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Role of Fungus-mediated Transport Mechanisms for Bacterial Activity under Environmental Stress

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Anja Worrich

Role of Fungus-mediated Transport Mechanisms for Bacterial Activity under Environmental Stress

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Dissertation

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Fungi and bacteria have evolved diverse types of ecological interactions that may provide benefits to cope with environmental stress.
The aim of this thesis was to assess the role of two fungus-mediated transport mechanisms, namely 'fungal highways' and 'fungal pipelines', under motility restricting or dormancy triggering conditions, respectively. We hypothesized that i) fungal networks facilitate bacterial dispersal over a broad range of environmentally relevant water potentials leading to improved biodegradation, ii) bacterial dispersal along fungal networks is a key process to achieve high functionality in heterogeneous systems exposed to osmotic stress, and iii) the transport of water and nutrients in fungal mycelia enables bacterial activity in a dry and oligotrophic environment.
A multiple microcosm setup was applied to test the influence of changes in the osmotic and matric potential on bacterial dispersal processes in agar matrices. Using glass fiber networks to mimic fungal mycelia, we could show that bacterial movement was facilitated at matric and osmotic potentials between 0 and -0.5 MPa, which drastically improved the biodegradation performance in the system. In a subsequent study, we focused on the relative importance of different spatial processes for biodegradation under osmotic stress. We systematically manipulated the microcosm setup and created scenarios that allowed us to assess the contribution of autonomous bacterial dispersal, network-based bacterial dispersal as well as substrate diffusion to overall biodegradation in the microcosms. We found that, in contrast to autonomous bacterial dispersal and substrate diffusion, network-based bacterial dispersal kept biodegradation almost consistently high regardless of the strength of the osmotic stress.
To test the influence of fungal resource translocation on bacterial activity, we designed a microcosm system based on the germination of *Bacillus subtilis* spores located in an otherwise dry and oligotrophic microhabitat. We found that the presence of mycelia enabled the germination and subsequent growth of bacterial spores near the hyphae. Using a unique combination of Time of Flight- and nanoscale Secondary Ion Mass Spectrometry (ToF- and nanoSIMS) coupled with stable isotope labeling, we could link spore germination to hyphal transfer of water, carbon and nitrogen.
The findings of the thesis propose an important ecological role of the two fungus-mediated transport mechanisms for bacterial activity and related ecosystem functioning under environmental stress. This might be highly relevant to assess the functional stability of ecosystems affected by prolonged drought periods predicted to increase with future climate change.

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SUMMARY

Bacteria in soils are major drivers of biogeochemical cycles and contribute to the provision of fundamentally important ecosystem services. However, they frequently experience conditions that are suboptimal for growth and reproduction. Especially near the earth surface, in the vadose zone, extremely harsh conditions can prevail that may affect bacterial activity in soil. The most common environmental stress for microbes in such terrestrial environments is water deprivation. Low water potentials impose physiological costs on bacteria, restrict their motility and reduce nutrient diffusion, which may cause starvation of bacteria. Other soil microorganisms, namely fungi, are more resistant to water and nutrient deprivation as they can efficiently translocate resources within their mycelium to achieve compensation between spatially separated source and sink regions. Moreover, fungi are less dependent on continuous water phases for active dispersal and nutrient acquisition. Their hyphae can cross air-water interfaces and easily bridge air-filled pores.

Fungi and bacteria co-inhabit a wide variety of habitats and bacterial microhabitats in soil are typically pervaded by fungal hyphae too. Probably caused by this co-occurrence and the strong selection pressure due to limited resources in soil, diverse types of ecological interactions between bacteria and fungi have evolved that may provide benefits to cope with environmental stress. In particular, two fungus-mediated transport mechanisms have been discovered in the past, and were proven to enhance bacterial biodegradation. First, fungal hyphae provide an infrastructure for fast and directed movement of bacteria towards pollutants ('fungal highways'). It is suggested that this accelerated dispersal along mycelia is an important mechanism for bacteria to overcome motility restrictions and invade habitats containing resources. Second, also pollutants are translocated within mycelia and thus become available to bacteria in remote areas ('fungal pipelines'). Generally, this substrate translocation probably becomes important when bacteria are inactive due to starvation and nutrient supply is necessary to enable their activity.

The aim of this thesis was therefore to assess the role of 'fungal highways' under motility restricting conditions (*i.e.* reduced osmotic and matric potentials), and the role of 'fungal pipelines' under conditions triggering dormancy (*i.e.* absence of water and nutrients) for ecosystem functioning. This functioning was evaluated in terms of bacterial growth, degradation activity or the bacterial transition from dormant to vegetative stages. Several experimental microcosm studies were conducted to challenge the hypotheses that i) fungal networks facilitate bacterial dispersal over a broad range of environmentally relevant water potentials leading to improved biodegradation, ii) bacterial dispersal along fungal networks is a key process to enhance functioning in heterogeneous systems exposed to osmotic stress, and iii) water and nutrients are transported in fungal mycelia, transferred to bacteria in otherwise dry and oligotrophic habitats, and subsequently enable bacterial activity.

A multiple microcosm setup was developed to microscopically analyze bacterial dispersal under defined osmotic and matric potentials covering a range of soil-typical val-

ues down to permanent wilting point conditions for plants. In addition to colony spreading, overall bacterial abundance and benzoate biodegradation were measured over time. Indeed, decreasing water potentials decelerated bacterial dispersal, growth and benzoate degradation in the system. Depending on which component of the overall water potential was changed, different critical thresholds were observed. For the matric potential, already slight reductions completely inhibited bacterial dispersal in the agar matrix leading to a drop in growth and biodegradation. For the osmotic potential, decreased values evoked a rather continuous decrease in the two attributes of functioning. The presence of glass fiber networks to mimic fungal mycelia increased bacterial dispersal at matric and osmotic potentials ranging from 0 to -0.5 MPa and considerably improved the biodegradation performance, especially for reduced matric potentials.

A subsequent study focused on the relative importance of different spatial processes for biodegradation under osmotic stress. To this end, the microcosm setup was systematically manipulated to create scenarios that allowed for autonomous bacterial dispersal, network-based bacterial dispersal or substrate diffusion. The contribution of these spatial processes to the overall biodegradation performance was assessed by comparison to the quasi-optimal scenario of homogeneously distributed bacterial cells. It could be demonstrated that, compared to substrate diffusion alone, autonomous bacterial dispersal clearly improved biodegradation at intermediate, but not at very low osmotic potentials. In contrast, network-based bacterial dispersal kept biodegradation almost consistently high regardless of the strength of the osmotic stress pointing to a key role of 'fungal highways' for functional ecosystem stability under stress. To test if resources translocated within mycelia can be transferred to and activate bacteria, another experimental microcosm system was designed. The presence of mycelia, connected to a separate resource patch, induced the germination and vegetative growth of *Bacillus subtilis* spores in an otherwise dry and oligotrophic habitat. This phenomenon was shown for two different fungi and one oomycete, respectively. Microscopic analysis revealed that vegetative cells occurred solely in direct vicinity of the hyphae pointing to the importance of the spatial organization of the organisms for their interaction. Thereupon, using a unique combination of Time of Flight- and nanoscale Secondary Ion Mass Spectrometry (ToF- and nanoSIMS) coupled with stable isotope labeling, spore germination could be linked to the hyphal transfer of water, carbon and nitrogen. This direct experimental evidence for the stimulation of bacterial activity by mycelial supply of scarce resources suggests a relevant contribution of this interaction to ecosystem functioning in natural stressed habitats.

In summary, the findings propose important ecological roles of the two investigated fungus-mediated transport mechanisms for bacterial activity and related ecosystem functioning under water and nutrient limitations. Therefore, they may be highly relevant for the functional stability of ecosystems affected by drought periods, which are anticipated to elongate and intensify with future climate change. Moreover, the findings of this thesis help to understand the dynamics of bacterial distribu-

tion and activity in spatially heterogeneous ecosystems exposed to fluctuating environmental conditions. The ability of mycelia to facilitate bacterial dispersal under motility restricting conditions and to redistribute and transfer resources under scarcity may help bacteria to access and occupy otherwise uninhabitable ecological niches. Hence, for assessing the dynamics and stability of microbial ecosystem functioning, fungi and bacteria need to be considered as interconnected entities rather than autonomously operating ecosystem components

ZUSAMMENFASSUNG

Bakterien im Boden treiben biogeochemische Kreisläufe an und tragen maßgeblich zur Bereitstellung wichtiger Ökosystemdienstleistungen bei. Allerdings herrschen im Boden häufig Umweltbedingungen, die bakterielles Wachstum sowie Reproduktion hemmen. Insbesondere in den oberen Bodenschichten, in der sogenannten ungesättigten Bodenzone, führen solche Umweltbedingungen häufig zu einer Verringerung der bakteriellen Aktivität. Tatsächlich stellt Wasserentzug und die damit einhergehende Austrocknung den wohl häufigsten Stress für Mikroorganismen in terrestrischen Habitaten dar. Physiologische Kosten für die Anpassung, Einschränkung der Beweglichkeit und verringerte Substratdiffusion, die zum Hungern der Zellen führen kann, sind einige der Konsequenzen, die eine Verringerung des Wasserpotentials im Boden mit sich bringt. Im Vergleich zu Bakterien besitzen Pilze eine höhere Resistenz gegenüber Wasser- und Nährstofflimitierung, da sie räumliche Unterschiede in der Ressourcenverfügbarkeit durch einen effektiven Transport in ihrem Myzel ausgleichen können. Außerdem sind Pilze für ihre Ausbreitung und die Erschließung neuer Substratquellen nicht auf kontinuierliche Wasserphasen angewiesen. Mit ihren Hyphen können sie Luft-Wasser-Grenzschichten überwinden und damit luftgefüllte Bodenporen durchqueren.

Pilze und Bakterien besiedeln häufig gemeinsame Habitate und auch bakterielle Mikrohabitate im Boden sind von Pilzhyphen durchzogen. Dieses Aufeinandertreffen und der starke Selektionsdruck, der häufig von Ressourcenlimitierung im Boden verursacht wird, haben wahrscheinlich zur Evolution diverser Formen der Interaktion zwischen Bakterien und Pilzen beigetragen, die unter Umständen bei der Bewältigung von Umweltstress helfen können.

Zwei pilzbasierte Transportmechanismen wurden entdeckt, die maßgeblich zur Steigerung des bakteriellen Schadstoffabbaus beitragen. Zum Ersten stellen Pilzhyphen eine Infrastruktur für die schnelle und gerichtete Ausbreitung von Bakterien hin zum Schadstoff dar (‚Pilzautobahnen'). Allgemein wird dieser beschleunigten Ausbreitung entlang der Hyphen eine Bedeutung für das Überwinden von Beweglichkeitseinschränkungen und dem Erschließen neuer Habitate und Substratquellen im Boden zugesprochen. Zum Zweiten wurde gezeigt, dass Schadstoffe in den Pilzhyphen zu den Bakterien transportiert werden, wo sie dann abgebaut werden können (‚Pilzpipelines'). Diese Ressourcentranslokation spielt wahrscheinlich vor allem dann eine Rolle, wenn sich Bakterien durch andauernde Limitierung in einem inaktiven Zustand befinden und die Bereitstellung von Nährstoffen für ein Wiedererlangen ihrer Aktivität notwendig ist.

Das Ziel der vorliegenden Arbeit war es deshalb, die Rolle der ‚Pilzautobahnen' und ‚Pilzpipelines' für die Ökosystemfunktionalität unter solchen Bedingungen zu testen, die zu einer Einschränkung der bakteriellen Beweglichkeit führen (verringerte osmotische und Matrixpotentiale) bzw. Dormanz auslösen (Abwesenheit von Wasser und Nährstoffen). Das bakterielle Wachstum, die Abbauaktivität sowie die Umwandlung von dormanten in vegetative Zellen dienten als Untersuchungsparameter um die Ökosystemfunktionalität zu bewerten. Es wurden die Hypothesen untersucht, dass i)

Pilznetzwerke die bakterielle Ausbreitung über einen weiten Bereich umweltrelevanter Wasserpotentiale unterstützen und damit zu einer Verbesserung der Abbauleistung im System beitragen, ii) die bakterielle Ausbreitung entlang der Pilznetzwerke eine Schlüsselrolle für die Funktionalität von heterogenen Ökosystemen einnimmt, die verringerte osmotische Potentiale aufweisen und iii) Wasser- und Nährstoffe in Pilzen transportiert werden und ihr Transfer zu Bakterien deren Aktivität in trockenen und nährstoffarmen Gebieten ermöglicht. Um diese Hypothesen zu testen, wurden verschiedene Mikrokosmenexperimente durchgeführt.

Ein Mikrokosmensystem, bestehend aus 24 Einzelmikrokosmen, ermöglichte die mikroskopische Untersuchung bakterieller Verteilungsprozesse bei definierten Wasserpotentialen, die über einen umweltrelevanten Bereich bis hin zum permanenten Welkepunkt variiert wurden. Außerdem wurden die bakterielle Abundanz sowie der Abbau von Benzoat über die Zeit gemessen, um die Auswirkungen verringerter Wasserpotentiale auf die Ökosystemfunktionalität zu erfassen.

Verringerte Wasserpotentiale verlangsamten die bakterielle Ausbreitung im System und verursachten eine Verzögerung im Wachstum sowie im Benzoatabbau. Je nachdem welche Teilkomponente des Wasserpotentials variiert wurde, konnten unterschiedliche kritische Schwellenwerte festgestellt werden. Variationen des osmotischen Potentials führten zu einer vergleichsweise kontinuierlichen Abnahme von Ausbreitung, Wachstum und Abbau, während bereits Matrixpotentiale von -0.25 MPa die bakterielle Ausbreitung im Agar komplett unterdrückten und damit einen deutlichen Einbruch des Wachstums und der Abbauleistung herbeiführten. Im Gegensatz dazu, fand bakterielle Ausbreitung entlang von Glasfasern, die uns als Modell für Pilznetzwerke dienten, bei osmotischen und Matrixpotentialen zwischen 0 und -0.5 MPa immer noch statt. Das führte zu einer deutlichen Verbesserung der Abbauleistung im System, insbesondere für die verringerten Matrixpotentiale.

In einer auf diese Ergebnisse aufbauenden Studie wurde die Bedeutung verschiedener räumlicher Prozesse für die Abbaueffizienz unter dem Einfluss von osmotischem Stress untersucht. Dafür wurde das Mikrokosmensystem systematisch abgeändert und damit der Einfluss von unabhängiger bakterieller Ausbreitung, netzwerkgestützter bakterieller Ausbreitung und Substratdiffusion auf den Substratabbau im System untersucht. Dazu wurden die Szenarien mit einem Referenzszenario verglichen, das eine homogene Verteilung der Bakterien im System aufwies. Bakterielle Beweglichkeit ergab einen deutlich verbesserten Substratabbau im System im Vergleich zum Szenario, in dem nur Substratdiffusion stattfinden konnte. Jedoch verschwand dieser Vorteil sukzessive mit sinkenden osmotischen Potentialen. Im Vergleich dazu, konnte die netzwerkvermittelte bakterielle Ausbreitung die initiale heterogene Bakterienverteilung im System nahezu vollständig ausgleichen unabhängig davon, um welchen Betrag das osmotische Potential variiert wurde. Das deutet auf eine Schlüsselfunktion der ‚Pilzautobahnen' für die funktionelle Ökosystemstabiltät unter Umweltstress hin.

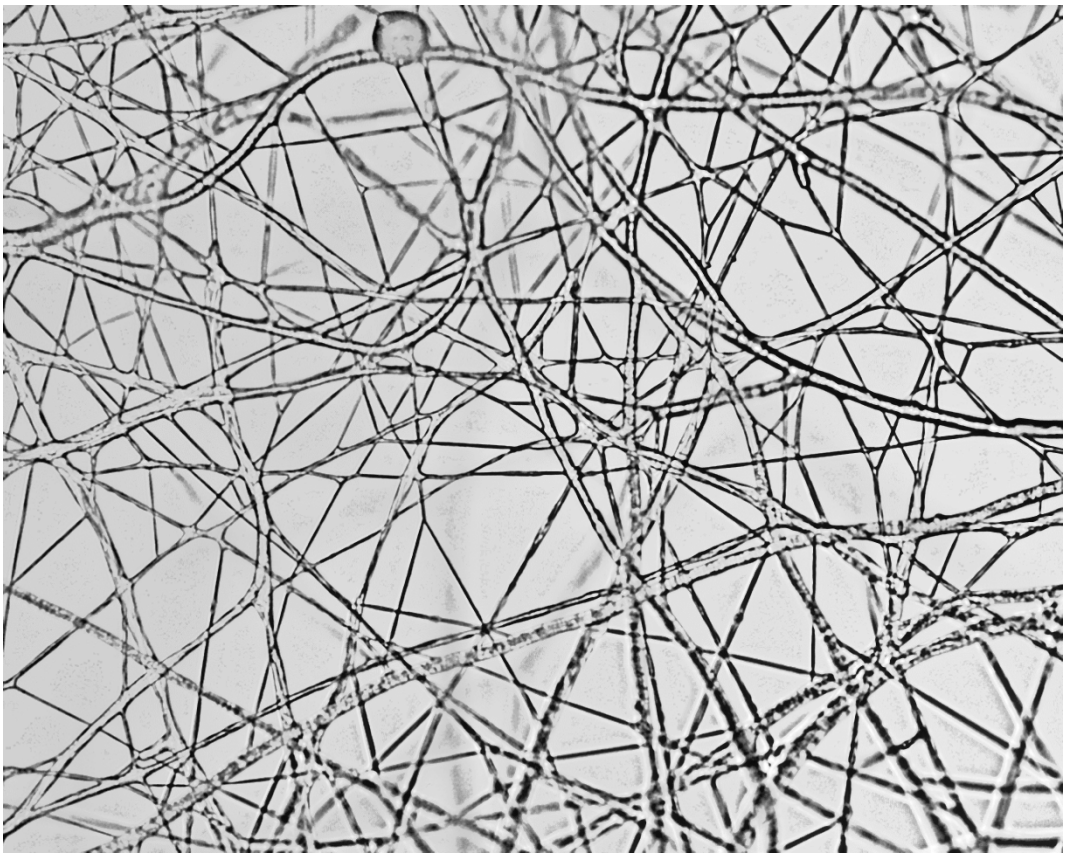
Um zu testen, ob Ressourcen in Pilzmyzelien transportiert und an Bakterien abgegeben werden, wurden spezielle Mikro-

kosmen entwickelt. In diesen wurde die Auskeimung von *Bacillus subtilis* Sporen in Gegenwart von Myzelien in trockenen und nährstoffarmen Gebieten untersucht. Sowohl Auskeimung als auch Wachstum konnte in Gegenwart von zwei Pilzen und einem Oomyceten nachgewiesen werden. Eine mikroskopische Untersuchung zeigte außerdem, dass vegetative Zellen ausschließlich in der direkten Hyphenumgebung auftreten. Das weist auf die Bedeutung der räumlichen Anordnung der interagierenden Organismen für die Aktivierung der Sporen hin. Mithilfe einer erstmalig erprobten Kombination von *Time of Flight*- und *nanoscale Secondary Ion Mass Spectrometry* (ToF- und nanoSIMS), die in Kombination mit stabiler Isotopenmarkierung durchgeführt wurde, konnte der Zusammenhang zwischen Sporenauskeimung und dem Transfer von Wasser, Kohlenstoff und Stickstoff in den Hyphen gezeigt werden. Damit wurde der direkte experimentelle Nachweis für die Stimulation bakterieller Aktivität durch die Hyphen-gestützte Versorgung bakterieller Zellen mit limitierenden Ressourcen erbracht. Dieser Mechanismus könnte entscheidend zur Aufrechterhaltung von Ökosystemfunktionen in von Umweltstress beeinflussten Habitaten beitragen.

