± 3	58 ± 28

* Mean maximum specific activity ± SD in $\mu\text{M NO}_2^-$ per mg of protein h^{-1}

** Mean saturation constant for activity ± SD in $\mu\text{M NO}_2^-$

Furthermore, some members of *Nitrospira* hold a vast variety of additional metabolic capabilities that all expand the niche of this genus beyond nitrite concentration. It has been shown that *Nitrospira* can use ammonium (Daims *et al.*, 2015; Pinto *et al.*, 2015; van Kessel *et al.*, 2015; Palomo *et al.*, 2016), hydrogen (Koch *et al.*, 2014), and urea (Koch *et al.*, 2015) as substrate for growth.

For example, in **Paper III** we explicitly tested the effect of nitrite loading on naturally occurring NOB community, dominated by complete ammonia oxidizing (comammox) *Nitrospira*. In that paper we showed that several comammox *Nitrospira* types were lost when nitrite became the sole energy source, suggesting a very different niche of these comammox *Nitrospira* compared to canonical *Nitrospira*.

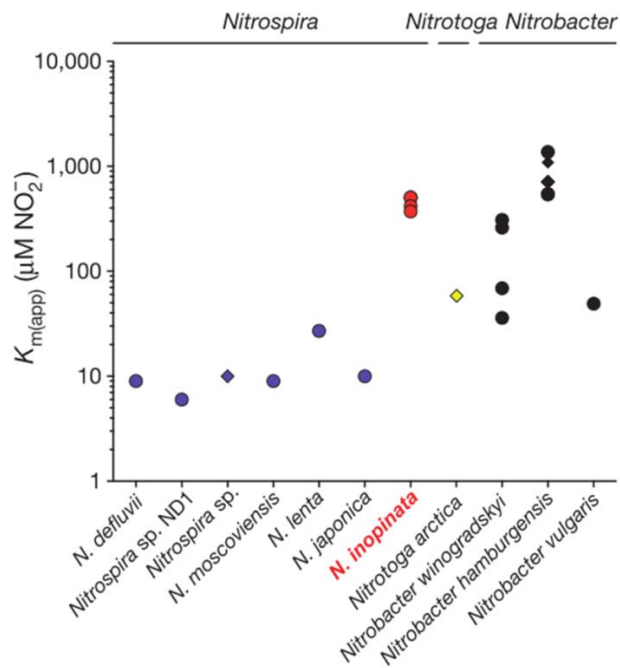


Figure 2.7 Comparison of the affinities of nitrite between comammox *N. inopinata* and pure cultures of known nitrite oxidizers according to Kits *et al.* (2017)

Unfortunately, the niche of *Nitrotoga* is still largely unexplained due to the low number of isolates. Based only on nitrite oxidation kinetics, *Nitrotoga* is considered to be more similar to *Nitrospira*, than any other NOB with known kinetic parameters (Figure 2.7). The kinetics of *Nitrotoga* have been measured in only one pure culture (Nowka *et al.*, 2015). These observations suggest that *Nitrotoga* has slightly higher maximum specific activity than most (known) lineage II *Nitrospira* but is lower than the activity of *Nitrobacter* strains. *Nitrotoga* is also capable of outcompeting sublineage II *Nitrospira* at higher nitrite concentrations (**Paper II**). With enrichments of new *Nitrotoga* isolates, the more diversity in *Nitrotoga* niches is suggested. For example, while all previously enriched *Nitrotoga* are psychrophilic and adapted to low nitrite environments [see *Ca. Nitrotoga arctica* (Alawi *et al.*, 2007), *Nitrotoga* enrichment cultures HAM-1 (Alawi *et al.*, 2009), HW29 (Hüpeden *et al.*, 2016) and AM1 (Ishii *et al.*, 2017)] then recently isolated and tentatively named *Ca. Nitrotoga fabula* tolerates higher nitrite concentrations (isolated from wastewater) and has a temperature optimum of 28°C (Kitzinger *et al. unpublished, personal correspondence*).

2.4.2 Lab-scale microcosms as model systems

For the purpose of this PhD, I developed a high-throughput continuous-flow microcosm system for growing replicate microbial biofilms of varying, but controlled, average thickness and associated community diversity. The main

hypothesis was that increased loading of a limiting growth-substrate – in this case nitrite – would result in larger microbial density (and thicker biofilms) due to larger carrying capacity of the community. Thus, by altering nitrite loading in model systems, we would be able to manipulate the strength of selection in community assembly (**Paper II**).

Table 2.6 Comparison of operational parameters of lab-scale microcosms and full-scale rapid sand filter used as a reference

Parameter	Unit	Full-scale RSF [*]	Low nitrite loading	High nitrite loading
Surface area	m^2/m^3	1628	3411	3411
Influent S	mg/L	S_{NH4+} 0.34	S_{NO2-} 0.5	S_{NO2-} 5
Q_{in}	ml/min	1.2×10^6	0.4	0.4
Surface loading rate	$g/m^2 d$	3.2	0.1	1.1
Operational time	days	-	60	60
Expected NOB	Cells in column	-	1.4×10^8	1.4×10^9

* data originating from Gulay *et al.* (2014) and Lee *et al.* (2014)

A system with 40 replicate biofilms was constructed using flow-through polypropylene columns housing a defined volume of commercial Filtralite® filter material supported by a stainless steel mesh (Figure 2.8). Filtralite® is commercial high quality filter media, manufactured from expanded clay material, used for filtration in drinking water treatment and in swimming pool filters. NOB were enriched from tap water source community by adding nitrite as sole energy source. Operational conditions were chosen to mimic full-scale rapid sand filter, with the exception of providing nitrogen in form of nitrite and not ammonia (see Table 2.6 for operational details).