Zusammenfassend weisen die Ergebnisse der Dissertation auf eine wichtige ökologische Funktion der untersuchten pilzbasierten Transportmechanismen für die Unterstützung der bakteriellen Aktivität und die daran geknüpfte Ökosystemfunktionalität unter Trockenheit und Nährstofflimitation hin. Relevanz besitzen die erhaltenen Erkenntnisse insbesondere in Bezug auf die Bewertung der funktionellen Stabilität von Ökosystemen, die von ausgedehnten Trockenperioden betroffen sind. Tatsächlich sind solche Trockenperioden im Verdacht durch den Klimawandel sowohl an Länge als auch in ihrer Frequenz deutlich zu zunehmen. Außerdem könnten die Ergebnisse dabei helfen, die Dynamik bakterieller Verteilung und Aktivität in heterogenen Umweltsystemen und unter fluktuierenden Umweltbedingungen besser zu verstehen. Wahrscheinlich unterstützt die Fähigkeit von Myzelien die bakterielle Ausbreitung zu fördern und Ressourcen zu transferieren außerdem die Erschließung neuer Nischen, die unter sonstigen Gegebenheiten für die Bakterien unzugänglich wären. Daher sollten Pilze und Bakterien eher als eine funktionale Einheit des Ökosystems betrachtet werden anstelle von unabhängig agierenden Komponenten des Ökosystems.

Chapter 1

SCOPE AND OUTLINE OF THE THESIS



Hyphae of Pythium ultimum on agar

1 SCOPE AND OUTLINE OF THE THESIS

Bacteria in soil are major drivers of biogeochemical cycles and contribute to the provision of fundamental ecosystem services. In terrestrial environments, bacteria frequently experience conditions that diminish their activity. Drought, for example, restricts the access to nutrients by suppressing bacterial dispersal and substrate diffusion. Two fungus-mediated transport mechanisms were proven to facilitate bacterial dispersal ('fungal highways') and to supply bacteria with substrates ('fungal pipelines'). However, the role of both mechanisms for bacterial activity and ecosystem functioning under common environmental stress scenarios remains elusive.

The main objective of the thesis was to investigate whether fungus-mediated transport mechanisms could support bacterial activity and ecosystem functioning under environmental stress, *i.e.* reduced water and nutrient availability. In detail, we proposed and tested the following hypotheses:

i. Mycelial networks facilitate bacterial dispersal over a broad range of environmentally relevant water potentials leading to improved pollutant biodegradation (H_i).

ii. Bacterial dispersal along fungal networks is a key process to achieve high functionality in heterogeneous systems exposed to osmotic stress (H_{ii}).

iii. Transport of scarce resources in fungal mycelia enables bacterial activity in dry and oligotrophic environments (H_{iii}).

Chapter 2 gives a general introduction into physical and biological factors influencing microbial habitats in soil. The importance of soil microbes for ecosystem service provisioning is briefly discussed with a focus on pollutant biodegradation by bacteria and fungi. Based on this, we present common ecological challenges for bacteria in soil that may restrict bacterial activity and ecosystem functioning. Special emphasis is given to bacterial-fungal interactions by elaborating different examples for cooperative and competitive interactions and presenting the role of fungi as transportation networks for bacteria ('fungal highways') and resources ('fungal pipelines'). At last, the use of synthetic microbial ecosystems as a tool to study operating ecological mechanisms in microbial ecology is introduced. The chapter ends with a summary of the major aims of the study, which formed the basis for the three research articles included in the thesis.

Chapter 3 describes the use of a synthetic microbial ecosystem to test the influence of different osmotic and matric potentials on bacterial dispersal, growth and biodegradation in presence and absence of artificial dispersal networks (H_i). Using a microscopy-based approach, we observed improved bacterial dispersal of *Pseudomonas putida* KT2440 in presence of glass fiber networks at lowered water potentials. This clearly enhanced bacterial growth as well as the degradation of benzoate in the microcosms.

In **Chapter 4,** systematically manipulated scenarios were created to unravel and quantify the impact of different spatial processes, *i.e.* autonomous bacterial dispersal, network-mediated bacterial dispersal, and substrate diffusion, for biodegradation under reduced osmotic potentials (H_{ii}). Network-mediated dispersal was found to keep biodegradation almost consistently high regardless of the strength of the osmotic stress thus pointing to its key role for efficient biodegradation in heterogeneous systems.

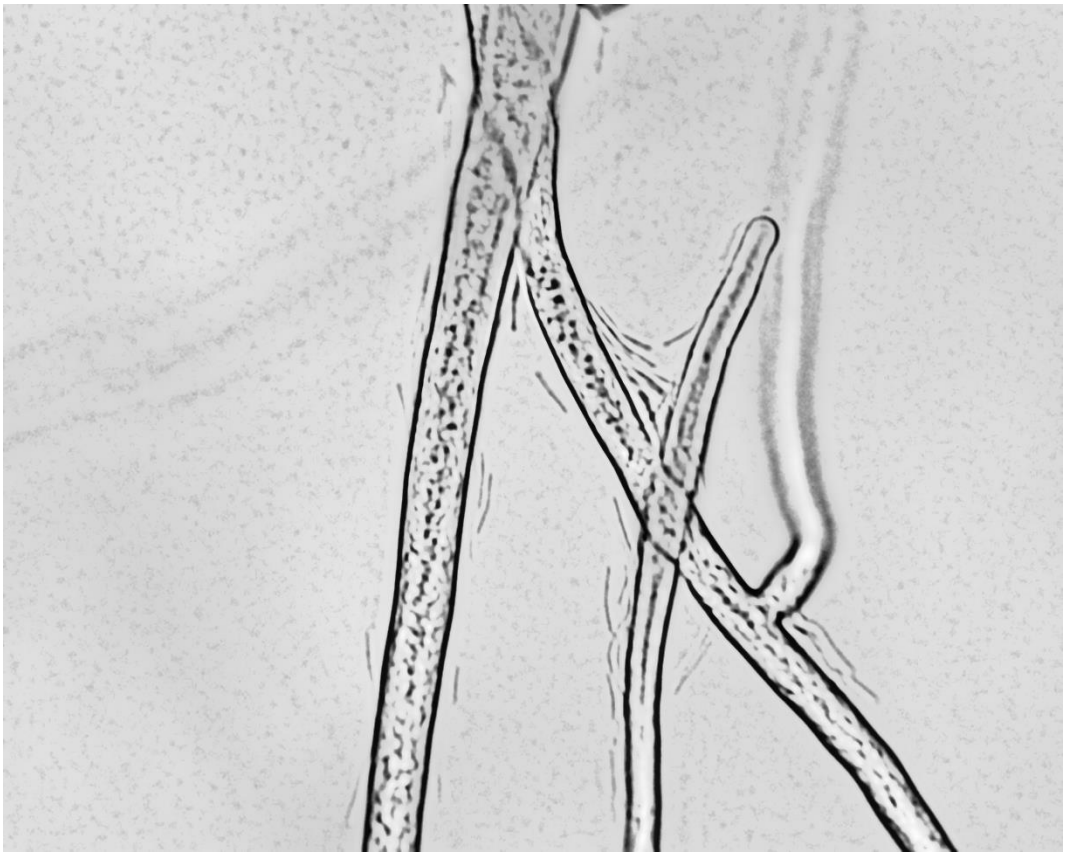
Chapter 5 studies the influence of mycelium-mediated resource transfer processes for bacterial activity (H_{iii}). Different fungi and an oomycete were demonstrated to induce germination of bacterial spores and to enable vegetative growth in a dry and oligotrophic environment. The results of a spatially resolved mass-spectrometric analysis (ToF- and nanoSIMS) revealed a transfer of water, carbon and nitrogen over several centimeters in the mycelium as the mechanism underlying the activation.

In **Chapter 6**, the main findings are summarized and discussed with respect to the major objective of this thesis and the relevance for natural soil systems. Moreover, ecological stability properties are introduced and the potential contribution of the investigated fungus-mediated transport mechanisms to functional ecosystem stability is debated. The chapter finishes with a critical assessment of the applied synthetic microbial ecosystems considering both strengths and limitations.

Chapter 7 presents a short conclusion and an outlook on additional experiments related to the research objective of this thesis. We propose further experiments to complement the 'fungal highway' and the 'fungal pipeline' study as well as an experimental possibility to test the combined effects of the two fungus-mediated transport mechanisms. Finally, further studies related to research questions in other contexts of microbial ecology are briefly addressed in which these mechanisms might also be highly relevant.

Chapter 2

INTRODUCTION



Bacillus subtilis along Pythium ultimum hyphae

2 INTRODUCTION

'Life would not long remain possible in the absence of microbes.'

- Louis Pasteur -

The last decades have led to unprecedented advances in our knowledge of the microbial world[1]. This was enabled because of the technical progress providing access to a wealth of information on the forces shaping bacterial communities, their responses to environmental change and their physicochemical and biological interactions or shortly – on microbial ecology[2].

Microbes sustain life on this planet because of their myriad associations and biogeochemical processes. Recently, Gilbert & Neufeld scrutinized Pasteur's assumption that life on this planet would not be possible if there were no microbes. They created various 'what-if' scenarios and, surprisingly, they concluded that in absence of microbes, life as an entity would persist but the quality and quantity would be reduced dramatically. Most biogeochemical cycles would cease, human and animal waste would accumulate and the limited biomass decomposition would result in a rapid exhaustion of available macro- and micronutrients in terrestrial and aquatic environments. Moreover, living food sources would be increasingly difficult to find. Due to nitrogen depletion, plants would cease photosynthesis and die. Most ruminant livestock would starve without microbial symbionts. Altogether, the absence of microbes would lead to an environmental, ecological, and humanitarian disaster[3].

In their thought experiment the biologists imagine that an antimicrobial wand removes all microbes. But is this just fiction? More than 20 years ago, the vast majority of the world's nations declared that human activity causes species loss at an alarming rate and transforms ecosystems into depauperate systems[4–6]. It is hypothesized that the anthropogenically induced loss and decline of species is a characteristic of the planet's 6th Great Mass Extinction[7]. The patterns and processes of biodiversity loss are still poorly understood. However, environmental and climate change are both anticipated to play a major role when changes in the abiotic conditions begin to exceed species' tolerance limits[8]. While responses of macroorganisms to novel conditions are often reported, we do not know if microbes follow the same patterns of responses[9]. Especially in terrestrial systems the response of heterotrophic microbes to stress and disturbances expected with climate change and the mechanisms controlling their structural and functional stability are insufficiently understood[10,11]. Developing such an understanding, however, is crucial to meet many of the challenges facing human society today – like, for example, the management of natural ecosystems and the mitigation of climate change[12].

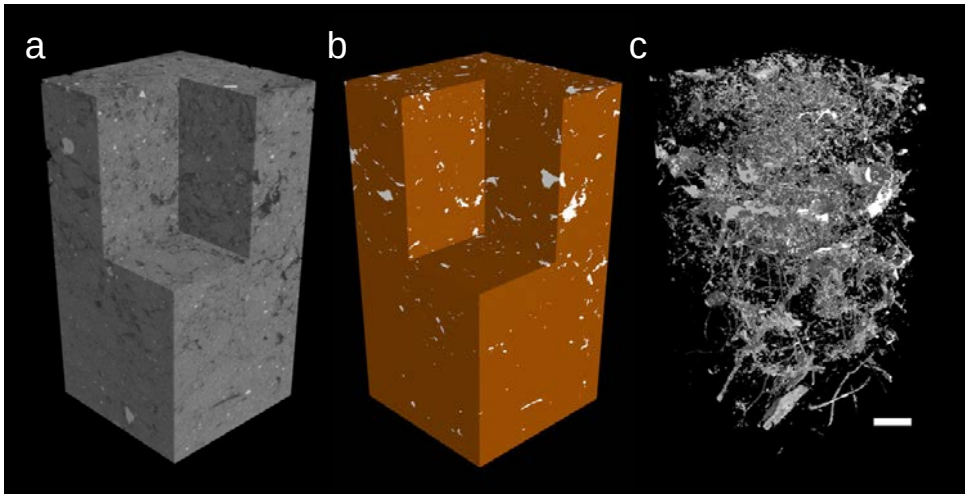


Figure 1 3D visualization of a reconstructed soil core. a) Grayscale density map of a soil core showing a virtual 'cut-out'. b) Thresholded 3D image highlighting solid material in brown and pore space in white. c) Visualization of the extracted pore structure in the soil core. Scale bar = 10 mm. Figure modified from[17].

2.1 The Soil-Microbe Complex

NASA's Curiosity rover finds first hints of microbial life in martian soil. This is hardly surprising, considering that soil supports all forms of life and constitutes the greatest reservoir of biodiversity on our own planet[13,14]. Indeed, just a handful of soil can harbor 10^{12} bacteria, 10^4 protozoa, 10^4 nematodes, 25 km of fungal mycelium, and countless other species[13].

Soil is not only the most diverse but also the most complicated biomaterial on the planet[13]. Reduced to the essential, it is the cover of the earth's surface composed of solids, water and air[15]. The solid material in soil accounts for more than half its volume[16] (Fig. 1a,b) and is comprised of inorganic materials such as sand, silt and clay particles and organic matter mostly derived from plant and animal inputs[18]. The aggregation of these components creates a highly complex environment with an intricate and tortuous three-dimensional network of water and air-filled pores[19] (Fig. 1c). Indeed, this labyrinth forms the physical framework in which all soil processes occur[18]. The pore network structure results in a complex distribution of water films and gradients in mobile resources and organic matter across distances of a few micrometers[16]. Due to this spatial heterogeneity soil is unique in the diversity of its microenvironments[13]. These microenvironments are highly variable over small timescales as the biological community grows, water flows or root growth changes the physical and chemical microstructure of the system[14]. Recently, Crawford et al.[20] provided experimental evidence for their conceptual model for self-organization of the soil-microbe complex.

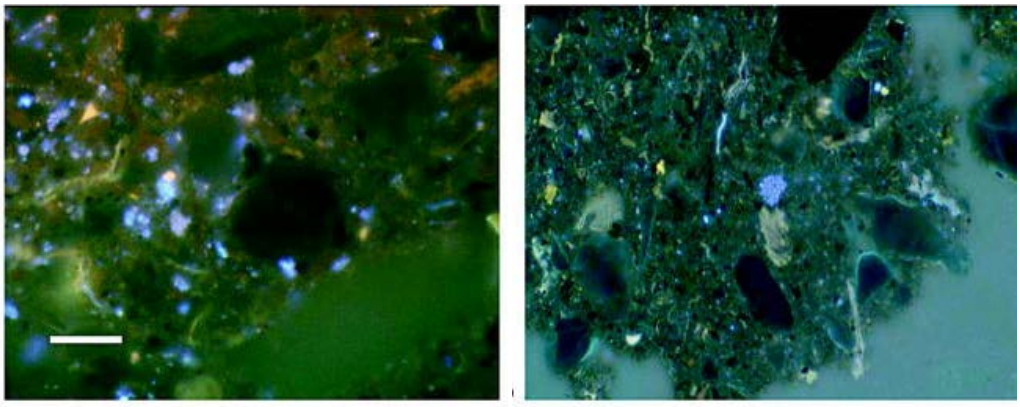


Figure 2 Microscopic images of fluorescent bacterial microcolonies embedded in sliced soil aggregates. Scale bar = 20 µm. Figure taken from[24].

They show that the porous structure arises spontaneously out of the interaction between microbial activity, particle aggregation and resource flows in soil[20].

Although soil is full of life, its surface is not homogeneously populated[21]. This is due to the tremendous internal soil surface area with 1 cm^3 of soil being equivalent to approximately 20 m^2. Consequently, even billions of cells distributed homogeneously over this area would cover only a tiny fraction of the available space[13,22]. However, the distribution of soil microorganisms is neither uniform nor random. Instead, different studies provide evidence that *e.g.* bacterial communities are arranged in micro-colonies, which show a patchy distribution within soil pores or soil particles[23] (Fig. 2). This spatial distribution is a result of multiple environmental drivers and intrinsic population processes such as dispersal, reproduction, mortality, competition or cooperation[22].

The connection between microhabitats in soil depends on the soil structure and the hydration status. Especially the hydraulic connectivity, *i.e.* the presence of continuous water films, has a substantial impact on the microbial activity as it controls the diffusion of nutrients and signaling molecules as well as the motility of bacteria in the soil matrix[16,25]. Based on the water content the soil compartment is distinguished into two different zones: the saturated zone beneath the ground water table and the vadose (or unsaturated) zone extending from the surface to the groundwater table. Water in the vadose zone is retained via a combination of adhesion and capillary action. Sizes of liquid elements and film thicknesses are determined by the water potential and are linked to the degree of dryness and pore space geometry[16]. In the vadose zone microbial growth and activity rapidly decrease with increasing depth due to reduced soil organic matter and nutrient concentrations and increase again in the capillary fringe located at the air-water intersect above the groundwater table[26,27].