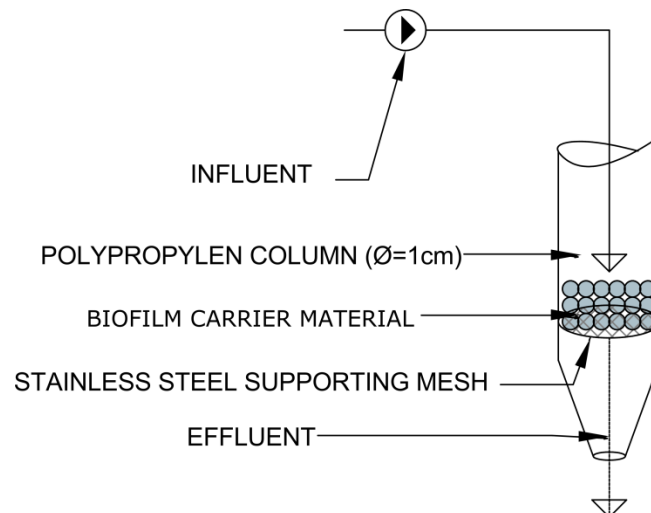


Figure 2.8 Schematic representation of lab-scale microcosms used for invasion experiment

The full-scale rapid sand filter in Islevbro waterworks (located in Copenhagen area, Denmark) was used as reference system to design the flow-

through experiments. This waterworks treats anaerobic groundwater, where main compounds to be removed are ammonium and reduced forms of iron and manganese (Lee *et al.*, 2014). Previously, *Nitrospira* dominance was observed in the rapid sand filters (Gülay *et al.*, 2016), suggesting that the operational conditions are favourable for NOB enrichment.

The establishment of NOB activity was determined by regular nitrite and nitrate measurements at the column influent and effluent. Complete removal of nitrite was observed from day 42 onwards (Figure 2.9), indicating successful establishment of active NOB community.

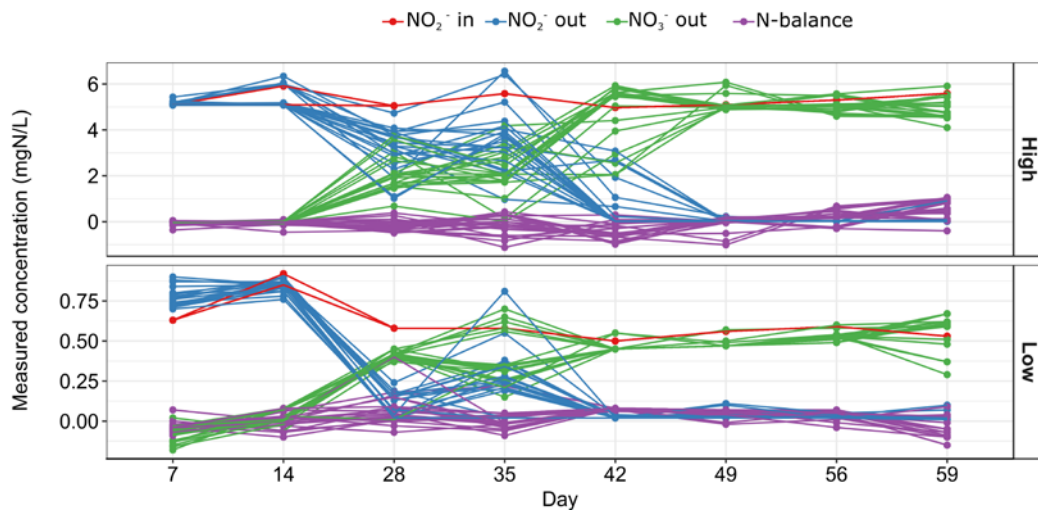


Figure 2.9 Nitrite oxidation dynamics in lab-scale microcosms operated under differential nitrite loading suggests establishment of an active NOB population on Filtralite® material (Paper IV)

3 Community assembly processes in model NOB guilds

Managing and steering complex microbial systems requires an understanding of microbial community assembly processes, as explained above. In this chapter, I present the experimental evidence on the governing processes of community assembly based on the Vellend (2010) framework, and using the NOB model communities.

There are four processes suggested to govern microbial community assembly: selection, drift, speciation and dispersal. However, most experiments testing community assembly processes favour selection and neglect the other three processes. This is because community diversity is used as proxy for selection, yet it could theoretically represent any of the above described assembly processes. It is also suggested that selection in given community can be noticeably shifted when the community is subjected to ecological disturbance (Shade *et al.*, 2012; Ma *et al.*, 2015) or sudden shift in environmental conditions (**Paper III**). Disturbances and changes in operational regime tend to decrease community density and diversity, possibly increasing the vacant niche space – in other words, briefly decreasing competition. More typically; however, numerous processes interact during community assembly and these processes cannot be easily uncoupled (Ofiteru *et al.*, 2010) unless focusing on newly assembled communities (Langenheder and Szekely, 2011).

Studies on newly assembled microbial communities, using batch microcosms, indicated that the initial colonization steps were critical, with an alleged role of priority effect, which has a high stochastic component (Burns *et al.*, 2015). Priority effect can be seen in the dominance of community members establishing first. However, when community succession was allowed to proceed under continuous dispersal from a source community, the importance of the (largely stochastic) initial assembly step declined and the influence of selection grew larger (Langenheder and Szekely, 2011).

In **Paper II** and **Paper IV**, I assembled communities comprising significant fraction of NOB and evaluated the contribution of selection compared to drift and dispersal in newly assembled communities. To do this, I postulated that differential nitrite loading creates conditions for differential selection. I applied two defined nitrite loadings and assembled replicate biofilms from a source community continuously immigrating from tap water. In addition, in **Paper IV**, I extended these observations and also used batch systems

inoculated from communities assembled under continuous regime to eliminate the possible effect of dispersal.