2.2 Microbial Contributions to Soil Ecosystem Services

'The nation that destroys its soil destroys itself.'

- Franklin Delano Roosevelt -

Soil provides ecosystem services (ES) with a high economic benefit[28]. Daily has defined ES as 'the conditions and processes through which natural ecosystems, and the species that make them up, sustain and fulfill human life'[29]. ES can be classified into four main categories: *Provisioning ES* are associated with the delivery of goods (*e.g.* food, fiber, fresh water). *Supporting ES* promote life on the planet (*e.g.* soil formation, nutrient cycling, flood control, pollination). *Regulating ES* are derived from benefits of the regulation of ecosystem processes (*e.g.* climate regulation, disease control, detoxification). *Cultural ES* are those not associated with material benefits (*e.g.* recreation, aesthetic and cultural uses)[30]. Soils with its inherent microbes contribute to all four different dimensions of ecosystem services. Different examples of soil ES belonging to the first three categories and the role of soil microbes are shown in Table 1.

Table 1 Ecosystem services provided by soil microorganisms (modified from [15]).

SOIL ECOSYSTEM SERVICE	ROLE OF SOIL MICROBES
Provisioning ES	
Biochemical, medicinal and pharmaceutical resources	Production of enzymes or antimicrobial agents
Genetic resources	Large portion of life's genetic diversity, genes for the production of a wide variety of compounds
Supporting ES	
Plant growth	Supply with nutrients, phytohormones, induced systemic resistance
Nutrient cycling	Drivers of carbon, nitrogen and phosphorus cycling
Regulating ES	
Carbon sequestration	Mineralization of soil carbon, determination of carbon storage capacity
Recycling of wastes and detoxification	Degradation and mobilization of contaminants

Attempts to assign a value to certain ecosystem services revealed that the economic benefit of waste recycling has the largest portion and accounts for $760 billion per year[31]. Organic matter in soil is decomposed mostly by fungi and bacteria[32]. The same holds true for the degradation of pollutants entering the soil compartment mostly due to human activity such as agricultural practice, industrial processes or the use of chemicals in many areas of our daily lives[33]. Bioremediation of chemical pollutants in soil is estimated to provide a global economic benefit of $121 billion per year[31].

2.2.1 *Soil Pollution*

Along with the rising level of industrialization, the production and use of chemicals drastically increased[34]. Soils are a natural sink for contaminants and a huge variety of chemicals released into the environment finally ends up in soil[33]. Over time, the accumulation of tiny amounts can reach considerable concentrations depending on environmental conditions and degradability of the compound[35].

Soil pollution is mainly caused by human activity. Chemicals can enter the soil via different routes[34,36] such as:

- Accidental spills and leaks during storage, transport or use of chemicals
- Foundry activities and manufacturing processes that involve furnaces
- Mining activities involving crushing and processing of raw materials
- Agricultural activities involving the spread of pesticides and fertilizers
- Transportation activities
- Dumping of chemicals
- Storage of wastes in landfills

The sources of soil pollution are not necessarily located close to the contaminated area. Depending on their physicochemical properties and reactivity they may travel over longer distances and pollute soil far away from the area where they were initially released[36]. Pollutants can be, for example, transported by wind (especially if they are associated to dusts) or by groundwater.

Soil pollution can affect human health via crops and plants that absorb pollutants from soil and pass these on to humans. Moreover, chemicals can leach into the groundwater, which is then consumed by humans without sufficient pretreatment. In addition, inhalation of vaporized soil contaminants and direct contact with contaminated soil endangers human health. Several pollutants are known to cause diseases or symptoms of poisoning in humans[37]. Examples are heavy metals like lead (Pb), mercury (Hg) and arsenic (As) as well as polycyclic aromatic hydrocarbons (PAH) and numerous insecticides and herbicides[38]. Soil pollution is also a threat to ecosystem functioning and thus to the provision of ecosystem services[39]. The ecological balance of ecosystems can severely be affected when plants as well as soil (micro-) organisms are unable to adapt to changes in the soil chemistry or the toxic effects of the pollutants. The consequences range from increasing soil erosion to diminished soil fertility thus making land unsustainable for agriculture[31].

2.2.2 *Microbial Biodegradation of Pollutants in Soil*

Without actively degrading microbes many of the pollutants would persist and might adversely affect the biosphere and contaminate our water resources[40]. Two main actors of the soil microbiome significantly contribute to the biodegradation of pollutants in soil – bacteria and fungi[41]. However, the ways in which they degrade pollutants are considerably different from each other.

Bacteria typically use the pollutant as a growth substrate and the enzymes involved in bacterial degradation pathways are specific[33]. If it increases their ecological success, bacteria adapt to new pollutants by extending or modifying existing pathways thus being able to degrade an almost unlimited variety of pollutants[42]. However, if the concentration, bioavailability or energy content of the pollutant is too low to cover their maintenance requirements, bacteria undergo a stringent response coupled to a reduced metabolic cell activity[33,43]. Thus, it is unlikely that specific pathways exist or will evolve for micropollutants. Their ability to use alternative electron acceptors enables bacteria to unfold activity in anoxic zones such as in deeper soil layers or after heavy rainfall events[42]. In general, bacteria are promising degraders for aliphatic and aromatic pollutants which have only few functional groups and occur at high concentrations[33]. Indeed, since the mid-1970s, the use of degrading bacteria is a widely used technology for *in-situ* bioremediation techniques aiming at the decontamination of polluted sites. Biostimulation and bioaugmentation are the two major strategies pursued in bioremediation. The first involves the supply of stimulating agents (nutrients, electron acceptors or donors) to indigenous microorganisms, whereas the second is based on the injection of pregrown cultures to subsurfaces in order to enhance the degradation capacity[44].

Filamentous fungi dominate the soil microbial biomass and form huge networks extending over up to hundreds of hectares[45] (cf. section 2.4). Almost all fungi degrade organic pollutants co-metabolically under aerobic conditions while using plant-derived carbon sources as primary growth substrates. Indeed, merely simple monoaromatics (*e.g.* phenol, *p*-cresol, toluene) or aliphatic (*e.g. n*-alkanes) compounds were shown to serve as growth substrates for fungi[33]. The nonspecific mechanisms used by a special group of saprotrophic wood-decaying fungi, the so-called white-rot fungi, include a particular potential for the co-metabolic mineralization of even complex mixtures of organic pollutants[46,47]. They secrete several unspecific oxidoreductases (*e.g.* lignin and manganese peroxidase, laccase) with a high redox potential, which play a central role in the degradation of recalcitrant biopolymers such as lignin[48]. However, despite their huge potential, fungi are still not the preferential organisms for bioremediation approaches[33]. The main reasons for that are the demand for oxygen and the costs of providing it to polluted soils. Moreover, fungi do not tolerate mechanical homogenization and thus remediation schemes that have been developed for bacteria do not work for fungi and must be adapted.

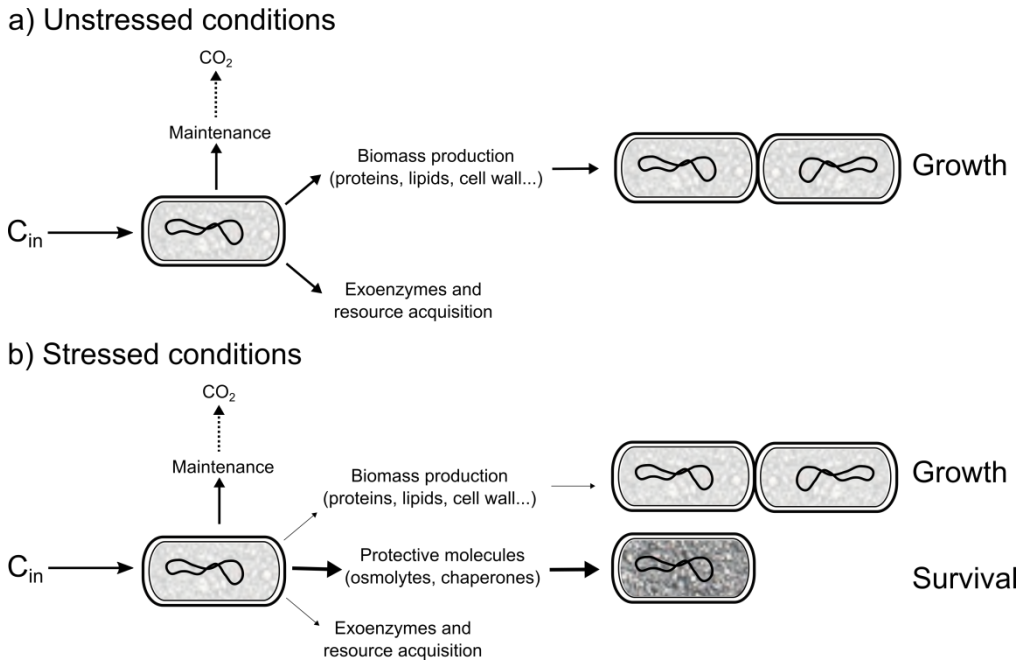


Figure 3 Resource allocation patterns in microorganisms. a) Under unstressed and (b) Under stressed conditions. Under stress there is a reallocation of resources from growth to survival pathways *i.e.* the production of protective molecules. Figure modified from[49].

2.3 Ecological Challenges for Bacteria in Soil

In soil, bacteria frequently experience conditions that are suboptimal for growth and reproduction. Especially near the earth surface, in the vadose zone, extremely harsh conditions can prevail that may affect bacterial habitats and activity[16]. Stress imposes costs on microorganisms that they must cover to survive and remain active[49]. Microorganisms acclimate to stress by altering their allocation of resources from growth to survival pathways via e.g. the production of protective molecules (Fig. 3)[43,49]. However, if the stress is too extreme they will enter dormancy or die[43,50]. Death and dormancy both remove bacterial functions from the soil, and thus may have severe consequences for the provision of ecosystem services[49]. Soils are influenced simultaneously by multiple stressors[51]. Drought, temperature fluctuations, changes in the pH value and anthropogenically induced contaminations with *e.g.* heavy metals or organic contaminants constitute only a small selection of the different factors that might affect bacterial activity in soil[51].

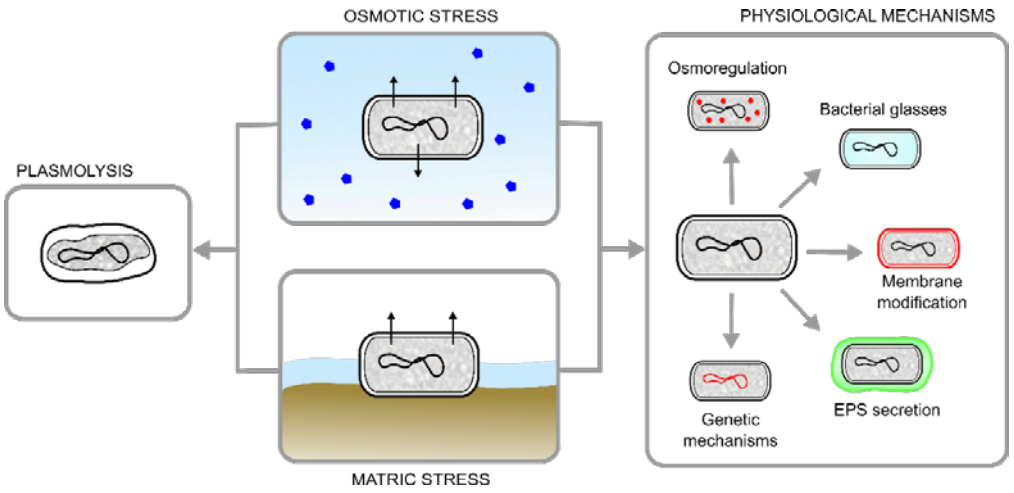


Figure 4 Consequences of osmotic and matric stress for bacterial cells. In absence of tolerance mechanisms water would flow out from the cell (indicated by the arrows) and the tugor pressure would decrease (Plasmolysis). Many bacteria can adapt to changes in the water potential by different physiological mechanisms, which help to maintain cell integrity.

2.3.1 *Soil Moisture Content*

Water depriviation is perhaps the most common environmental stress that bacteria in soil experience[49] and one of the major factors controlling rates of bacterially mediated biogeochemical processes[9,52]. According to prediction on environmental change, future climates will be characterized by more variable rainfall patterns. Both, the duration of drought periods and the size of rainfall events is expected to increase[53]. The thermodynamic availability of water is described by the water potential (Ψ_w), which is a measure of the energetic state of water in a system[54]. It describes the work necessary to move 1 mol of water from some point in a system to a pool of pure water at atmospheric pressure and at the same temperature as the system under consideration. If the Ψ_w differs between these two locations, water is not in equilibrium and there will be a net tendency for water to flow toward the region where Ψ_w is lower[54]. The Ψ_w is typically expressed as energy per unit volume of water (*i.e.* a pressure) and is always subatmospheric (negative)[55,56]. In topsoils, the Ψ_w often falls down to -1.5 MPa, which represents the permanent wilting point for plants (*i.e.* the minimal soil moisture content a plant needs to maintain its tugor pressure).

The two largest components of Ψ_w in soil are the osmotic (Ψ_o) and the matric (Ψ_m) potential[57]. According to Potts[54], the major distinction between Ψ_o and Ψ_m for bacteria (and other soil organisms) is the immediate environment to which they are exposed. Under matric stress the surfaces of the cell walls are exposed to the gas phase, while cells under osmotic stress are bathed in a solution of diminished water activity[a] (Fig. 4). In water saturated soils, Ψ_w is determined almost exclusively by Ψ_o, which depends on the amount of solutes in the aqueous phase[58]. Several factors could increase the solute concentration in the soil solution to

[a] A measure of water that is available for microbial growth, expressed as the decimal fraction of the amount of water present when the substrate is in equilibrium with a saturated atmosphere[33].

levels that may induce hyperosmotic[b] stress. These include a concentration of salts due to low rainfall and high evapotranspiration, salt input via irrigation, fertilizer amendment or root exudation[16,59]. Indeed, low Ψ_o are a major problem in irrigated agriculture and globally, 10^8 ha of arable land contain salt at concentrations that cause hyperosmotic stress in plants and microorganisms[60].

As soil dries and the pores drain, Ψ_m becomes the major contributor to Ψ_w[61]. It determines the sizes of liquid elements and film thicknesses via the strength of adsorptive and capillary forces acting upon water held in soil pores[16,61]. At low Ψ_m, water films can become too thin to support a complete immersion of the bacterial cell and thus may cause cell desiccation.

Different studies have proven that low Ψ_w exert negative effects on bacterial biomass development[62,63], amino acid uptake and protein synthesis[64], and respiration[59,65]. The reasons are the physiological stress associated to the active acclimatization to low water potentials or the indirect effects, which arise as a consequence of low hydration conditions such as restricted bacterial motility or reduced nutrient fluxes that may cause starvation.

2.3.2 *Physiological Stress*

Changes in the soil water potential can induce physiological stress in bacteria[66]. At low Ψ_w, water tends to flow out from the cell, intracellular turgor pressure decreases and finally cells plasmolyse (Fig. 4). Some bacteria may tolerate this stress because they possess adaptive traits, like a thick cell wall, which helps to withstand osmotic pressure[49]. Others have developed physiological strategies to maximize their chances of survival under certain environmental conditions. To maintain homeostasis and to avoid dehydration, bacteria commonly accumulate osmolytes to reduce their internal water potential (=osmoregulation)[66]. Osmolytes also stabilize native protein structures via preferential exclusion of harmful solutes from the protein's surface[67]. Microorganisms use simple organics with a good balance of high solubility and limited direct physiological effects[68]. Typically, amino compounds such as proline, glutamine, and glycine betaine serve as osmolytes in bacteria[68]. Moreover, non-reducing disaccharides such as sucrose and trehalose maintain membrane fluidity by interacting with the polar head groups of the phospholipids[69]. Beyond that, several other physiological mechanisms exist by which bacteria respond to reduced Ψ_w. Some examples like membrane modifications, EPS secretion and genetic mechanisms are presented in Figure 4. A detailed description of these mechanisms can be found in the outstanding review on desiccation tolerance in prokaryotes by Potts[54].

All these physiological mechanisms require a certain amount of energy. The synthesis of osmolytes, for example, can account for up to 30 – 110 ATP per molecule, which exceeds the costs for cell wall synthesis[63,70]. These energy requirements go to the expense of the growth rate, which was found to decrease linearly with increasing external osmolarity in *Escherichia coli*[54]. In addition, extended lag periods after the addition of a carbon source were observed at high salinity levels[71].

[b] Higher extracellular solute concentration causing an outflow of water

In general, investments in stress-tolerance mechanisms results in reduced relative fitness of the population. Some bacteria have evolved bet-hedging strategies to reduce their temporal variance in fitness by entering reversible states of low metabolic activity or dormancy[43]. There is evidence that at least 25 % of all microbial soil genomes contain genes for transitioning out of a dormant state[72]. Dormancy generates a seed bank of resting bacterial cells with the potential to be resuscitated in response to favorable changes in the environmental conditions[43]. Thus, dormancy contributes to the resilience of ecosystems as surviving cells form the basis for recovery after rewetting[43]. Nevertheless, dormancy is not a cost-free strategy as it requires bacteria to invest resources into resting structures (*i.e.* spores) and the machinery that is needed for the transition into and out of a dormant state[73].

2.3.3 Limited Dispersal and Nutrient Availability

Dispersal and migration are fundamental ecological processes that allow organisms to explore new habitats and pools of resources, to escape from adverse local conditions and to reduce competition[74,75]. Moreover, bacterial dispersal is considered a key factor for efficient biodegradation as it helps to overcome spatial separation, thus leading to increased contact probability between bacteria and contaminants in a spatially heterogeneous environment like soil[76]. Already in 1676, Antoni van Leeuwenhoek was delighted by the teeming mass of bacteria bustling around on the needle of his single-lens microscope[77]:

> *'I must say, for my part, that no more pleasant sight has ever yet come before my eye than these many thousands of living creatures, seen all alive in a little drop of water, moving among one another, each several creature having its own proper motion.'*

Bacterial cells can disperse in liquid environments randomly via Brownian motion and passively via convective flow[16]. However, both options have been shown to be inefficient modes of dispersion for nutrient acquisition[78]. Leeuwenhoek postulated in a charming phrase:

> *'…I can make out no paws… [yet] I am persuaded that they too are furnished with paws withal.'*

Indeed, most bacterial species have evolved active self-locomotion mechanisms such as swimming, swarming, twitching and gliding powered by flagella[c], pili or focal-adhesion complexes[79–81].

[c] Motor organelle for bacterial propulsion[81]

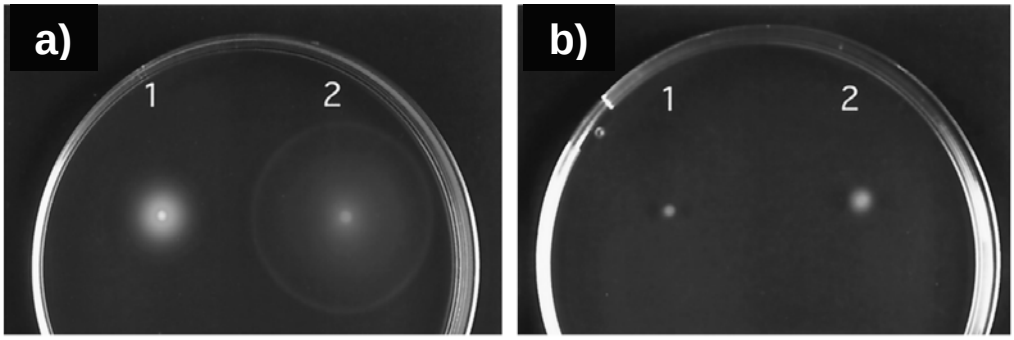


Figure 5 Swimming motility of *Pseudomonas* strain Y1000 (1) and *Enterobacte*r strain 22 (2) at a) 25 °C and b) 25 °C in the presence of 500 mM NaCl. A reduced osmotic potential repressed swimming motility in both strains. Figure taken from[85].

Water content is the primary factor controlling bacterial movement in soil. At low matric potentials especially flagellum-driven motility (*i.e.* swimming and swarming) is physically constrained by the size and fragmentation of aqueous habitats and their connectedness by micrometric aqueous films[16,82]. It has been shown, for example, that *Pseudomonas* flagellar motility on rough surfaces is only possible in water films > 1.5 µm[83]. Indeed, already at field capacity[d] conditions, the thickness of water films on a smooth mineral surface is estimated to be only 10 nm[16] and thus flagellar motility in unsaturated soils most likely is restricted to relatively rare and short-lasting wetting events (*e.g.* rainfall)[16,84]. In addition, also low Ψ_o may limit mobility in soil (cf. Fig. 5) via downregulation of structural genes involved in flagellum synthesis as it was recently shown for *Pseudomonas*, *Bacillus* and *Enterobacter* strains[85,86].

Changes in the water potential drastically influence the diffusional capacity and the pathways of gaseous and nutrient fluxes. In dry soils, liquid pathways are more fragmented, which decreases liquid and nutrient transport and diffusion rates particularly at the microscale of individual bacterial populations. Nutrient supply in soils is thus not permanent and bacteria must cope with frequent changes in nutrient availability and periods of starvation.

[d] Bulk water content retained in soil at –0.033 MPa.

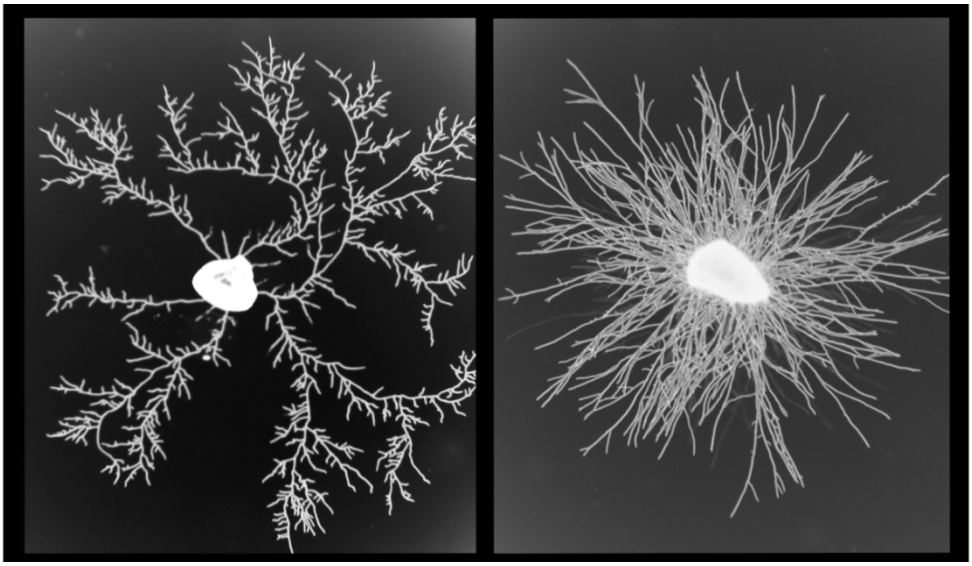


Figure 6 Mycelium of *Pythium ultimum* (oomycete) and *Fusarium oxysporum* (ascomycete). Scale bar = 1 mm. Pictures were acquired microscopically and colors inverted.

2.4 Ecological Features of Fungi

2.4.1 Fungal Morphology and Life in Soil

Fungi are eukaryotes and possess a heterotrophic lifestyle. Typically they develop a branched and spatially extensive mycelium network (cf. Fig. 6) and reproduce via the production of spores. Although some of the largest living organisms on earth are fungi, their thread-like hyphae usually have diameters of only 2 - 10 µm[33,45]. Fungi account for up to 75 % of the soil microbial biomass and the length of hyphae can be up to 10^2 - 10^4 m per g of soil depending on the soil type[87,88]. The development of fungi in soil is facilitated by the absence of mechanical disturbances such as shear forces, which would disrupt the mycelium[33]. Indeed, fungi are well adapted to life in the physically structured and heterogeneous soil environment. Their hyphae can grow into small soil pores (down to 2 µm in diameter) and penetrate rock matrices[89]. Moreover, mycelia directly influence soil structure via electrostatic, adhesive and enmeshment mechanisms[90] and organic matter decomposition[91] and thus act as 'ecosystem engineers'. Some fungi are extremely resistant to harsh environmental conditions and can grow at low and high temperatures (-5 to +60 °C) or pH values (1 to 9), low O_2 partial pressures and at a water activity of only 0.65[33].

However, one of the most important ecological advantages of fungi over bacteria is that they are less dependent on continuous water phases for active dispersal and nutrient acquisition. Their hyphae can cross air-water interfaces and easily bridge air filled pores[18]. Moreover, fungi effectively adapt their growth to the prevailing environmental conditions, showing sparse explorative growth under oligotrophic[e] conditions and dense explorative growth in presence of nutrients[18]. To be able to grow through nutrient-impoverished zones and air, fungi distribute nutrients, water and carbon sources in their mycelium between spatially separated regions by means of diffusive and active translocation[92–94]. This is supported by an efficient uptake of resources from the environment due to a high surface to volume ratio of the mycelium[95].

2.4.2 Interactions with Bacteria

Bacteria and fungi co-inhabit a wide variety of environments and also bacterial microhabitats in soil are pervaded by fungal hyphae[96]. Different studies revealed the presence of bacteria on top of fungal hyphae and spores or in association with fungal fruiting bodies[97]. In the so-called mycosphere[98] (*i.e.* the microhabitat that surrounds fungal hyphae in soil), interactions between the organisms can be either ecologically neutral, competitive or cooperative and may vary with the organisms' ecophysiology and the local environmental conditions[96]. Bacterial-fungal interactions (BFI) are not unilateral as both partners actively shape the conditions in the microhabitat and thereby influence each other. Indeed, early studies on mycorrhizal[f] fungi have shown that mycorrhizosphere-inhabiting bacteria are profoundly influenced by the presence of mycorrhizal fungi and, *vice versa*, soil bacteria also locally affect the fungus[99].

The physical associations between fungi and bacteria can range from seemingly disordered polymicrobial communities to highly specific symbiotic associations[100]. On top of fungal hyphae, bacteria can occur in the planktonic lifestyle or in biofilms. The latter differs from the planktonic lifestyle, as in a biofilm the microbes are arranged in a more structured fashion held together by an extracellular matrix of microbe-derived macromolecules[101]. Mixed biofilms can be formed by complexes secreted by both organisms or fungi provide the surface needed for bacterial biofilm establishment[102,103]. The most intimate form of the physical associations is when bacteria colonize the cytoplasm of the hyphae or, in some cases, also fungal spores[104].

[e] An environment with low nutrient levels

[f] Mutualistic symbioses between plant and fungus

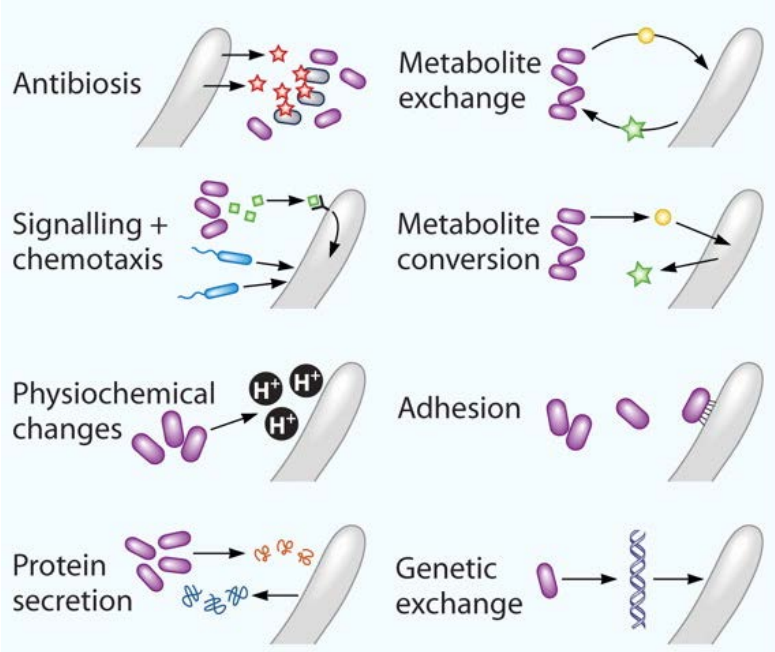


Figure 7 Molecular interactions between bacteria and fungi are mediated by different mechanisms. Figure taken from[100].

Multiple molecular communication mechanisms underlying BFI have been reported in literature for a variety of different fungal and bacterial strains. In their review, Frey-Klett et al.[100] summarized the different types of molecular communication occurring in BFI (Fig. 7). Antibiosis involves a chemical warfare based on the diffusion of deleterious and often chemically complex substances from one partner to the other[100]. Antibiotics constitute one of the most prominent examples of such a competitive type of interaction. Environments in which antibiotics are present exert a strong selection pressure on sensitive microbes, which do not possess effective resistance mechanisms[100]. Other molecules have more subtle effects compared to antibiotics. They act as signaling molecules to induce certain processes in the partner or attract bacteria towards the hyphae in a process called chemotaxis[g,100]. Moreover, communication in BFI may occur via modulation of the physicochemical environment such as the alteration of the pH value. Interactions via protein secretion and gene transfer likely play an important role in the context of bacterial virulence towards higher eukaryotes by directly delivering proteins or DNA into the host cytoplasm[106–108]. Adhesion might be an important mechanism for contact-dependent BFI and extracellular polysaccharides probably play a role for the attachment of bacteria to fungal hyphae[109]. Cell-cell contact can change the physiology of the partners and has been shown to be modulated by the environmental conditions[100].

[g] Directed movement of bacteria towards or away from a chemical[105]

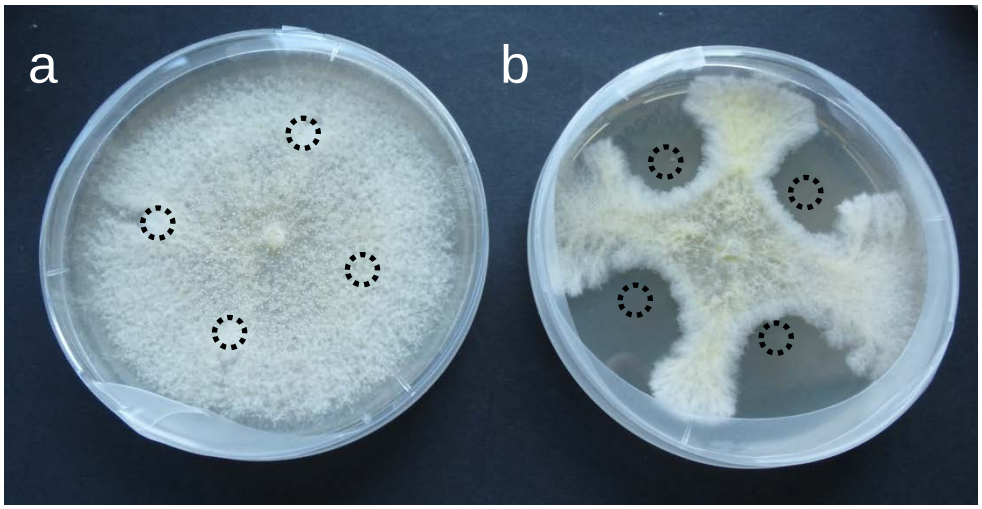


Figure 8 Different patterns of BFI between a) *Pseudomonas putida* and b) *Bacillus subtilis* with the basidiomycete *Gloeophyllum trabeum* identified by growth inhibition tests on agar plates. Dashed black circles indicate the position where the bacteria were inoculated. *B. subtilis* inhibited the growth of the fungus in vicinity to the colony, while *P. putida* did not.

Within microbial communities competitive and cooperative interactions are often related to the excretion and uptake of metabolites from involved partners[110–112] (cf. Fig 8). Fungi and bacteria can benefit from the exchange of specific compounds that are produced by the other partner if they are unable to produce them on their own. An extension of that concept is the exchange of metabolites leading to the formation or degradation of a molecule that none of the partners can perform alone[100].

BFI can have profound consequences on the physiology, life cycles and survival of the involved organisms (Table 2, 3)[100]. It is known that bacteria can influence fungal development and spore production to the benefit or the detriment of the fungus. Moreover, bacteria can influence fungal pathogenicity. This mechanism has been successfully applied in agriculture via biocontrol[h] strains, which prevent plants from fungal infestation (cf. Table 3). Observations of the influence of fungi on bacteria are less described due to the small size and single cell nature of prokaryotes[100]. However, especially in fungal-bacterial biofilms fungi may promote physiological differences in bacteria, such as resistance to antibiotics or other types of environmental stress as it has been shown for clinically relevant BFI[113,114]. The 'omics' techniques contributed to an advanced understanding to what happens to bacteria on the subcellular level during BFI[115,116]. Moreover, the recent advances in sequencing technology allow for studying BFI involving even more complex microbial communities[100].

[h] Control of pests by introducing a natural enemy or a pathogen into the environment

Table 2 Examples for cooperative BFI in terrestrial environments. Asterisks indicate which partner is positively influenced by the interaction.

FUNGUS	BACTERIUM	BFI	REMARKS	REF.
Cooperative Interactions				
*Glomus intraradices**	*Paenibacillus validus*	Stimulation of fungal spore germination	AMF[i]	[117,118]
*Rhizopus microspores**	*Burkholderia* sp.	Facilitation of vegetative reproduction of the fungus	Endobacterium	[119]
*Agaricus bisporus**	*Pseudomonas putida*	Induction of mushroom production	-	[120,121]
*Amanita muscaria**	*Streptomyces* sp.	Effect on cytoskeleton organization	EMF[j]	[122]
*Laccaria bicolor**	*Pseudomonas fluorescens**	Bacterium promotes fungal viability, fungus promotes bacterial growth via trehalose excretion	EMF	[123,124]
Gaeumannomyces graminis	*Pseudomonas fluorescens**	Improved bacterial growth; influence on gene regulation	Plant pathogenic fungus	[125]
Fusarium oxysporum	*Achromobacter* sp.*	Mobilization of bacteria	-	[126]
Pythium ultimum	*Pseudomonas putida* KT2440*	Facilitation of horizontal gene transfer between bacteria	Oomycete	[127]
Pythium ultimum	*Bdellovibrio bacteriovorus**	Effective foraging of prey bacteria along mycelium	Oomycete and predatory bacterium	[128]
Laccaria proxima	*Pseudomonads**	Bacterial feeding on fungal exudates	Potential fungal exudates	[129]
*Glomus mosseae**	*Stenotrophomonas sp.*, *Pseudomonas* sp., *Arthrobacter* sp.	Promotion of mycorrhization and growth inhibition of pathogens	AMF	[130]
*Agaricus bisporus**	*Pseudomonas putida*	Removal of self-inhibiting compounds	-	[121]
*Tuber brochii**	*Pseudomonas fluorescens*, *Bacillaceae*	Sporocarp weakening via chitinolytic and cellulolytic activity	EMF	[131]
Lyophyllum karsten	*Burkholderia terrae**	Bacterial translocation along hyphae via apical growth	Biofilm formation	[132]
*Gigaspora margarita**	*Actinomycetes*	Stimulation of spore germination	Bacterial volatiles	[133]

[i]Arbuscular mycorrhizal fungus
[j]Ectomycorrhizal fungi

Table 3 Examples for competitive BFI in terrestrial environments. Asterisks indicate which partner is negatively influenced by the interaction.