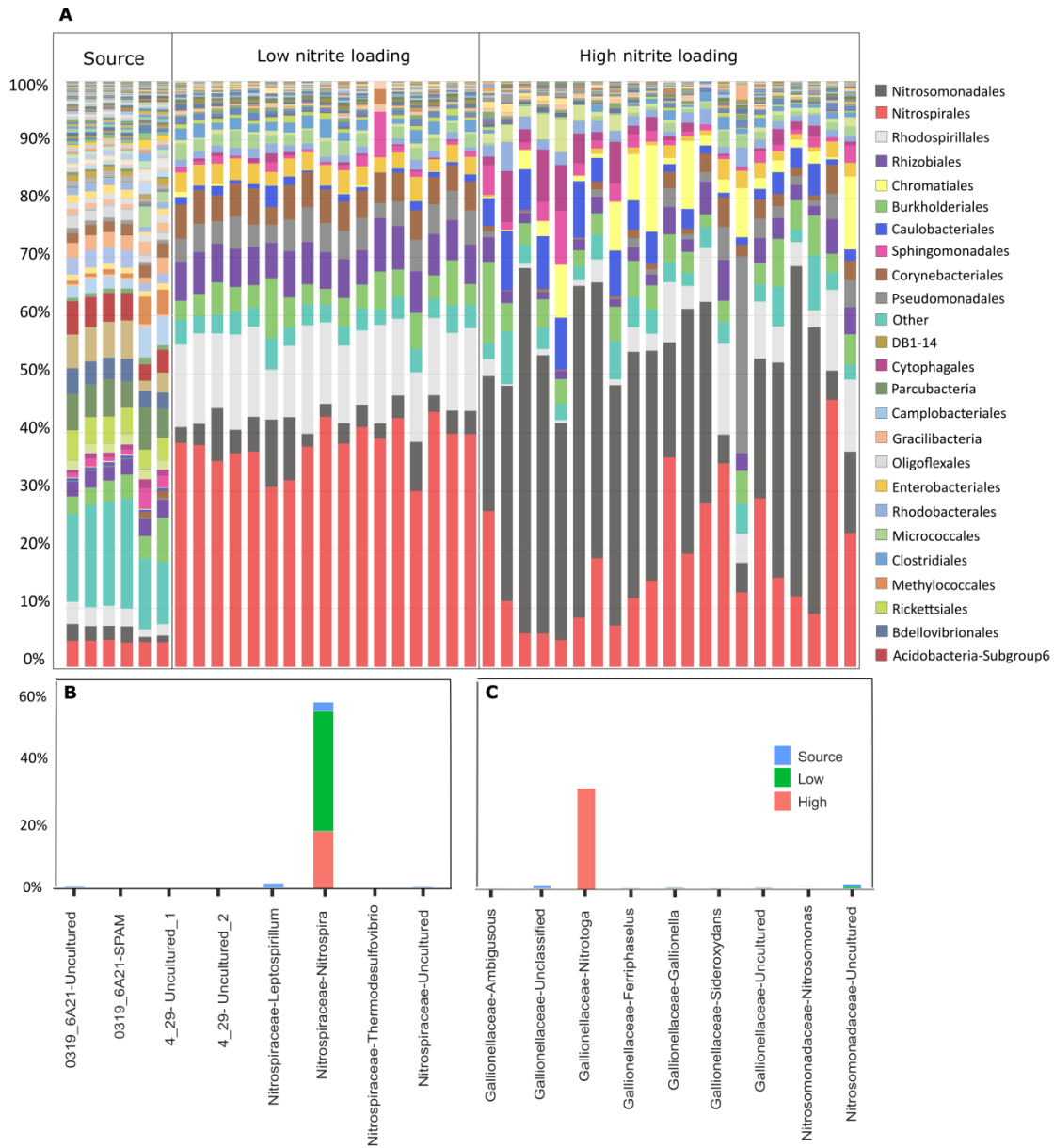


Figure 3.1. Community composition of all replicate communities (a) (source, high- and low- nitrite loading biofilms) displayed as relative abundance of the 16S rRNA gene sequences classified at the order level against Silva123 prokaryotic reference database and genus level breakdown of two most abundant orders: *Nitrospirales* (b) and *Nitrosomonadales* (c) (Paper II).

In Table 3.1 I provide an overview of the conceptual evaluation of the importance of selection, drift and dispersal in newly assembled model NOB guilds. I neglect speciation due to the short timeframe of the experiment that makes it unlikely that new types will arise and achieve significant abundance.

The interpretations regarding the strength of processes in these NOB guilds are based on qualitative evaluation of the community composition and the conceptual synthesis of community ecology (Vellend, 2010).

Table 3.1. Conceptual overview of the strength of community assembly processes in resident communities assembled under different conditions based on qualitative evaluation of community composition, and the conceptual synthesis of community ecology (Vellend, 2010), and the average observed phylogenetic diversity of resident NOB guild (Paper IV)

	COLUMNS		BATCH	
	Low nitrite	High nitrite	Low nitrite	High nitrite
SELECTION	+	+++	++	++
	(<i>Nitrotoga</i> / <i>Nitrospira</i>)	(<i>Nitrotoga</i>)	(<i>Nitrospira</i>)	(<i>Nitrospira</i>)
DRIFT	++	+	+++	+++
Ratio of Selection/Drift	++	++++	+	+
DISPERSAL	++	+	0	0
SPECIATION	0	0	0	0

First, I interpreted strong dispersal only in flow-through columns open to the environment and receiving constant background immigration from the inoculum tap water. Although, it has been shown before that dispersal can impact the diversity, composition, as well as functioning of a microbial community (Zha *et al.*, 2016); here, I observed that the effect of dispersal was low, compared to the ratio of selection over drift in newly assembled NOB guilds from tap water (Figure 3.2). These observations are based on the large dissimilarity of the two newly assembled NOB guilds and the source community. If dispersal would dominate, the composition of newly assembled communities would be highly similar to that of the source community. Hence, I will focus on the relative importance of selection and drift from here on.

I found evidence to support the presence of both, selection, as well as drift. First, in **Paper II** the importance of selection was evident from the deterministic effect of nitrite loading on NOB guild assembly. *Nitrospira* and *Nitrotoga* were both at a low abundance in the source community, but the NOB guild was consistently skewed towards *Nitrospira* dominance at low nitrite loading and *Nitrotoga* dominance at high nitrite loading (**Figure 3.1**). The contribution of selection was also evident from the large and consistent dissimilarities between the source and assembled communities (**Figure 3.2**). In **Paper IV** I observed strongest effect of selection from the high similarities of replicate communities assembled at high nitrite loading in flow-through systems (Figure 3.3).

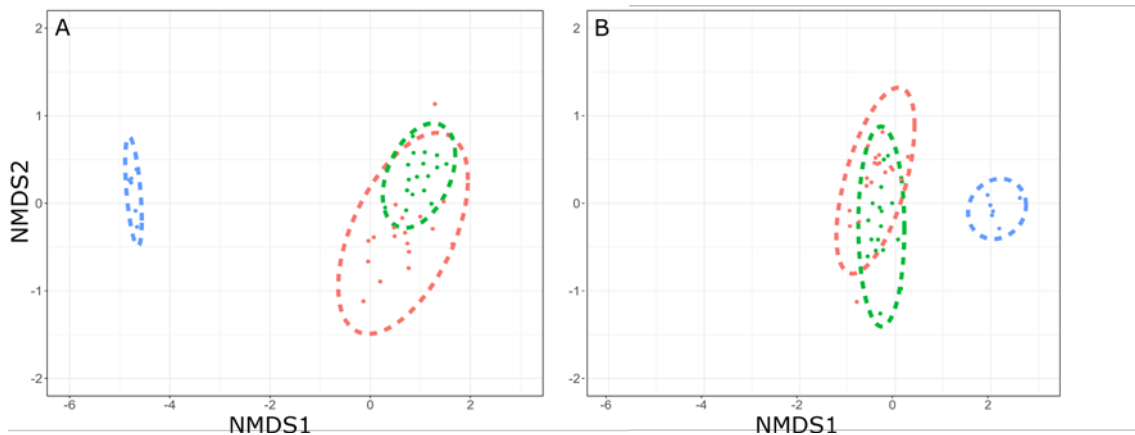


Figure 3.2. Nonmetric multidimensional scaling ordinations of replicate communities based on Bray–Curtis distances showing total community (a) and NOB guild (b) biofilm communities at low (green) and high (red) nitrite loadings compared to source community (blue, tap water community) (Paper II).

Second, the influence of drift was inferred from the increased within-group dissimilarities of repeatedly assembled high nitrite loading and low nitrite loading communities, surpassing that of the repeatedly sampled source community (**Figure 3.2**). Within-group dissimilarities have been previously used to highlight stochastic contribution to community assembly (Evans *et al.*, 2017). Interestingly, these dissimilarities were smaller at guild level than total community level (**Figure 3.2**), and were increased in the batch systems compared to the flow through systems (**Figure 3.3**). In the batch systems, half of the community was regularly removed evoking randomness in replacement of removed community members. This, however, is often not discussed when testing community assembly in batch systems.

In **Paper II** we compared the biofilm community compositions to those expected based on the neutral community assembly model (Sloan *et al.*, 2006) by using two different approaches for defining the source community. First, we used only the sequence variants that were common to the source and the biofilm communities. This method, commonly used in NCM, excludes the sequences that are not present in both the source and assembled communities from the analysis. In my case, this resulted in a loss of more than half of the sequences. To avoid that my assessment of the NCM is only based on a fraction of the data obtained from amplicon sequencing, I also used a second approach, where I included all sequence variants detected in any of the biofilms to be also part of the source, despite not being detected (from here on, I refer to this as the extended source community). I assigned the lowest observed average relative abundance in the source community (0.001%) to those sequence variants in the source community. This approach is possible

due to the nature of my experimental system. All biofilms were newly assembled from the same source community; hence community members below detection limit in the source but found in the biofilms must have originated from the tap water source community.

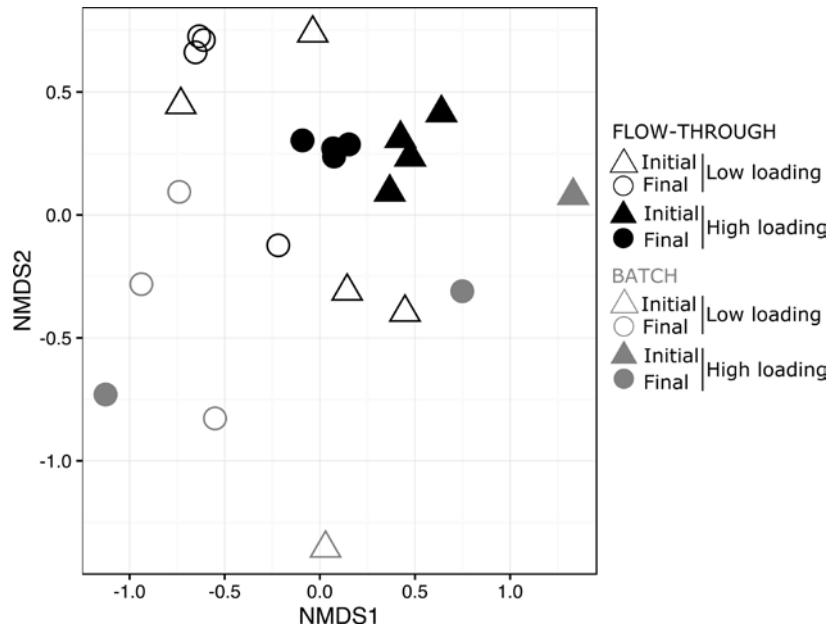


Figure 3.3. Nonmetric multidimensional scaling ordinations based on Bray-Curtis distances showing similarities between repeatedly assembled resident communities in columns and batch systems under differential nitrite loadings (Paper IV)

When I focused only on the common sequence variants, I clearly rejected the hypothesis that there is a significant correlation between the model prediction and the observed composition of NOB guilds (Figure 3.5a,b). Although, I estimated high correlation coefficient for the low nitrite loading treatment ($\rho_{\text{spearman}}=0.46$), this correlation was statistically not significant due to low number of sequence variants that were common between the biofilm and the source community (7 sequence variants). Only after extending the source community (Figure 3.5c,d) did I see significant support for neutral assembly ($p<0.001$).

Losing the support for the NCM model when focusing only on the common sequence variants can be as a result of too few data points remaining for analysis, or it can suggest that the rare taxa in the source community are more affected by drift. Although other studies testing the fit of the NCM report much higher significant correlations, it is important to note that in all these studies, the composition of the source community is not directly measured but estimated by averaging the composition of multiple local communities

(Sloan *et al.*, 2006; Ofiteru *et al.*, 2010; Burns *et al.*, 2015; Roguet *et al.*, 2015).