FUNGUS	BACTERIUM	BFI	REMARKS	REF.
Competitive Interactions				
*Cladosporium cladosporioides**	*Streptomyces griseoruber*	Inhibition of fungal spore germination	VOC[k]	134
*Rhizoctonia solani**	Various bacterial strains (*e.g. Stenotrophomonas, Pseudomonas*)	Inhibition of mycelial growth	VOC	135
*Fusarium oxysporum**	*Serratia*, *Achromobacter*, *Bacillus*, *Stenotrophomonas*	Repressed expression of two virulence genes in the fungus	VOC	136
*Rhizoctonia solani**, *Gaeumannaomyces graminis**	*Bacillus* spp.	Reduced infection of plants by the fungi	Biocontrol strain	137
Several crop pathogens* (*Fusarium, Penicillium, Rhizopus*)	*Myxobacteria*	Production of chitinases for fungal cell wall degradation	Community screening	138
*Fusarium oxysporum**	*Pseudomonas putida*	Supression of fungus in iron deficient soils	Siderophore[l] production	139
*Fusarium oxysporum**	*Pseudomonads fluorescens*	Competition for nutrients and niches	Plant root colonization	140
*Fusarium solani**	*Pseudomonas stutzeri*	Lysis of fungal cell walls and outflow of cytoplasm	-	141
*Agaricus bisporus**	*Pseudomonas tolaasi*	Disruption of fungal membranes	Tolaasin[m] secretion	142
*Botrytis cinerea**	*Cupriavidus, Pseudomonas, Bacillus, Variovorax, Ralstonia*	Bacterial degradation of fungal pathogenicity factors	Oxalic acid	143

[k] Volatile organic compounds
[l] Small, high-affinity iron chelating compounds
[m] Cause of bacterial 'brown blotch disease' of edible mushrooms

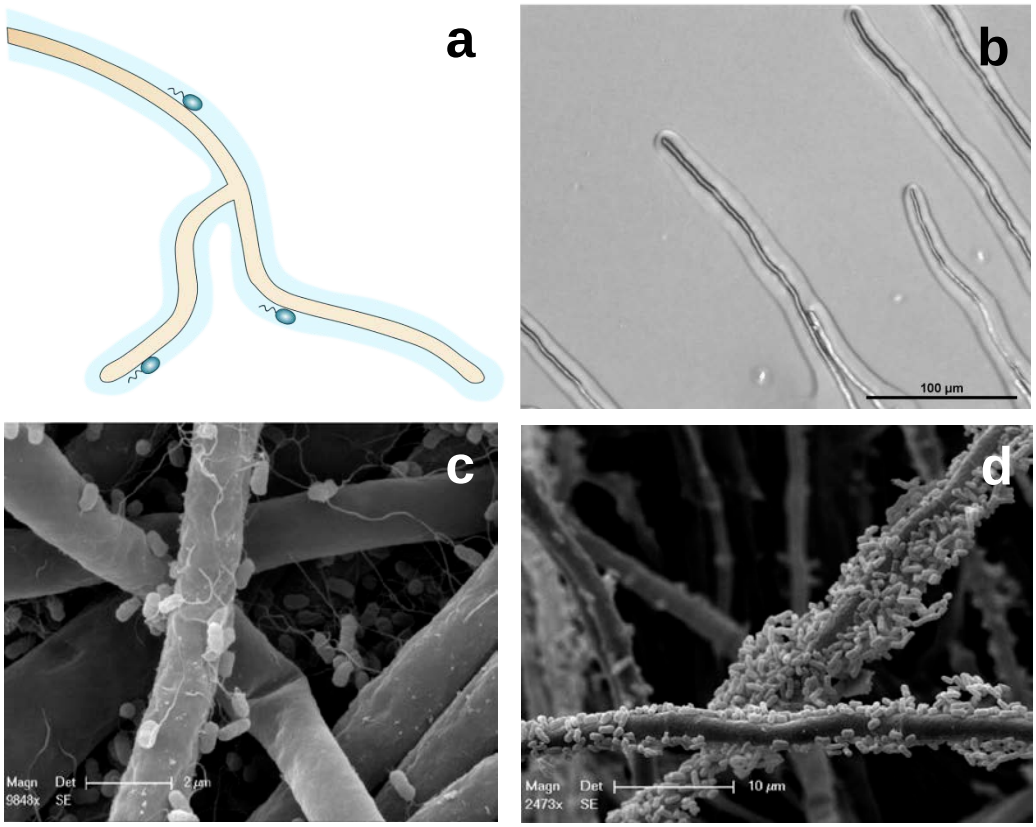


Figure 9 The 'fungal highway'-principle. a) Simplified scheme of bacteria moving in the water films surrounding fungal hyphae. Figure reproduced from[33]. b) Micrograph showing the liquid films around hyphae of *Coprinopsis cinerea* on top of an agar plate. c) and d) SEM images of bacteria moving along fungal hyphae of *Fusarium* species[144].

2.5 Fungi as Transportation Networks

2.5.1 'Fungal Highways'

As described in chapter 2.3.3, bacterial movement in soil is only possible in presence of continuous water pathways and in liquid films immersing bacterial cells[79] and thus, active movement of bacteria is strongly limited in water-unsaturated soils[16]. Microcosm experiments have demonstrated that fungal mycelia can be used as paths for the active dispersal of bacteria[126]. Bacteria were shown to move in the liquid film formed around the hyphae, which thus act as 'fungal highways' (Fig. 9)[126]. In addition to active movement, other studies found that translocation of bacterial strains along fungal mycelia occurred via apical transport of attached bacteria[132]. Hence, bacteria may profit from the presence of fungal networks under unsaturated conditions by facilitated dispersal. In this context, the accessibility of contaminants by bacteria in unsaturated soil microcosms has been studied[145–147].

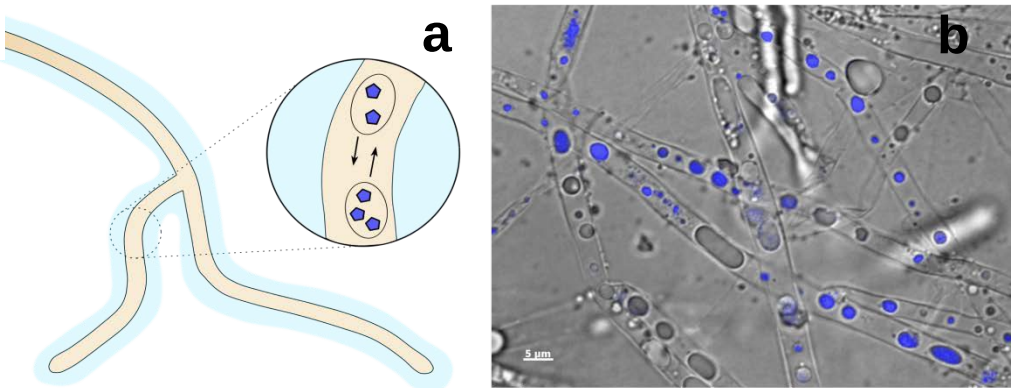


Figure 10 The 'fungal pipeline'-principle. a) Simplified scheme of bidirectional vacuolar transport of nutrients and carbonaceous compounds in fungal hyphae. Figure modified from[33]. b) Micrograph of the mycelia-forming oomycete *Pythium ultimum* transporting the contaminant phenanthrene in vesicles that fluoresce in blue. Photo: Susan Schamfuß/UFZ.

It was also shown that bacterial dispersal along fungal mycelia was driven by chemotaxis and could significantly increase the degradation of PAH in physically separated regions[147,148]. Modeling studies revealed that fungal dispersal networks enhance the biodegradation performance under motility restricting conditions and limited bioavailability of the contaminants also in systems, which usually exhibit spatially heterogeneous conditions like soil[149,150]. However, experiments were performed with different agar concentrations to restrict bacterial motility, which rather represents a physical hindrance for bacterial motility. In this thesis, I therefore investigated the influence of different osmotic and matric potentials, as the two major components of the total water potential in soil (cf. section 2.3.1), on bacterial dispersal in presence and absence of 'fungal highways' and the subsequent effects on the biodegradation performance by bacteria **(Publication 1)**. Especially the osmotic potential was often neglected with respect to its influence on bacterial dispersal and biodegradation in soil. Thus, we carried out a detailed investigation of the importance of 'fungal highways' for biodegradation in comparison to autonomous bacterial dispersal and substrate diffusion at different osmotic potentials using a newly developed quantification approach **(Publication 2)**.

2.5.2 *'Fungal Pipelines'*

Fungi redistribute nutrients between spatially separated source and sink regions via diffusion or active translocation[92–94]. This mechanism is well studied for nutrient transport by mycorrhizal fungi, which efficiently take up nutrients from the soil and transfer them to the plant symbiont in exchange for photosynthates[151]. AM fungi actively translocate plant-derived lipids at speeds of up to 11 mm per s, or at a rate of up to 1.3 mg lipids per h per runner hypha[152]. Translocation of substances in non-mycorrhizal fungi has been investigated far less, but it is suspected that all fungi use similar mechanisms. Centimeter-range transport in hyphae occurs in vacuoles that can move in both directions even against the mass flow that supports the turgor-driven tip extension of hyphae[94,153] (Fig. 10a).

Recently it was shown that mycelium-forming oomycetes[n] transport PAH in enriched vesicles with an estimated contaminant transport rate as high as 5 ng per h by a single runner hypha[154,155] (Fig. 10b). This mechanism might become especially important when bacteria starve and substrate transport is necessary to enable their activity[156].

Although the transport of water and nutrients in fungal mycelia is generally assumed, the explicit transport and subsequent transfer of those resources to other soil organisms has not yet been tested. Indeed, the 'fungal pipeline'-principle has been established for hydrophobic contaminants, which are not utilized by the transporting oomycete. Thus, it is not clear whether essential resources like water or carbon and nitrogen compounds are also transferred to bacteria. Moreover, the 'fungal pipeline'-mechanism was discovered under almost optimal conditions for both, the fungus and the bacteria. However, it is not known whether such transfer processes also emerge in dry and oligotrophic regions, which are suboptimal for both partners, and how this influences bacterial activity. In this thesis, I therefore investigated the role of 'fungal pipelines' for bacterial activity under water and nutrient limitation and directly examined the transfer of water, carbon and nitrogen **(Publication 3)**.

[n] Filamentous protists once classified as fungi

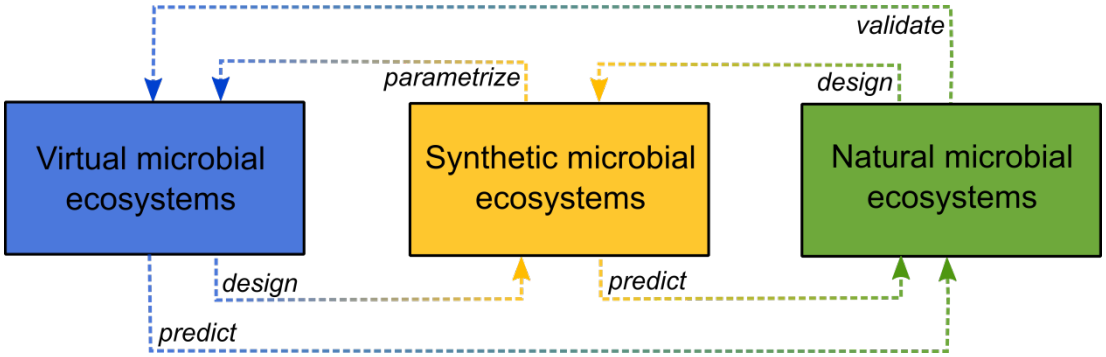


Figure 11 Flow of information between virtual, synthetic and natural microbial ecosystems. Regarding criteria such as complexity, realism, reproducibility and controllability, synthetic microbial ecosystems represent an intermediate state. SME can provide valuable information on ecological mechanisms that help to predict natural systems and also can be used to parametrize ecological models. Figure modified from[157].

2.6 Synthetic Microbial Ecosystems as a Tool in Microbial Ecology

Significant progress has been made in the description of the microbial communities and their functionality in almost every conceivable environment. However, to gain insights into ecological mechanisms and factors determining ecosystem behavior and functionality, it is necessary to go beyond pure descriptions of the diversity and abundance of microbes. One of the major obstacles for studying these mechanisms and factors is the complexity of natural ecosystems in which many abiotic and biotic factors simultaneously influence microbes and their activity[157]. Moreover, observing microorganisms in nature is difficult as they often have few distinguishing morphological features and many of them cannot be cultivated in the laboratory[12].

Synthetic microbial ecosystems (SME) are a powerful tool as they possess a reduced complexity and higher controllability compared with natural ecosystems[12] (Fig. 11). SME can be tailored to enable easy observation of ecological processes and the studied microorganisms can be isolated from their natural environment or assembled artificially. Artificial assemblages may offer a huge potential for analyzing and explaining microbial ecosystem dynamics due to the wealth of genetic and physiological information available for many commonly used microorganisms[158]. Their small sizes and short generation time allow for running and replicating many experiments at the same time. SME enable comprehensive variation of biotic and abiotic parameters, while excluding or holding other influencing factors constant[157]. This reduction is essential for uncovering operating ecological mechanisms[158], which then might help to also understand the dynamics of other microbial ecosystems. By combining data and information obtained from natural systems and SME, *in silico* models representing 'virtual ecosystems' can be created, parametrized and validated[157]. Moreover, the outcome of the simulations could also provide rationales for the design of new SME.

SME represent a valuable tool to prove microbial interactions and to unravel the underlying mechanism. Indeed, many of the studies leading to significant progress in understanding BFI and fungus-mediated transport mechanisms have been carried out in SME. Recently, multiwell agar microcosms were applied to demonstrated that the 'fungal highways' represent focal points for horizontal gene transfer[127]. Moreover, a sophisticated microcosm system developed by Otto et al.[128] provided evidence for effective foraging of a predatory bacterium along the 'fungal highway'. In addition, the system allowed to assess the consequences of bacterial predation on the biodegradation efficiency of a desorbing contaminant[159].

In literature there are various other examples where the usefulness of SME has already been demonstrated.

Table 4 Examples in which SME have been successfully applied to study ecological principles and mechanisms.

SME	PRINCIPLE/MECHANISM	REF.
Agar plates with diffusion barriers	Limited diffusive flux of growth substrate enables coexistence of bacterial species	160
Microfluidic device	Defined spatial structure stabilizes multispecies bacterial community	161
Culture flasks (shaken vs. static)	Heterogeneity fosters adaptive radiation of *Pseudomonas*	121
Porous surface model	Hydration status controls bacterial motility	58,83
Agar plates with hot spot of solid naphthalene	Chemotaxis occurs along vapor-phase gradients of naphthalene	162
Quartz sand microcosms	Filamentous growth of bacteria is beneficial at low hydraulic conductivity	163
Agar plates	Resource limitation drives spatial organization in microbial groups	164
Culture flasks (shaken vs. static)	Disturbance modulates the effect of spatial heterogeneity on biological diversity	165
Soil-agar microcosms	Mycelia can be used to isolate degrading bacteria	166
Microfluidic device	Physical attachment modulates the antagonistic interaction between *Bacillus subtilis* and *Coprinopsis cinerea*	167
(MEGA[o])–plate	Evolution of resistance in bacteria along an antibiotic gradient	168
Agar plates	Self-organized patchiness facilitates survival in a cooperatively growing *Bacillus subtilis* population	169
Glass bead columns	Water content, bacterial spatial distribution and motility control biodegradation efficiency	170

[o] Microbial evolution and growth arena

2.7 Aims of This Study

Although the benefits of fungus-mediated transport mechanisms for bacterial biodegradation have been demonstrated in the past, we still do not know how these mechanisms contribute to the functional stability of soil ecosystems under environmental stress. Based on the 'state of the art' presented in the previous sections, this doctoral thesis aims at understanding the role of both, 'fungal highways' and 'fungal pipelines' for the maintenance of bacterial activity under drought and nutrient limitation. Experiments were performed in tailored synthetic microbial ecosystems of high experimental controllability in order to meet the following objectives:

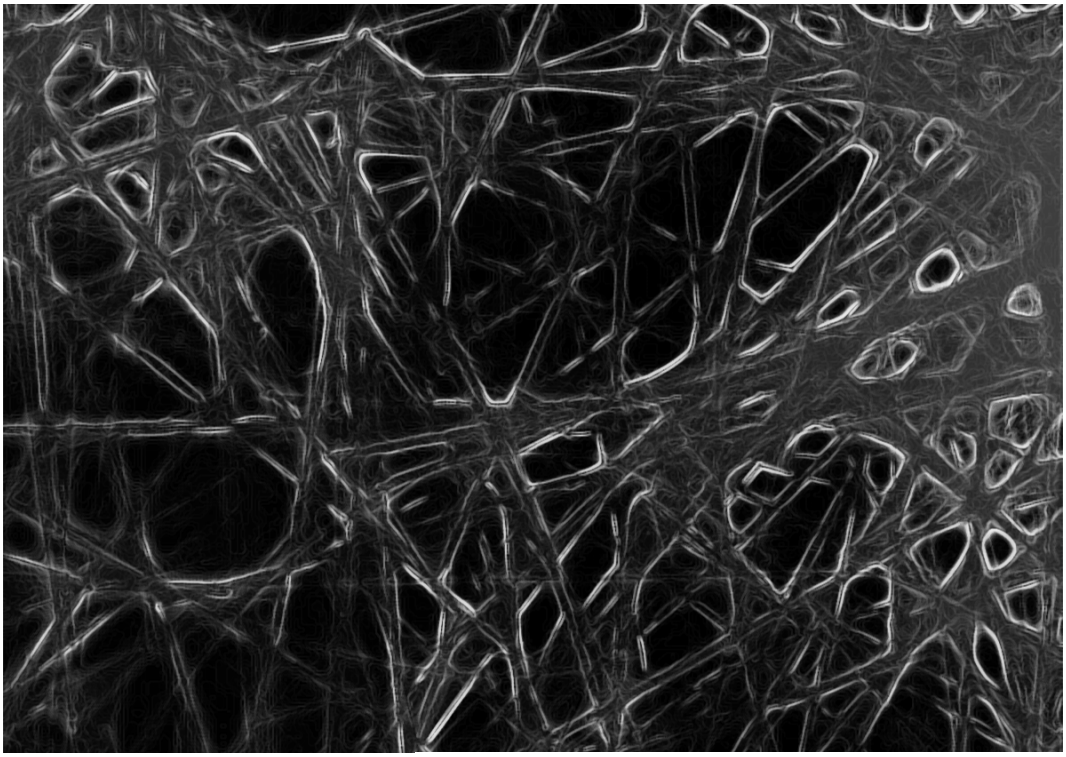
To investigate bacterial dispersal at different water potentials and the subsequent effects on growth an biodegradation efficiency in presence and absence of 'fungal highways' simulated by glass fiber networks. The model strain used throughout this work was the motile, soil-dwelling bacterium *Pseudomonas putida* KT2440, which degrades the aromatic compound benzoate serving as bacterial growth substrate. Specific aims were the development of agar-based SME exhibiting a range of defined osmotic or matric potentials, the microscopic analysis of bacterial colony expansion as a measure of bacterial dispersal, the cultivation-based determination of bacterial abundance to characterize bacterial growth and the analytical determination of benzoate to assess the biodegradation performance.

To evaluate the importance of 'fungal highways' for ecosystem functioning at different osmotic potentials. The previous study clearly pointed towards a key role of 'fungal highways' for the maintenance of bacterial biodegradation processes under osmotic stress. To quantify the relative importance of network-mediated dispersal for biodegradation at different osmotic potentials, scenarios with glass fiber networks should be compared to a reference scenario with a homogeneous bacterial cell distribution. Moreover, the role of other spatial processes, namely autonomous bacterial dispersal and substrate diffusion, for biodegradation efficiency should be assessed. To this end, a systematic variation of the previously developed SME is necessary.

To examine the effects of 'fungal pipelines' on bacterial activity in a dry and oligotrophic environment. In a specially designed SME, germination of *Bacillus subtilis* spores should serve as an indicator for the stimulation of bacterial activity in presence of mycelia. To this end, it was intended to test two different fungi and an oomycete for their ability to activate bacterial spores inoculated to a resource-deficient environment. To reveal the transfer of metabolites as the mechanism underlying the activation, a combination of ToF- and nanoSIMS together with stable isotope labelling should be applied on the SME.

Chapter 3

'FUNGAL HIGHWAYS' AT LOW WATER POTENTIALS



Gfp-tagged Pseudomonas putida KT2440 on glass fiber network

3 FUNGAL HIGHWAYS AT LOW WATER POTENTIALS

3.1 Mycelium-like Networks Increase Bacterial Dispersal, Growth and Biodegradation in a Model Ecosystem at Varying Water Potentials

Worrich, A., Konig, S., Miltner, A., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Kastner, M. & Wick, L. Y. Mycelium-like Networks Increase Bacterial Dispersal, Growth and Biodegradation in a Model Ecosystem at Varying Water Potentials. *Appl. Environ. Microbiol.* (2016). doi:10.1128/AEM.03901-15

Significance: Fungal mycelia were shown to facilitate bacterial dispersal under unsaturated conditions, but their effects on bacterial dispersal and ecosystem performance at environmentally relevant water potentials have not been evaluated yet. This paper reflects a laboratory microcosm study and presents evidence that bacterial dispersal along mycelia-like dispersal networks is not restricted to the previously reported critical thresholds for bacterial motility. We could also show that the improved dispersal along the networks led to an increased functional performance examined via bacterial growth and degrading activity. We therefore propose, that fungal-bacterial interactions may provide an important buffer for unfavorable environmental conditions in soil supporting the maintenance of ecosystem functioning.

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Mycelium-Like Networks Increase Bacterial Dispersal, Growth, and Biodegradation in a Model Ecosystem at Various Water Potentials

Anja Worrich,[a,b] Sara König,[a,c] Anja Miltner,[b] Thomas Banitz,[c] Florian Centler,[a] Karin Frank,[c,d,e] Martin Thullner,[a] Hauke Harms,[a,d] Matthias Kästner,[b] Lukas Y. Wick[a]

Department of Environmental Microbiology, UFZ-Helmholtz Centre for Environmental Research, Leipzig, Germany[a]; Department of Environmental Biotechnology, UFZ-Helmholtz Centre for Environmental Research, Leipzig, Germany[b]; Department of Ecological Modelling, UFZ-Helmholtz Centre for Environmental Research, Leipzig, Germany[c]; German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany[d]; University of Osnabrück, Institute for Environmental Systems Research, Osnabrück, Germany[e]

Fungal mycelia serve as effective dispersal networks for bacteria in water-unsaturated environments, thereby allowing bacteria to maintain important functions, such as biodegradation. However, poor knowledge exists on the effects of dispersal networks at various osmotic (Ψ_o) and matric (Ψ_m) potentials, which contribute to the water potential mainly in terrestrial soil environments. Here we studied the effects of artificial mycelium-like dispersal networks on bacterial dispersal dynamics and subsequent effects on growth and benzoate biodegradation at $\Delta\Psi_o$ and $\Delta\Psi_m$ values between 0 and −1.5 MPa. In a multiple-microcosm approach, we used a green fluorescent protein (GFP)-tagged derivative of the soil bacterium *Pseudomonas putida* KT2440 as a model organism and sodium benzoate as a representative of polar aromatic contaminants. We found that decreasing $\Delta\Psi_o$ and $\Delta\Psi_m$ values slowed bacterial dispersal in the system, leading to decelerated growth and benzoate degradation. In contrast, dispersal networks facilitated bacterial movement at $\Delta\Psi_o$ and $\Delta\Psi_m$ values between 0 and −0.5 MPa and thus improved the absolute biodegradation performance by up to 52 and 119% for $\Delta\Psi_o$ and $\Delta\Psi_m$, respectively. This strong functional interrelationship was further emphasized by a high positive correlation between population dispersal, population growth, and degradation. We propose that dispersal networks may sustain the functionality of microbial ecosystems at low osmotic and matric potentials.

Population dispersal is considered a key factor of ecosystem functioning, enabling bacteria to invade new habitats and to harness new pools of resources (1). Consequently, it is also a prerequisite for efficient biodegradation in soils because it increases the contact probability for bacteria and contaminants (2). However, in terrestrial environments, bacterial surface motility is usually restricted due to limited water availability, which is anticipated to become an even more important constraint in future due to prolonged drought periods (3, 4). Water availability is described by the concept of the water potential (Ψ_w), which is a measure of the energetic state of water in a system (5). In water-saturated soils, Ψ_w is determined almost exclusively by the osmotic potential (Ψ_o), which refers to the amount of solutes in the aqueous phase. In unsaturated environments, the matric potential (Ψ_m) also becomes important due to increasing capillary and adsorptive forces between water and the soil matrix (5, 6). Several studies evaluated the effects of different water potentials on the biodegradation performance of bacteria, mainly over time, without consideration of spatial processes (7, 8). However, both the spatial and temporal dynamics are crucial for a system's biodegradation performance, as substrates and bacteria in soil are typically distributed heterogeneously (2, 9). In a sand matrix adjusted to different Ψ_m values, degradation has been shown to depend strongly on the initial distribution and the dispersal of degrading bacteria (10). Researchers have revealed various bacterial strategies to cope with the direct physiological effects of low water potentials by, for example, accumulating compatible solutes or changing the lipid content of the membranes (11), but mechanisms to overcome the motility restrictions in thin water films are still poorly understood.

In contrast to bacteria, fungi do not rely on continuous water pathways to grow through the soil matrix. They can bridge air-water interfaces due to efficient resource translocation in the mycelium, thus connecting fragmented soil habitats (12, 13). Flagellated bacteria can actively move in the liquid films surrounding hydrophilic hyphae and thus overcome motility restrictions in unsaturated environments (14). Different studies using soil column experiments and simulation modeling showed that this phenomenon can enhance biodegradation of phenanthrene and glucose, respectively (15–17). However, studies to assess the benefits of dispersal networks were carried out with culture plates with different agar concentrations routinely used in standard bacterial motility assays (18–20). Indeed, these concentrations represent only a narrow range of Ψ_m values, which is insufficient for studying relevant environmental conditions, in particular in soils, where Ψ_m often falls to −1.5 MPa (21). Salt accumulation caused by irrigation or fertilization is a major threat in agricultural soils, and globally, 10^8 ha (5%) of arable land are affected by salt at a level that causes osmotic stress (22). Furthermore, soil drying and soil surface proximity are often accompanied by high solute concentrations (9), which have been shown to cause a repression of

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Address correspondence to Matthias Kästner, matthias.kaestner@ufz.de.

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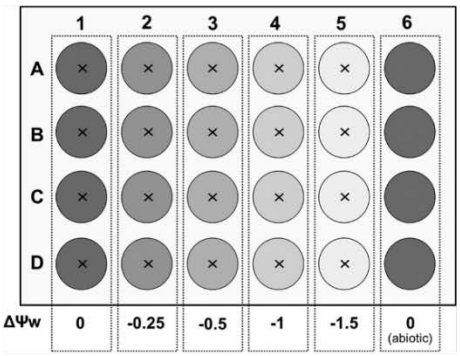

FIG 1 Scheme of the multiple-microcosm setup to assess the effects of five different Ψ_w values on bacterial dispersal, growth, and biodegradation. The water potential in each column was adjusted with different concentrations of NaCl or PEG 8000. *P. putida* KT2440-gfp was inoculated into the center (×) of each well, and one column served as an abiotic control.

motility genes, leading to an impairment of movement in *Pseudomonas* and *Bacillus* strains (23, 24). However, no study has yet evaluated the effects of dispersal networks at different Ψ_o values.

In the present study, we tested the hypothesis that dispersal networks facilitate bacterial dispersal over a broad range of environmentally relevant Ψ_w values and hence improve the ecosystem's functional performance in terms of bacterial biodegradation. To obtain a mechanistic understanding of the effects of different water potentials and dispersal networks on bacterial population dispersal, we developed highly controlled laboratory microcosms in which we measured the radial colony expansion at different Ψ_w values in both the presence and absence of an artificial dispersal network. By varying either Ψ_o or Ψ_m, we aimed to disentangle the different modes of action as well as the capability of dispersal networks to improve the systems' performance for the two main descriptors of Ψ_w in soil. The systems' performance was assessed by following population growth of a green fluorescent protein (GFP)-tagged derivative of the well-studied soil bacterium *Pseudomonas putida* KT2440 and the biodegradation of benzoate as a model for water-soluble and therefore well-accessible substrates.

MATERIALS AND METHODS

Organisms and culture conditions. A benzoate-degrading, GFP-tagged derivative of the soil bacterium *Pseudomonas putida* KT2440 was chosen for the experiments because of its well-known motility behavior (4, 25). It was cultivated in FAB minimal medium (26) supplemented with 50 mM sodium benzoate (FAB-50; Sigma-Aldrich, Munich, Germany) at room temperature with rotary culture flask movement at 150 rpm. For long-term cultivation, bacteria were transferred weekly to FAB-50 plates containing 1.5% (wt/vol) agar and incubated at room temperature.

Multiple-microcosm approach. Laboratory systems were designed to investigate bacterial motility behavior at five water potentials in a miniaturized system allowing parallel and semicontinuous microscopic observation of 24 microcosms. Therefore, we used clear, sterile, flat-bottom 24-well microtiter plates covered with low-evaporation lids (Orange Scientific, Braine-l'Alleud, Belgium) and filled the wells of each column with 0.3% (wt/vol) FAB agar. The whole system contained four replicates per $\Delta\Psi_w$ treatment and for the abiotic control (Fig. 1).

Adjustment of $\Delta\Psi_o$ and $\Delta\Psi_m$. To change the Ψ_o value of the FAB-50 medium by −0.25, −0.5, −1, or −1.5 MPa, we added 3.2, 6.4, 12.8, or 19.2 g liter^{-1} of sodium chloride, respectively, to the agar prior to autoclaving (7). Agar without sodium chloride served as a control treatment ($\Delta\Psi_o$ = 0 MPa). Each well was then filled with 1 ml molten agar in a laminar flow cabinet. Comparable changes of the Ψ_m value were obtained by overlaying 1 ml FAB double-strength agar containing 100 mM sodium benzoate directly in the well with 1.5 ml polyethylene glycol (PEG) solution containing 250, 392, 584, or 704 g PEG 8000 (Carl Roth, Karlsruhe, Germany) per liter of distilled water (27). For controls ($\Delta\Psi_m$ = 0 MPa), the agar was overlaid only with distilled water. After 72 h of equilibration, solutions were discarded and residues were carefully removed by pipetting. Before use, all plates were dried with open lids in a laminar flow cabinet for 5 min.

Microcosm inoculation. Cells were harvested from liquid culture by centrifugation at 8,000 × *g* for 10 min after 16 h of cultivation. The pellet was washed once with 10 mM potassium phosphate buffer (PB) at pH 7.2 and adjusted to an optical density of 50. Microcosms were inoculated with a 0.2-μl bacterial suspension (approximately 2.44 × 10^6 CFU) in the center of each well by use of a microliter syringe. Abiotic controls were left uninoculated. In experiments with the abiotic dispersal networks, a glass fiber mat (Mühlmeier Composite, Bärnau, Germany) with an area weight of 14 g m^{-2} was cut into circular pieces with a diameter of 1.2 mm, heat sterilized at 450°C for 4 h in a muffle furnace, and placed on top of the agar in the microcosms before inoculation with bacteria. The addition of the glass fiber mat had no effect on water volumes in the microcosms. Plates were incubated in plastic containers at room temperature in the dark.

Bacterial dispersal measurement. Directly after inoculation, a plate was placed under a microscope equipped with an Hg vapor lamp and a black-and-white camera (AZ 100 Multizoom; Nikon, Amsterdam, Netherlands). Colony images were captured at intervals of 30 min for 24 h at respective *x-y* positions, using the 4D module and GFP filter settings. Picture stacks were imported into ImageJ (28) and converted to binary images after applying an intensity threshold of 40. Original image size information was included via the set scale command before the colony area was measured.

Bacterial growth measurement. Cell numbers in replicate plates were determined after 6, 24, 30, and 48 h. The agar in each well was suspended in 2 ml PB and transferred to a sterile Falcon tube. Detachment was carried out by vortexing and subsequent ultrasonication treatment for 1 min. Culturable cells were analyzed by the number of CFU. Therefore, bacteria were spread on FAB-50 plates by using the drop plate method as described earlier (29). Briefly, 10-fold dilution series of the supernatants were prepared directly in 96-well microtiter plates, and 5-μl samples of consecutive dilutions were dropped on an agar plate. Plates were incubated at 25°C for 48 h. Droplets containing between 5 and 30 single colonies were used to determine CFU numbers.

For further analyses, 1.5 ml of supernatant obtained from cell detachment was fixed by incubation with 500 μl of a 12% (wt/vol) formaldehyde solution for 24 h at 4°C. To verify the results obtained from the CFU counts, we tested whether the change in Ψ_o or Ψ_m had an influence on the culturability of *P. putida* KT2440. Therefore, 0.5-μl droplets of the fixed samples obtained from treatments giving Δ0 and Δ−1.5 MPa after 48 h were spotted on individual boxes of a cellulose acetate grid filter (0.45 μm; Sartorius, Goettingen, Germany). Afterwards, cells were stained by embedding the filter in 4′-6-diamidino-2-phenylindole (DAPI)-amended mountant containing 9 parts Citifluor mountant (Citifluor, Leicester, United Kingdom), 1 part PB, and 1 μg ml^{-1} DAPI (30). Filters were stored at −20°C until analysis. For cell number determination, the size of each sample drop was first measured under transmission light by using a 1× objective. Subsequently, images of the bacteria were recorded at 5 random positions within the same drop by using a 20× objective and DAPI filter settings. Cells were counted using NIS Elements software (Nikon, Amsterdam, Netherlands). Therefore, an appropriate threshold was chosen to separate cells from background fluorescence. The ratios of the numbers of cells and CFU were calculated and compared by performing a two-sample *t* test for the two different water potentials and for Ψ_o and Ψ_m.

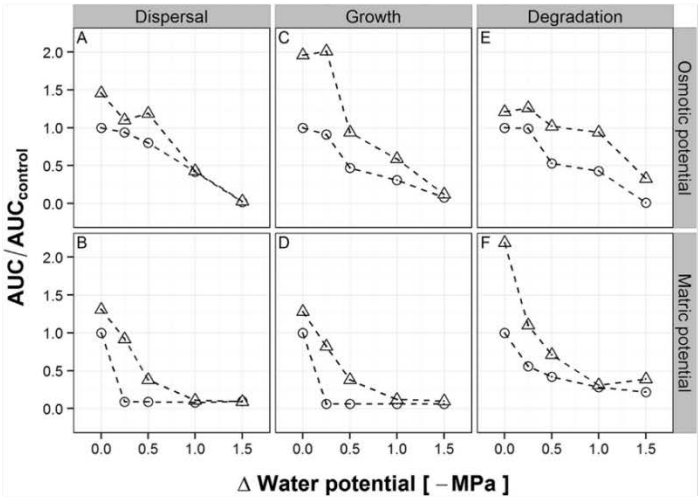

FIG 2 Relative dispersal (A and B), growth (C and D), and degradation (E and F) of *P. putida* KT2440-gfp for the different $\Delta\Psi_o$ (A, C, and E) and $\Delta\Psi_m$ (B, D, and F) values in the presence (triangles) and absence (circles) of dispersal networks, as determined by AUC calculation (see Material and Methods for details). Dispersal was measured microscopically by following the colony expansion based on the GFP signal in the single wells every 30 min for 24 h. Growth and degradation were assessed by the increase in the number of CFU per milliliter and the decrease in benzoate concentration after 6, 24, 30, and 48 h. Performance is given as the AUC relative to the AUC for the control ($\Delta\Psi_w$ = 0 MPa) without dispersal networks.

Bacterial degradation measurement. One milliliter of fixed supernatant obtained from the detachment procedure was filtered through a 0.22-μm syringe filter (Carl Roth, Karlsruhe, Germany) to remove bacteria. The benzoate concentration was determined with a high-pressure liquid chromatography (HPLC) system equipped with a C_{18} reverse-phase column (250 by 4 mm) and a photodiode array detector (PDA) set at 271 nm. The system was operated at a flow rate of 1.2 ml min^{-1}, with a 10-μl injection volume and a mobile phase consisting of 80% sodium acetate (50 mM; pH 4.5) and 20% methanol (MeOH) (31). Benzoate had a retention time of 13.8 min under these operation conditions.

Evaluation of dispersal, growth, and degradation data. For evaluation of the dispersal, growth, and degradation data for *P. putida* KT2440-gfp, we calculated the areas under the curve (AUC) for the time span over which the respective data were experimentally obtained (24 h for dispersal and 48 h for growth and degradation). The AUC rises when a curve increases either earlier or to a higher level. Hence, it serves as an aggregate measure of the extent and temporal performance of the respective characteristics. The AUC values were calculated using the trapezoidal method. This numerical approximation of the time integral allows for nonequidistant time data and thus is not hampered by missing measurements for certain points in time. Relative AUC values were calculated by dividing the respective value by the $\Delta\Psi_o$ (0 MPa) or $\Delta\Psi_m$ (0 MPa) AUC value for the control treatment without network presence. To evaluate the correlation strength between population dispersal, population growth, and benzoate degradation, we calculated pairwise Pearson's product moment correlation coefficients (ρ) between AUC values at the different $\Delta\Psi_o$ and $\Delta\Psi_m$ values. Therefore, we combined the AUC values for the experiments with and without dispersal networks.