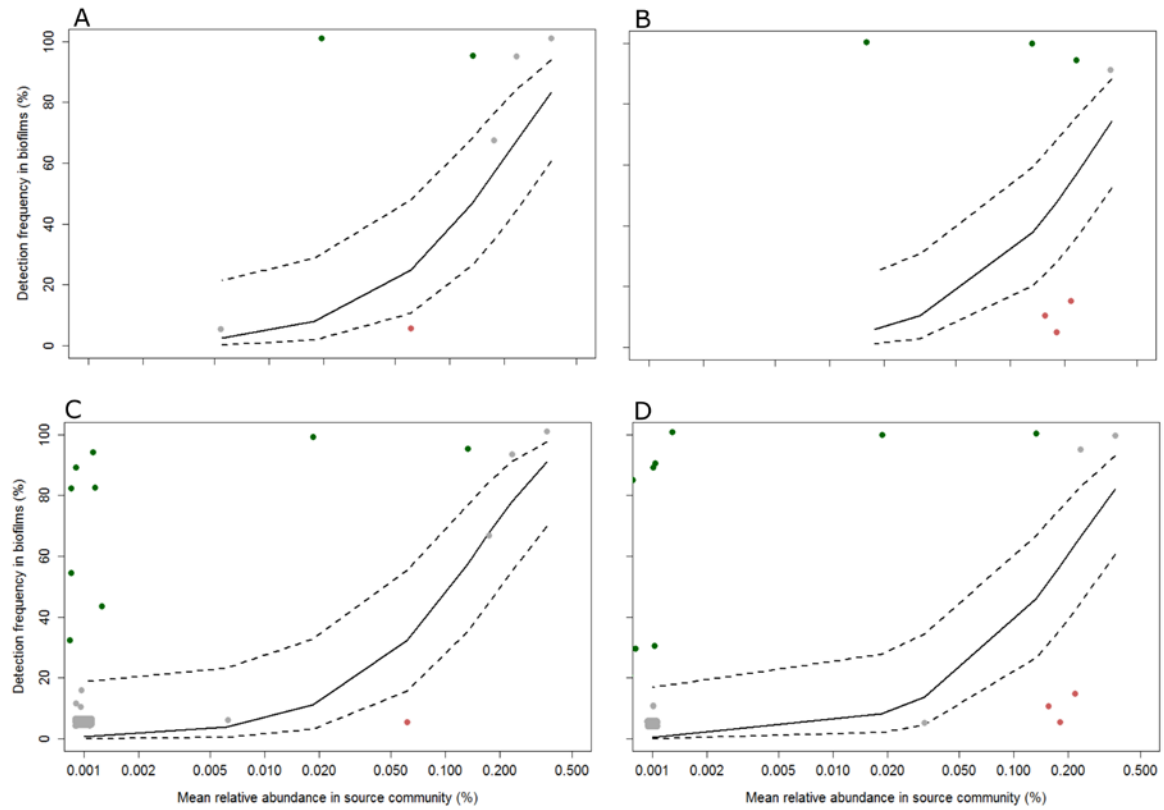


Figure 3.4. Fit of the predicted neutral model to observed NOB guild assembly. The predicted occurrence frequencies for only shared sequence variants in LNL biofilm (a) and HNL biofilm (b) communities and the predicted occurrence frequencies with detection limit (0.001%) applied in the source community in low (c) and high-loading (d) biofilms. Exact sequence variants that occur significantly more frequently than predicted by the model are shown in green, while those that occur less frequently than predicted are shown in red. All symbols are jittered over x-axes. Dashed lines represent 95% confidence intervals around the model prediction (black line) (Paper II)

In conclusion, I showed, for the first time, how selection caused by elevated nitrite loading resulted in the dominance of *Nitrotoga* over *Nitrospira* in nitrite-oxidizer guild. Although the newly assembled NOB guilds exhibited signs of selection, the component of drift cannot be ignored. Neutral processes were able to explain nearly half of the observed guild composition. These observations provide additional support for community assembly being the result of combined processes.

4 Determinants of invasion in model NOB guilds

By presenting invasion research in a community ecology framework, it is apparent that some processes have received more attention than others. A selection focus has overwhelmed invasion research, while other processes shaping community dynamics are rarely considered. After thorough analysis of the processes governing NOB guild assembly, I subjected these newly assembled communities (from now on referred to as resident communities) to invasion by a *Nitrotoga* HW29 culture at three different propagule pressures (**Paper IV**). I tested the invasion success in situations where invader was phylogenetically similar to the resident community that varied in phylogenetic diversity. I hypothesized that when the direction of selection is similar for the resident community members and invader then invasion success is influenced by propagule pressure. Hence the effect of drift on invasion success increases with decreasing phylogenetic distance between invader and the members of the resident community.