RESULTS

Population dispersal at different $\Delta\Psi_o$ and $\Delta\Psi_m$ values in the presence and absence of dispersal networks. The radial colony expansion graph revealed two-phasic behavior for most of the $\Delta\Psi_w$ treatments without a network presence. The initial phase is a result of the accumulation of cells due to growth taking place within the clearly delimited droplet on the agar surface formed during the inoculation procedure. The second phase reflects the swimming of the cells through the pores of the agar matrix away from the injection site. In the presence of dispersal networks, the biphasic behavior vanished because bacterial cells did not accumulate in a colony on the surface but immediately used the network to move away from the injection site. This dispersal, however, was not measured during the first hours of the experiments because we needed to adjust the detection threshold for image analysis to a constant value for all experiments, and we selected a value optimized for measuring the bulk of cells dispersing, which we consider to be responsible for efficient degradation (see Fig. S1 in the supplemental material). The population dispersal performance characterized by the AUC remained nearly constant (94% of the control) at a $\Delta\Psi_o$ of −0.25 MPa but decreased continuously at lower $\Delta\Psi_o$ values (Fig. 2A). At a $\Delta\Psi_o$ of −1.5 MPa, only 2% of the AUC compared to that of the control ($\Delta\Psi_o$ = 0 MPa) remained. In contrast, changing the $\Delta\Psi_m$ diminished the AUC for population dispersal by at least 90% for all treatments, due to a complete repression of bacterial movement through the agar matrix (Fig. 2B). The remaining 8 to 10% of the AUC for the $\Delta\Psi_m$ treatments stemmed from bacterial colony growth on the surface of the agar resulting in a passive push-forward effect.

A glass fiber mat was used as an abiotic model of mycelium-like dispersal networks to test whether they could maintain bacterial dispersal processes. This glass fiber network accelerated the dispersal of *P. putida* KT2440 between $\Delta\Psi_o$ and $\Delta\Psi_m$ values of 0 and −0.5 MPa (Fig. 2A and B). The highest benefits compared to the treatments without network presence were found at a $\Delta\Psi_o$ value of 0 MPa and a $\Delta\Psi_m$ value of −0.25 MPa, with 46% and 83% absolute differences of the AUC, respectively.

Population growth at different $\Delta\Psi_o$ and $\Delta\Psi_m$ values in the presence and absence of dispersal networks. Bacterial population growth was analyzed after detachment of the cells from the

TABLE 1 Pairwise Pearson's product moment correlation coefficients (ρ) and corresponding *P* values

Pairwise combination	Data for Ψ_o		Data for Ψ_m	
	ρ value	*P* value	ρ value	*P* value
Growth vs degradation	0.86	0.002	1	<0.001
Growth vs dispersal	0.87	0.001	0.93	<0.001
Degradation vs dispersal	0.88	<0.001	0.92	<0.001

agar matrix at the four different time points. The AUC for CFU-based population growth of *P. putida* KT2440 remained nearly stable at a $\Delta\Psi_o$ value of −0.25 MPa but dropped at lower water potentials, by up to 92% at a $\Delta\Psi_o$ value of −1.5 MPa relative to the control (0 MPa) (Fig. 2C). For $\Delta\Psi_m$, population growth was reduced by 94% relative to the control treatment at all potentials tested (Fig. 2D). The presence of dispersal networks resulted in an increase of the AUC for population growth for all treatments, with an absolute difference of 110% for a $\Delta\Psi_o$ value of −0.25 MPa and 76% for a $\Delta\Psi_m$ value of −0.25 MPa compared to the corresponding treatments without network presence (Fig. 2C and D). However, the networks' effects at a $\Delta\Psi_o$ value of −1.5 MPa and a $\Delta\Psi_m$ value of −1 MPa and below were negligible (between 4 and 6% differences in the AUC values) (see Fig. S2 in the supplemental material).

We further tested if a change of the water potentials led to different proportions of culturable cells in all cells. We did this in order to exclude biases potentially occurring due to a viable but not culturable state of the bacteria, which is often related to the occurrence of environmental stressors (32). Therefore, we microscopically counted cells obtained from the incubations at $\Delta\Psi_o$ and $\Delta\Psi_m$ values of 0 MPa and −1.5 MPa and compared the proportions of CFU in all cells for the two water potentials. There was no significant difference in these proportions for $\Delta\Psi_o$ (32.4% ± 4.6% at a $\Delta\Psi_o$ value of 0 MPa and 30.1% ± 4.6% at a $\Delta\Psi_o$ value of −1.5 MPa) and $\Delta\Psi_m$ (36.7% ± 3.3% at a $\Delta\Psi_m$ value of 0 MPa and 33.2% ± 8.6% at a $\Delta\Psi_m$ value of −1.5 MPa), which supports the results obtained from the measurement of culturable cells.

Benzoate degradation at different $\Delta\Psi_o$ and $\Delta\Psi_m$ values in the presence and absence of dispersal networks. Sodium benzoate was the sole carbon and energy source in the agar, and its decrease in concentration was monitored by HPLC analysis. Degradation performance was not affected at a $\Delta\Psi_o$ value of −0.25 MPa (99% of the AUC remained), decreased by 47% at a $\Delta\Psi_o$ value of −0.5 MPa, and almost disappeared at a $\Delta\Psi_o$ value of −1.5 MPa (1% of the AUC remained) (Fig. 2E) relative to the performance at a $\Delta\Psi_o$ value of 0 MPa. In contrast, at a $\Delta\Psi_m$ value of −0.25 MPa, the degradation performance had already decreased by 44%, but at a $\Delta\Psi_m$ value of −1.5 MPa, it decreased by only 78% (Fig. 2F). The presence of dispersal networks was beneficial for biodegradation for all treatments. For $\Delta\Psi_o$ treatments, absolute AUC improvements ranged from 21% at 0 MPa to 52% at −0.5 MPa compared to the corresponding treatments without network presence (Fig. 2E). For $\Delta\Psi_m$ treatments, AUC values varied much more, leading to increases of 2% at −1 MPa and 119% at 0 MPa (Fig. 2F). Population dispersal, population growth, and benzoate degradation were found to be positively correlated for both $\Delta\Psi_o$ ($\rho > 0.86$) and $\Delta\Psi_m$ ($\rho > 0.92$) (Table 1).

DISCUSSION

Effects of dispersal networks on bacterial dispersal at different $\Delta\Psi_o$ and $\Delta\Psi_m$ values. In this study, we tested the hypothesis that network-based dispersal increases the functional performance of bacterial population growth and biodegradation by facilitating bacterial motility at environmentally relevant $\Delta\Psi_o$ and $\Delta\Psi_m$ values. The motile soil bacterium *P. putida* KT2440 was chosen for the experiments because its motility under unsaturated conditions has been studied extensively (4, 33).

In the absence of the dispersal networks, colony expansion was found to decrease with lowering $\Delta\Psi_o$ values, down to −1.5 MPa. A similar effect was reported for *Pseudomonas* and *Bacillus* strains at salt concentrations which correspond to $\Delta\Psi_o$ values between −1.8 and −2.3 MPa (23, 24), likely as a result of downregulation of motility genes to avoid energetic disadvantages due to flagellar system formation. In contrast to $\Delta\Psi_o$, a reduction of $\Delta\Psi_m$ to −0.25 MPa (as induced by PEG 8000 addition) already resulted in drastically reduced dispersal, which is in good agreement with several studies pointing out that bacterial motility is restricted to a narrow range of high matric water potentials (4, 9, 33, 34). Dechesne et al. also reported a drastically reduced lateral colony expansion in response to insufficient water film thickness for *P. putida* KT2440 at Ψ_m values as low as −0.0036 MPa on a porous surface model system (25).

The use of PEG 8000 to change the matric component of $\Delta\Psi_w$ is still debated, as different gene expression profiles were observed, unlike the case with directly applied $\Delta\Psi_m$ (35). However, for plant cells, PEG 8000 was shown to evoke the same effects as soil drying, causing cytorrhysis rather than plasmolysis, without toxic effects of the PEG itself (27). Furthermore, the method of adjusting the Ψ_m by adding PEG 8000 has a distinct advantage over the use of different agar concentrations (36), as the latter imposes a physical hindrance rather than a decrease of the Ψ_m (37).

The presence of glass fiber networks clearly improved bacterial dispersal until the $\Delta\Psi_w$ and $\Delta\Psi_o$ values reached −0.5 MPa. Glass fibers were used to simulate hyphae surrounded by liquid films (16, 18) and to exclude effects of hyphal activities on bacterial growth and nutrition (38–41) to avoid additional complexity that might mask effects attributable to the promotion of bacterial dispersal at a lowered Ψ_m.

From the experimental data, we cannot infer that −0.5 MPa is a critical threshold below which dispersal along the glass fibers is completely restricted. Population dispersal was observed for 24 h because the microscopic observation area for the control treatments without network presence was completely colonized in this time span. Most, but not all, scenarios with lower $\Delta\Psi_w$ values showed an improvement by use of dispersal networks within this time span. However, the shape of the population dispersal curves for the remaining three scenarios (see Fig. S1 in the supplemental material) suggests that benefits from the dispersal networks would probably occur soon after 24 h. We hypothesize that the dispersal network-mediated benefits for bacteria may compensate the energetic costs of flagellar maintenance and hence prevent the downregulation of motility genes. Moreover, our results indicate that bacterial dispersal in soil in the presence of dispersal-enabling mycelia is probably more dynamic than previously assumed and may not be restricted to the generally accepted Ψ_w of −0.05 MPa, a water potential which corresponds to high soil humidity (21).

Effects on bacterial growth and biodegradation. The presence of dispersal networks resulted in an increase in population growth relative to the situation without network presence for $\Delta\Psi_w$ values down to −1 MPa. This points to a dispersal network-mediated benefit also occurring at values below −0.5 MPa, which occurred with a delay of more than 24 h but within the 48-h observation period used for growth analysis. Such an observation is consistent with a previous study showing that faster dispersal is accompanied by increased bacterial abundance (10).

Biodegradation activity was assessed by following the consumption of sodium benzoate in the microcosms (see Fig. S3 in the supplemental material). The good water solubility of benzoate makes it a suitable model compound for highly accessible and bioavailable contaminants. With the developed microcosm system, we could clearly demonstrate that the network-mediated benefit for bacterial dispersal also led to an accelerated biodegradation over a wide range of $\Delta\Psi_o$ and $\Delta\Psi_m$ values. This is highly relevant for soil, for which bacterial dispersal was identified as one of the key factors for efficient biodegradation (17, 42–44). Interestingly, the observed negative influence of salt exposure on biodegradation is inconsistent with a study showing no influence of Ψ_o on degrading activity in liquid culture experiments (7). This discrepancy is presumably a consequence of the completely different conditions of the experimental test systems. Shaken liquid culture systems are virtually homogeneous regarding bacterial cell and substrate distribution, leading to short diffusion pathways. In contrast, in the agar systems applied here, bacteria are initially concentrated at the inoculation spot (Fig. 1), and restricted dispersal causes diffusion-limited degradation. Moreover, differences may arise from the use of degradation rates to compare effects at different $\Delta\Psi_w$ rates for liquid culture experiments. Degradation rates are probably not a robust measure for comparing effects at different $\Delta\Psi_w$ values because abrupt osmotic shifts are known to increase lag times (45), which are not considered for rate calculation but are well represented by the AUC.

Relevance for soil ecological functions. The dispersal of microorganisms is recognized as a key factor for soil ecological functions (1, 46–48), such as the promotion of microbial diversity or the turnover of chemicals. Bacterial dispersal along fungal mycelia has been shown for numerous bacterial species and fungi (18, 49, 50) and hence may be of special relevance in unsaturated soil systems. Fungi constitute up to 75% of the soil microbial biomass, with a length of 10^2 to 10^4 m hyphae per g of soil (12, 51). Moreover, fungi have a unique lifestyle that is adapted to environmental changes, and they were shown to possess a marked resistance to desiccation in the field (52, 53). Because of the ubiquity, high abundance, and adaptive capacity of fungi, interactions between fungi and bacteria may substantially affect soil environmental dynamics and should therefore not be neglected (54).

The overall level and spatiotemporal variability of soil moisture have long been described as some of the primary environmental regulators of soil microbial activity (55, 56). Indeed, desiccation is a frequent physiological stress for soil microbial communities and is anticipated to gain further importance during future climate change (21). Simulation models predict an increased risk of drought in the 21st century (57). Furthermore, soil salinization is also a rising problem, especially in agriculture, due to irrigation and fertilizer amendment (58). However, the steadily increasing global population necessitates land use changes toward agriculture, which will also increase the amount of pesticides applied to the soil. Thus, to remain efficient in future, bioremediation approaches have to consider the effects of drought and salts on bacterial dispersal, growth, and degradation performance, which were shown to react drastically to changes in Ψ_o and Ψ_m in this study.

Obviously, the developed microcosm system reflects natural conditions in a highly simplified manner. However, such simplifications are also a strength of microbial model systems, which are necessary to better understand single aspects of natural systems (59, 60), such as the observed beneficial effects of dispersal networks on bacterial degradation performance at various water potentials.

The use of gel media to study bacterial dispersal in soil is discussed controversially (33). Scanning electron microscopy pictures revealed that soil surfaces, on top of which bacterial dispersal occurs, are not just bare mineral surfaces but rather are covered by patchy materials most likely originating from broken cell envelopes (61). The role of these sponge-like structures on bacterial motility in soils is completely neglected. However, they are probably better represented by agar matrices than by ceramic or quartz surfaces. Further investigations, in particular with fungi in a real soil system, are advisable to extrapolate our findings and to further elucidate the combined effects of fungal-bacterial interactions on contaminant degradation. Nevertheless, our findings strongly indicate that fungi may act as promoters of biodegradation under low-hydration and high-salinity conditions, thus improving the functional performance of microbial ecosystems.

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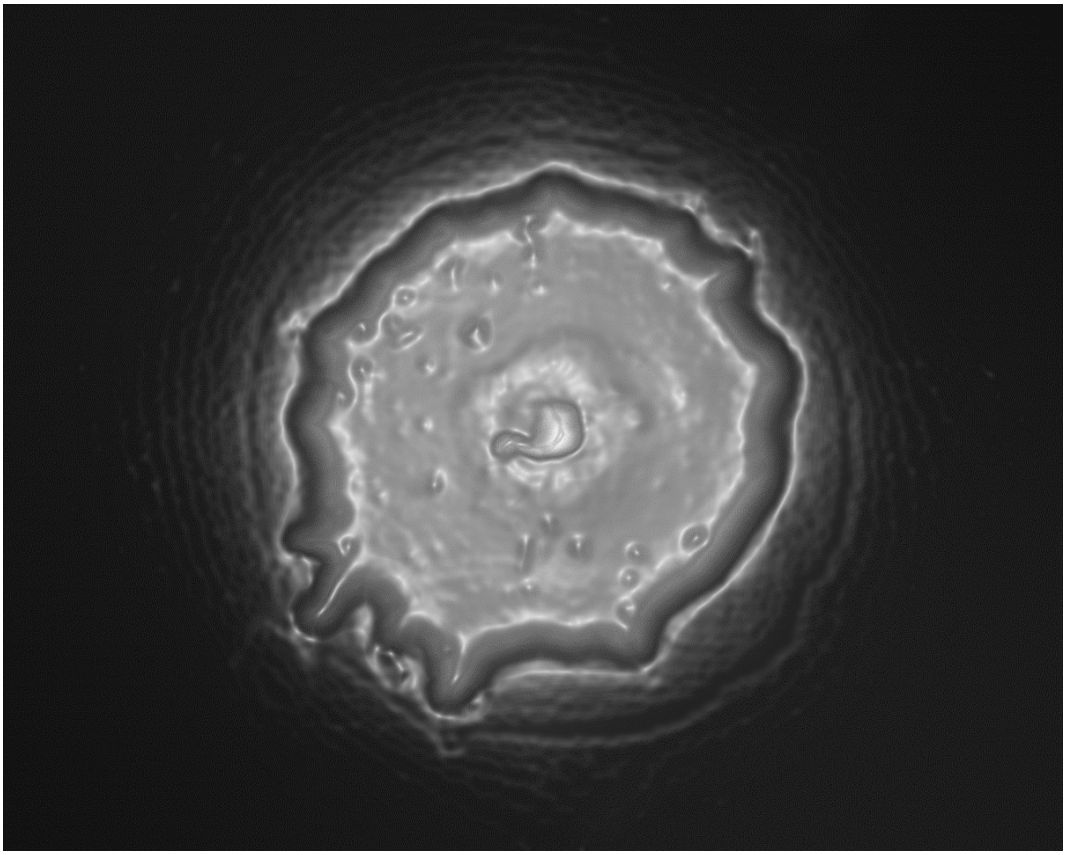
Chapter 4

BACTERIAL DISPERSAL UNDER OSMOTIC STRESS



Colony of Pseudomonas putida on agar of lowered Ψ_o

4 BACTERIAL DISPERSAL UNDER OSMOTIC STRESS

4.1 Bacterial Dispersal Promotes Biodegradation in Heterogeneous Systems Exposed to Osmotic Stress

Worrich, A., König, S., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Miltner, A., Wick, L. Y. & Kästner, M. Bacterial Dispersal Promotes Biodegradation in Heterogeneous Systems Exposed to Osmotic Stress. *Front. Microbiol.* **7,** 1214 (2016).

Significance: Bacterial activity in soil is characterized by high intrinsic spatial variations arising from the communities' ecological features and the shaping forces of environmental factors. However, the compounded effects of this spatial heterogeneity and common environmental stressors such as salinization on biodegradation processes are poorly understood. Using a microbial model system, we tested, in how far different spatial processes (*i.e.* substrate diffusion and autonomous as well as mediated bacterial dispersal) are able to counteract the disadvantages of spatial degrader heterogeneity at different osmotic stress intensities. We found that dispersal is essentially important for contaminant fate because biodegradation was drastically reduced when substrate diffusion was the sole spatial process.