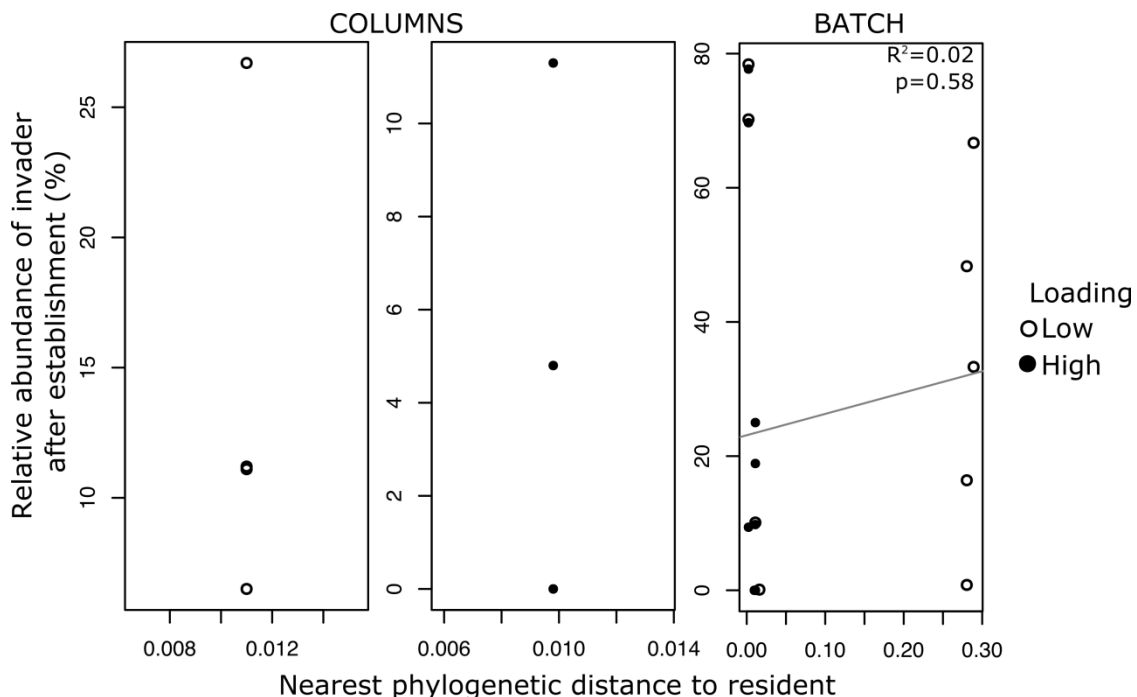


Figure 4.1 Correlation between the invader relative abundance and the phylogenetic distance to the nearest member of the resident community (Paper IV)

I did not detect invasion success based on quantification of total *Nitrospira* and *Nitrotoga* cells (targeted qPCR), because there was no significant change in NOB cell numbers. This, however, is preferred. It indicates that the resident communities had reached its carrying capacity by the time of invasion and that the invader establishment would require the displacement of some of the members of the resident community. As the drift in the resident communities is relatively high, this already indicates, that random displacement can facilitate invasion success, as well as cause invader to fail after introduction. In order to monitor the establishment of the invader I used amplicon sequencing of 16S rRNA of total community. This works because the invader *Nitrotoga* was originally absent from the resident community. I observed that the invader *Nitrotoga* strain was established in the flow-through systems only at high propagule pressure, whereas in batch systems, establishment was observed at almost all propagule pressures, although at different relative abundance as estimated by its sequence read abundances.

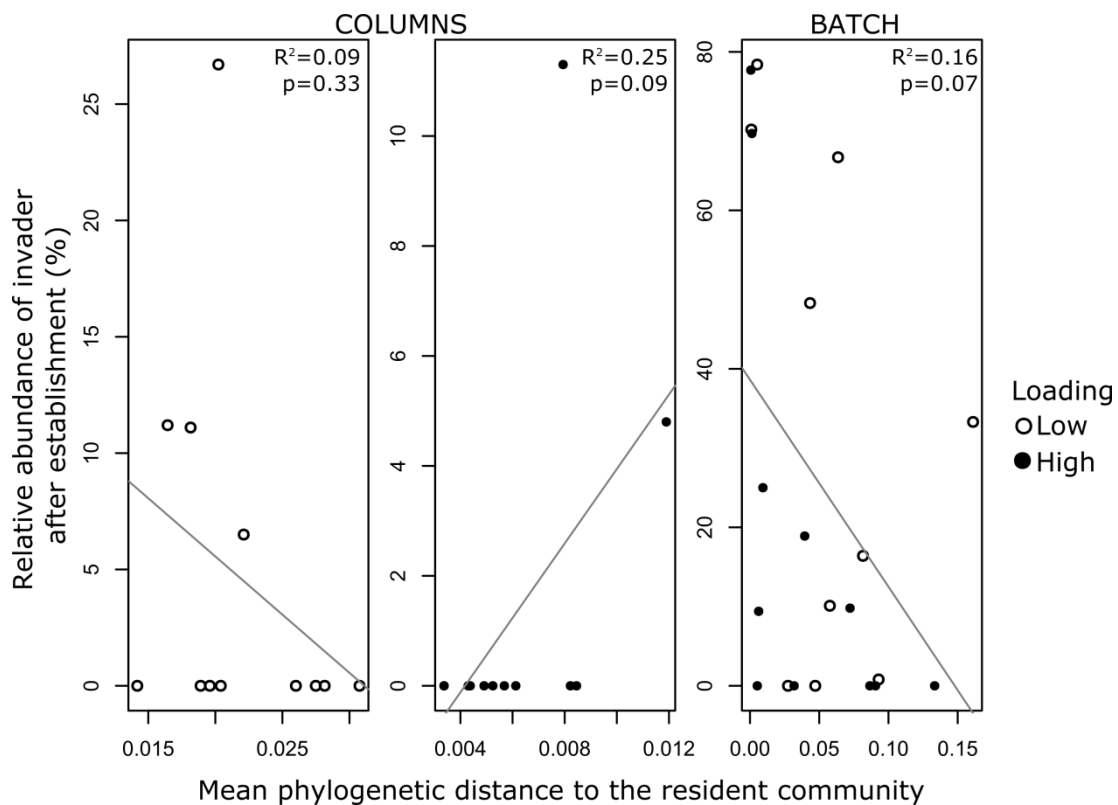


Figure 4.2. Correlation between the invader relative abundance and the mean phylogenetic distance to the entire resident community (Paper IV)

To determine the governing processes of invasion I also challenged the current state-of-the-art observations with my data. I tested the hypothesis of phylogenetic relatedness as well as the diversity-invasibility relationships

described above. However, I need to highlight that correlation between community properties (diversity etc.) and invasion success can be representative only when communities assembled by similar processes are compared. In flow-through systems, communities assembled at high loading and low loadings were shown to be determined by different processes. Therefore, merging the replicates from different treatments would encourage false conclusions of what governs invasion success. This is why I determined correlations separately for high loading and low loading columns, but combined all batch samples.

First, I observed no competition between the invader and the phylogenetically closest resident community member determined from the absence of correlations between nearest phylogenetic distance and invader relative abundance (Figure 4.1). I then postulated that perhaps invader competes with all resident NOB (see Table 2.2 for basis of these hypothesis). This, however, is not supported by the experimental observations either (Figure 4.2). A negative correlation trend between invader relative abundance and resident community phylogenetic diversity can be seen in when resident communities have lower selection to drift ratio.

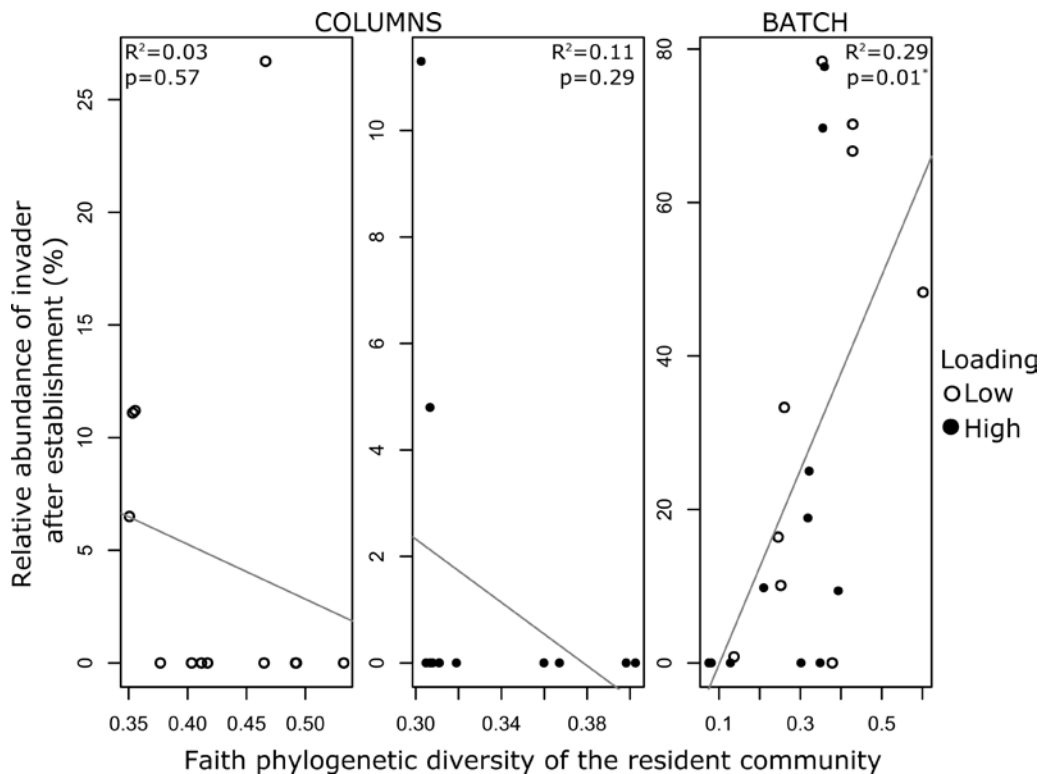


Figure 4.3. Relative abundance of the invader after establishment is significantly positively correlated with phylogenetic diversity of the resident community in low nitrite loading columns and batch systems. Statistical significance of regression line is determined by ANOVA and R^2 represents the linear regression correlation coefficient (Paper IV).

Although not statistically significant (probably due to low number of data points), I detected negative correlation between resident community diversity and invader relative abundance after establishment only flow-through systems (Figure 4.3). This was expected from a system where selection dominates over drift. I observed significant positive correlation in batch systems, where drift dominates and no correlation was expected. My observations, however, highlight that due to absence of consistent pattern in observations, phylogenetic diversity is not a universal predictor for invasion success in microbial communities.

So, I failed to see an effect of selection as governing process of invasion based on the absence of consistent correlation between the mean distance from the resident community and invader relative abundance. Instead, I observed a clear effect of propagule pressure on invasion success. Invasion frequency increased with increasing propagule pressure suggesting strong effect of drift on invader establishment (Figure 4.4).

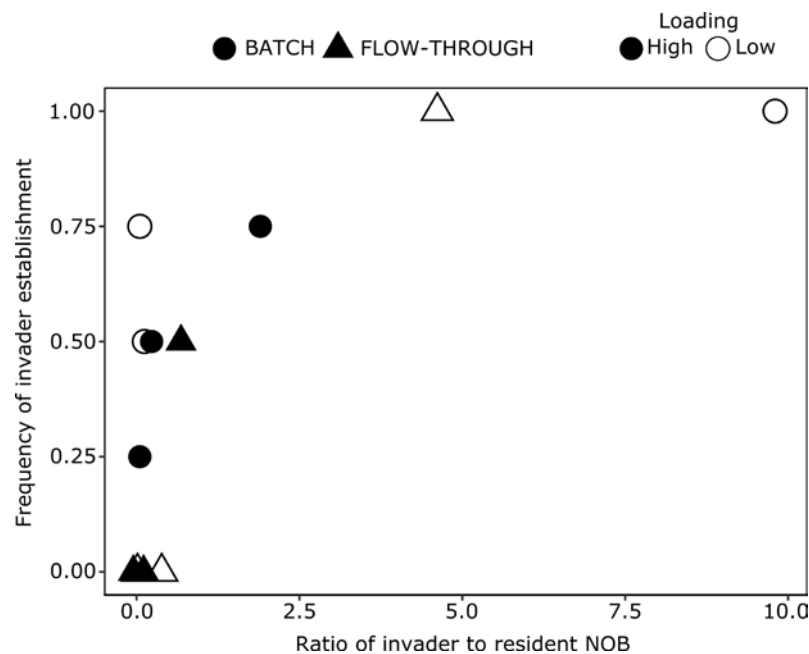


Figure 4.4 The effect of propagule pressure, quantified as the ratio of invader cells to resident NOB cells, to the frequency of invader establishment within four biological replicates

It needs to be noted that these observations are made using model communities where phylogenetic distances between community members and the invader are very low, compared to most other invasion experiments where phylogenetic distances are up to 10-fold higher (Naughton *et al.*, 2015; Tan *et al.*, 2015). However, one other study used synthetic communities of

heterotrophs with similarly low phylogenetic diversity and low phylogenetic distance between invader and resident. In line with my observations, they concluded that propagule pressure increased invasion success and observed no effect of phylogenetic diversity in explaining invasion success (Ketola *et al.*, 2017).

In conclusion, I showed that in situations when the phylogenetic distance between resident community members and a possible invader is very low, neutral processes govern invasion success. My observations provide support to earlier work that reached similar conclusions (Acosta *et al.*, 2015; Ketola *et al.*, 2017). In addition, my results contradict the interpretation of studies explicitly supporting the resident community diversity as a key factor governing the invasion success (van Elsas *et al.*, 2012; Mallon *et al.*, 2015a). However, my initial hypothesis stated that when phylogenetic distance between invader and resident is high in communities where the ratio of selection over drift is high then selection dominates invasion success. Therefore, several studies observing the effect of resident community diversity might actually observe the effect of negative selection towards the invader (Hodgson *et al.*, 2002).

5 Conclusions

In this thesis, I focused on unravelling the mechanisms behind microbial community assembly in order to understand the significance of these processes towards invasion in the microbial communities. I used drinking water microbial communities as a model system from where I enriched nitrite-oxidizing bacteria as model guild. This work has provided the first extensive interpretation of invasion success in view of community assembly processes in a well-defined microbial guild. The main findings of this work are as follows:

Framework for interpreting invasion of microbial communities:

Current observations on microbial invasions do not adhere to a coherent framework. Therefore, comparison of observations across different ecosystems is challenging. Here, I provided a new conceptual framework to study r invasion in microbial communities based on community assembly processes. This framework will, hopefully, encourage the scientific community to extend their interpretations on invasion beyond selection processes.

Novel experimental system to test community assembly processes and invasion:

I developed a novel flow-through system to assemble replicate biofilms under defined conditions in order to test community assembly processes, as well as invasion. This system allows assembling microbial communities from natural inocula as well as synthetic communities under flow-through conditions.

I showed that community assembly processes in flow-through biofilm systems are different than in batch suspended-growth systems. More precisely, the newly assembled communities in the flow-through biofilm systems were more governed by selection than the batch systems. In batch systems, drift dominated community assembly, and there was no significant effect of differential nitrite loading on community composition in batch systems as opposed to what was observed in the flow-through biofilm systems.

Differential enrichment of nitrite-oxidizing bacteria is governed by combination of selection and drift:

Enrichment of the newly assembled NOB guilds in the flow-through biofilm systems revealed that *Nitrotoga* is favoured over *Nitrospira* at increasing nitrite loadings. Furthermore, this selective enrichment altered the community diversity such that lower diversity was associated with stronger selection. The newly assembled NOB guilds showed large stochastic components. More precisely, I observed a significant fit of the NCM to the assembled NOB guilds when using an extended source community approach. A large contribution of drift on community assembly was also inferred by large within-group dissimilarities between repeatedly assembled communities. These observations suggest that even though strong environmental filtering is enforced through adding nitrite, the deterministic aspect of nutrient loading is nearly equal to that of stochastic processes.

Propagule pressure increases invasion success in NOB model communities, suggesting the dominance of drift in determining invasion outcome:

There was no correlation between the average phylogenetic distance nor the nearest phylogenetic distance of the invader strain to the resident community and the relative abundance of the invader strain after establishment. These observations suggest a smaller effect of selection on invasion success compared to earlier studies.

The phylogenetic diversity of the resident community showed a negative correlation trend in flow-through system and a significant positive correlation between diversity and invasion success in batch system. These observations suggest that community diversity should not be used as a universal predictor of community invasibility. When the relative importance of selection versus drift in a resident community is high, invasion success decreases with increasing resident community diversity. However, when the relative importance of selection versus drift is low, resident community diversity is positively correlated with invasion success. Finally, if invader and resident community are phylogenetically similar then drift governs invasion success and relative abundance after establishment.

6 Recommendation for future research

The conceptual framework I developed as a part of this PhD project allows comparing the experimental observations of invasion in very different ecosystems. Here, I demonstrated how this framework could be applied on NOB enrichments from a drinking water metacommunity. Revising previous observations in terms of a given framework and referring to the framework when interpreting new experimental observations could possibly identify additional properties of discussed community assembly processes.

Community assembly processes are often studied using assembly from a mixture of pure cultures. While this allows possible simplification of the system by eliminating the effect of certain processes completely, the applicability to real world natural systems is unclear. Hence, additional experimental as well as modelling efforts are needed. Ideally, pilot scale experiments with, for example, wastewater and drinking water treatment systems would allow increased complexity between microbial interactions while maintaining the opportunity to manipulate abiotic factors that possibly influence community assembly processes.

Here, I used phylogenetically similar resident communities and well-characterized invader strain. In future studies, I suggest to increase the phylogenetic dissimilarities between invader and resident community until conditions are reached where selection dominates over drift. In terms of NOB this would mean more differential nitrite loading conditions and different invaders in view of phylogenetic similarity to the resident. For example, *Nitrospira* dominating communities could be invaded with *Nitrobacter* and vice versa. Furthermore, based on this work, the competition between *Nitrospira* and *Nitrotoga* showed a different outcome based on the experimental system. Hence, both batch and flow-through experiments should always be considered.

Last, during this PhD project the success of single invader in view of resident community assembly was studied. Additionally, conceptual framework to include multiple invaders and the interactions between invaders and resident community should be considered. For example, if two invaders have mutualistic or commensalistic interactions, how would that affect invasion success in terms of possible competition with the resident community? Or would all studies, if interpreted appropriately, always conclude the dominance of neutral processes?

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Papers

- I Kinnunen, M.**, Dechesne, A., Proctor, C., Hammes, F., Johnson, D., Quintela-Baluja, M., Graham, D., Daffonchio, D., Fodelianakis, S., Hahn, N., Boon, N., Smets, B.F (2016) A conceptual framework for invasion in microbial communities. *The ISME Journal* **10**: 2773–2775.
- II Kinnunen, M.**, Gülay, A., Albrechtsen, H.-J., Dechesne, A., and Smets, B.F. (2017) *Nitrotoga* is selected over *Nitrospira* in newly assembled biofilm communities from a tap water source community at increased nitrite loading. *Environmental Microbiology* **19**: 2785–2793.
- III Kinnunen, M.**, Palomo, A., Fowler, J., Albrechtsen, H.-J., Dechesne, A., and Smets, B.F. Switching from ammonium to nitrite feeding induces a differential loss of comammox *Nitrospira* from a rapid sand filter community. *Submitted to FEMS Microbial Ecology*
- IV Kinnunen, M.**, Albrechtsen, H.-J., Dechesne, A., and Smets, B.F. Neutral processes govern invasion success in microbial communities when invader is phylogenetically similar to the resident community. *Manuscript in preparation*

In this online version of the thesis, paper **I-VI** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

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