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Bacterial Dispersal Promotes Biodegradation in Heterogeneous Systems Exposed to Osmotic Stress

Anja Worrich[1,2], Sara König[1,3], Thomas Banitz[3], Florian Centler[1], Karin Frank[3,4,5], Martin Thullner[1], Hauke Harms[1,4], Anja Miltner[2], Lukas Y. Wick[1] and Matthias Kästner[2]*

[1] UFZ - Helmholtz Centre for Environmental Research, Department of Environmental Microbiology, Leipzig, Germany, [2] UFZ - Helmholtz Centre for Environmental Research, Department of Environmental Biotechnology, Leipzig, Germany, [3] UFZ - Helmholtz Centre for Environmental Research, Department of Ecological Modelling, Leipzig, Germany, [4] German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany, [5] Institute for Environmental Systems Research, University of Osnabrück, Osnabrück, Germany

Contaminant biodegradation in soils is hampered by the heterogeneous distribution of degrading communities colonizing isolated microenvironments as a result of the soil architecture. Over the last years, soil salinization was recognized as an additional problem especially in arid and semiarid ecosystems as it drastically reduces the activity and motility of bacteria. Here, we studied the importance of different spatial processes for benzoate biodegradation at an environmentally relevant range of osmotic potentials ($\Delta\Psi_O$) using model ecosystems exhibiting a heterogeneous distribution of the soil-borne bacterium *Pseudomonas putida* KT2440. Three systematically manipulated scenarios allowed us to cover the effects of (i) substrate diffusion, (ii) substrate diffusion and autonomous bacterial dispersal, and (iii) substrate diffusion and autonomous as well as mediated bacterial dispersal along glass fiber networks mimicking fungal hyphae. To quantify the relative importance of the different spatial processes, we compared these heterogeneous scenarios to a reference value obtained for each $\Delta\Psi_O$ by means of a quasi-optimal scenario in which degraders were *ab initio* homogeneously distributed. Substrate diffusion as the sole spatial process was insufficient to counteract the disadvantage due to spatial degrader heterogeneity at $\Delta\Psi_O$ ranging from 0 to −1 MPa. In this scenario, only 13.8–21.3% of the quasi-optimal biodegradation performance could be achieved. In the same range of $\Delta\Psi_O$ values, substrate diffusion in combination with bacterial dispersal allowed between 68.6 and 36.2% of the performance showing a clear downwards trend with decreasing $\Delta\Psi_O$. At −1.5 MPa, however, this scenario performed worse than the diffusion scenario, possibly as a result of energetic disadvantages associated with flagellum synthesis and emerging requirements to exceed a critical population density to resist osmotic stress. Network-mediated bacterial dispersal kept biodegradation almost consistently high with an average of 70.7 ± 7.8%, regardless of the strength of the osmotic stress. We propose that especially fungal network-mediated bacterial dispersal is a key process to achieve high functionality of heterogeneous microbial ecosystems also at reduced osmotic potentials. Thus, mechanical stress by, for example, soil homogenization should be kept low in order to preserve fungal network integrity.

Keywords: spatial processes, heterogeneity, biodegradation, dispersal networks, *Pseudomonas putida*, osmotic stress, diffusion, contaminants

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****Correspondence:***
Lukas Y. Wick
lukas.wick@ufz.de

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INTRODUCTION

Bacterial degradation of contaminants prevents their persistence in the environment as well as the contamination of water resources due to leaching (Arias-Estévez et al., 2008). However, the distribution of bacterial degraders in soils shows a distinct spatial heterogeneity in both horizontal (Vinther et al., 2008) and vertical (Badawi et al., 2013) direction. For nearly a century, spatial heterogeneity was considered only a large-scale phenomenon (Dechesne et al., 2014), and microbial ecologists stuck to the "Everything is everywhere" paradigm suggesting that microorganisms are mostly cosmopolitan and almost homogeneously distributed (Cho and Tiedje, 2000). Owing to intensive work on spatial heterogeneity in soil, the endemism hypothesis for bacteria is now generally accepted (Cho and Tiedje, 2000; Fulthorpe et al., 2008) and there is increasing awareness that heterogeneity is not only a field-scale phenomenon, but similarly occurs at the micro-scale (Vieublé Gonod et al., 2006). Studies on the distribution of 2,4-dichlorophenoxyacetic acid (2,4-D) degraders in soil and soil column systems confirmed the existence of soil regions comprising several millimeters right up to a few centimeters, which are devoid of 2,4-D degrading activity (Pallud et al., 2004; Vieublé Gonod et al., 2006; Pinheiro et al., 2015). However, the majority of publications dealing with spatial heterogeneity of bacterial degraders in soils are rather descriptive, whereas the consequences of these small-scale spatial heterogeneities on biodegradation efficiency are difficult to assess and thus have hardly been evaluated so far (Dechesne et al., 2014).

Bacterial dispersal and substrate mass transfer are considered key factors for efficient biodegradation as both processes help to overcome spatial separation, thus leading to increased contact probability between bacteria and contaminants in a spatially heterogeneous environment like soil (Semple et al., 2007). Motility is a highly conserved trait among bacteria and more than two thirds of the sequenced species are motile, indicating that it provides bacteria with an essential ecological advantage (Czaban et al., 2007; Wei et al., 2011). Motile bacteria, for example, can actively disperse in the pore water and spread along dense mycelial networks formed by fungi leading to improved phenanthrene biodegradation (Wick et al., 2007).

Besides being often contaminated, soils are highly affected by salt accumulation due to low rainfall and high evapotranspiration (Rengasamy, 2006). Globally, more than 831 million hectares of land are affected by salt at levels causing osmotic stress in bacteria (Martinez-Beltran and Manzur, 2005). Recently, it was shown that the presence of mycelium-like networks improved dispersal, growth, and biodegradation under environmental stress conditions induced by lowered water potentials (Worrich et al., 2016). However, to what extent spatial processes like bacterial dispersal, network-mediated bacterial dispersal, or substrate diffusion may counteract the disadvantage due to a heterogeneous degrader distribution at different osmotic potentials remains unclear. Indeed, it is highly relevant to investigate whether and under which environmental conditions the spatial heterogeneity of degrading bacteria has to be taken into account in order to (i) improve models of microbial biodegradation in soils, (ii) predict contaminant fate in the environment, and (iii) derive strategies for risk assessment and management (Holden and Firestone, 1997; Soulas and Lagacherie, 2001).

Here, we aimed at quantifying the effect of different spatial processes (i.e., bacterial dispersal and substrate diffusion) on biodegradation efficiency at a range of environmentally relevant osmotic potentials, which are known to cause restrictions in bacterial growth and motility. We tested the hypothesis that to some extent autonomous bacterial dispersal but especially the dispersal along mycelia is crucial to counteract the disadvantages evoked by spatial degrader heterogeneity as sufficient compensation cannot be achieved by substrate diffusion alone. Furthermore, we wanted to assess whether there is a certain threshold at which not the spatial processes but rather the physiological limitations control biodegradation efficiency. In a microbial model system, we created different scenarios permitting solely substrate diffusion, or substrate diffusion and autonomous bacterial dispersal, or the two aforementioned processes plus network-mediated bacterial dispersal. A comparison of the respective scenarios to a reference scenario exhibiting a homogeneous degrader distribution allowed for a relative quantification of the biodegradation promoting effects for the different spatial processes.

METHODS

Organisms and Culture Conditions

Experiments were carried out with a benzoate-degrading, GFP-tagged derivative of the soil bacterium *Pseudomonas putida* KT2440. It was cultivated in FAB minimal medium (Hansen et al., 2007) supplemented with 50 mM sodium benzoate (FAB-50, Sigma-Aldrich, Munich, Germany) at room temperature with 150 rpm rotary culture flask movement. For strain maintenance, bacteria were transferred weekly to FAB-50 plates containing 1.5% (w/v) agar and incubated at room temperature.

Bacterial Population Growth Kinetics under Osmotic Stress

For a detailed analysis of the effects of different sodium chloride concentrations on bacterial growth kinetics, we measured the increase in optical density at 600 nm in 96-well microtiter plates (OD_{600}) in a plate reader (SpectraMAX 250, Molecular Devices, California U.S.) set to 25°C. The wells were filled with 200 µl FAB-50 supplemented with either 3.2, 6.4, 12.8, and 19.2 g l^{-1} of sodium chloride, which corresponds to $\Delta\Psi_o$ of −0.25, −0.5, −1, or −1.5 MPa (Holden et al., 1997). FAB-50 medium without sodium chloride served as $\Delta\Psi_o = 0$ MPa control treatment. To minimize edge effects, outer wells were filled with potassium phosphate buffer (PB, 10 mM, pH 7.2) and not used for growth analysis (Johnsen et al., 2002). Cells from a liquid culture were harvested by centrifugation at 8000 g for 10 min. The pellet was washed once with PB and adjusted to an optical density of 20 with FAB-50 medium. Each well was inoculated with 2 µl using a multichannel pipette. Plates thus contained 12 replicates per osmotic stress treatment. Measurements were carried out

every 30 min over a total of 86 h. Maximum specific growth rates (μ_{max}) were determined by exponentially fitting the sections of highest increase in the growth curves. Maximum biomass yields were calculated from the maximum OD_{600} values of each replicate using a calibration curve for the correlation between dry biomass and OD_{600}. Samples exhibiting different OD_{600} values were filtered through weighed cellulose nitrate membrane filters with a pore size of 0.22 µm (Sartorius, Goettingen, Germany). After drying at 60°C for 48 h, filters were weighed again with the increase determining dry biomass. Lag times were derived from the x-axis intercept of the straight line in the maximum slope of a non-parametric spline fit function of the "grofit"-package in R (Kahm et al., 2010; R Core Team, 2014).

Microcosm Setup

To investigate the role of the different spatial processes, four different scenarios were created. The wells of clear and sterile 24-well flat-bottom microtiter plates were filled with either 1 ml of FAB-50 minimal medium agar of 1% (w/v) to allow for diffusion but completely restrict bacterial dispersal (D_{dif}) or 0.3% (w/v) to allow for bacterial dispersal and substrate diffusion (D_{dis}). To test the influence of mediated bacterial dispersal (D_{net}), a glass fiber net with an area weight of 14 g m^{-2} (Mühlmeier composite, Bärnau, Germany) was placed on top of 0.3% (w/v) agar (Supplementary Figure 1). The glass fiber net was cut into circular pieces of Ø 1.2 cm using a cork borer. Subsequently, the pieces were heat sterilized at 450°C for 4 h in a muffle furnace and placed in the microcosms before inoculation with bacteria. To create the reference scenario with a homogeneous cell distribution (D_{hom}), wells were filled with liquid FAB-50 minimal medium. Osmotic stress was induced similarly to the growth kinetics experiment. Different amounts of sodium chloride were added to the FAB-50 liquid medium or agar prior to autoclaving. Agar and liquid medium without additional sodium chloride served as $\Delta\Psi_o = 0$ MPa control treatments. Before use, all agar plates were dried in a laminar flow cabinet for 5 min.

Microcosm Inoculation

Cells were harvested from liquid culture by centrifugation at 8000 g for 10 min after 16 h of cultivation. The pellet was washed once with PB and adjusted to an optical density of 50. Microcosms were inoculated in the center of each well with 0.2 µl bacterial suspension [$\sim 2.4 \cdot 10^6$ colony forming units (CFU)] using a microliter syringe. The needle was pricked deep into the agar (~0.8 cm; cf. **Figure 1**) but did not touch the bottom of the microcosms during inoculation. Abiotic control treatments were left uninoculated. To achieve a homogeneous distribution of bacterial cells in the D_{hom} scenarios, plates were shaken for 1 min at 300 rpm on a rotary shaker. Plates were incubated under static conditions in plastic containers at room temperature in the dark.

Bacterial Population Growth and Degradation Measurement

Cell numbers and benzoate concentrations were determined after 6, 24, 30, and 48 h in replicate multiwell plates. Well contents were carefully mixed with 2 ml PB. For the agar experiments, the PB-agar slurry was subsequently transferred to a sterile Falcon tube and detachment of bacterial cells was carried out by vortexing and ultrasonication in a water bath with a frequency of 35 kHz (Sonorex Super RK 255H, Bandelin, Berlin) for 1 min. Culturable cells were analyzed as CFU. To this end, bacteria were spread on FAB-50 plates using the drop plate method as described earlier (Chen et al., 2003). Briefly, 10-fold dilution series of the supernatant were prepared directly in 96-well microtiter plates and 5 µl of consecutive dilutions were dropped on the agar plate. Plates were incubated at 25°C for 48 h. Droplets giving rise to

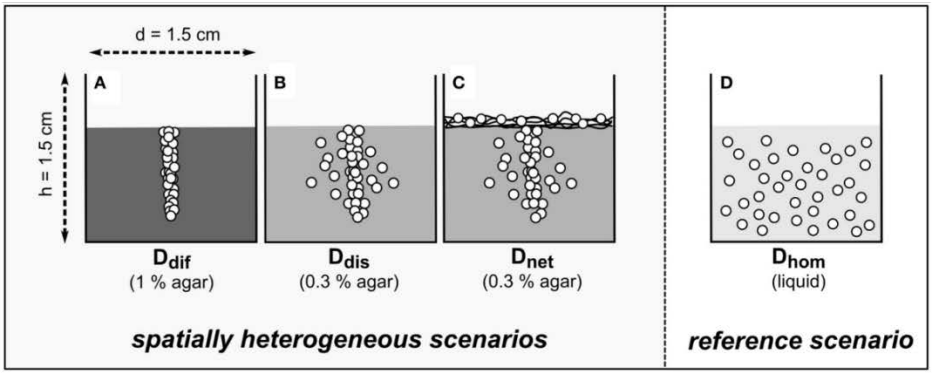

FIGURE 1 | Scheme of the model ecosystems used to assess the counteracting effects of substrate diffusion (A), substrate diffusion and autonomous bacterial dispersal (B), and substrate diffusion, autonomous bacterial dispersal, and network-mediated bacterial dispersal (C) on population growth and biodegradation in systems exhibiting a heterogeneous degrader distribution in comparison to a homogeneous reference scenario (D). White circles indicate the anticipated distribution of bacteria in matrices with increasing physical hindrance (indicated by shades of gray). Black lines **(C)** depict the glass fiber network used to simulate mycelial networks. In each treatment the osmotic potential $\Delta\Psi_O$ was varied between 0 and −1.5 MPa using sodium chloride.

between 5 and 30 single colonies were used to determine CFU numbers.

For benzoate measurements, the supernatant obtained from cell detachment was fixed with 4% (w/v) formaldehyde and filtered through 0.22 µm syringe filters (Carl Roth, Karlsruhe, Germany) to remove bacteria. The benzoate concentration was determined with an HPLC system equipped with a C_{18} reverse phase column (250 × 4 mm) and a Photodiode Array Detector (PDA) set at 271 nm. The system was operated at a flow rate of 1.2 ml min^{-1}, 10 µl injection volume and a mobile phase consisting of 80% sodium acetate (50 mM, pH 4.5) and 20% MeOH (Warikoo et al., 1996). Benzoate had a retention time of 13.8 min under these operation conditions.

Determination of the Relative Counteracting Effects of Bacterial Dispersal and Substrate Diffusion at Different Osmotic Potentials

The extent to which bacterial dispersal and substrate diffusion are able to counteract the disadvantage caused by a heterogeneous degrader distribution at different osmotic potentials and thus to maintain benzoate biodegradation was analyzed by comparing the performance of the respective scenarios (D_{dif}, D_{dis}, and D_{net}) with the performance of the D_{hom} setup exhibiting a quasi-optimal homogeneous distribution of the degraders. We calculated the areas under the curve (AUC) for the time courses of benzoate biodegradation for the different distribution scenarios at the different osmotic potentials. The AUC serves as an aggregated measure of the temporal biodegradation performance and was calculated using the trapezoidal method in R. This numerical approximation of the time integral allows for non-equidistant time data and, thus, is not hampered by missing measurements for certain points in time. The relative counteracting effects in terms of biodegradation performance (RCE) for each scenario D_i in dependence of the osmotic potential $\Delta\Psi_o$ were calculated according to:

$$\mathrm{RCE}_{D_i,\Delta\Psi_o} = \frac{\mathrm{AUC}_{D_i}}{\mathrm{AUC}_{D_{hom}}}; (D_1 = D_{dif}; D_2 = D_{dis}; D_3 = D_{net}). \quad (1)$$

RESULTS

Bacterial Population Growth Kinetics under Osmotic Stress

The obtained bacterial growth curves (Supplementary Figure 2) show a distinct response to different levels of osmotic stress created by the addition of sodium chloride. With decreasing $\Delta\Psi_o$, the maximum specific growth rates were found to gradually decrease from 0.26 h^{-1} at $\Delta\Psi_o = 0$ MPa to 0.14 h^{-1} at $\Delta\Psi_o = -1$ MPa and then dropped to 0 h^{-1} at $\Delta\Psi_o = -1.5$ MPa (**Figure 2A**). A similar behavior was observed for maximum biomass, which showed only minor changes between $\Delta\Psi_o = 0$ MPa and $\Delta\Psi_o = -1$ MPa (reduction from 0.32 to 0.3 mg) but markedly decreased to 0.03 mg at $\Delta\Psi_o = -1.5$ MPa, which corresponds to the inoculated biomass (**Figure 2B**). Lag times exhibited a different pattern as a drastic prolongation to 59 h was observed already at $\Delta\Psi_o = -1$ MPa compared to 7.3 h at $\Delta\Psi_o = 0$ MPa. At $\Delta\Psi_o = -1.5$ MPa, lag times could not be determined as no growth was observed within the duration of the experiments (**Figure 2C**).

Effects of the Spatial Degrader Distribution on Biodegradation and Population Growth

The spatial distribution of the bacterial population had a drastic impact on benzoate biodegradation and bacterial population dynamics. In the D_{hom} scenario, the total amount of benzoate in the system was already degraded after 24 h, whereas in the D_{dif} scenario, only 11.1% of the total benzoate was consumed at this time point (**Figure 3A**, dotted and dot-dashed line). At the end of the experiment (after 48 h), 26.1% of the benzoate was consumed on average in the D_{dif} scenario (i.e., when bacteria were trapped at the inoculation point, **Figure 3A**, dot-dashed line). In the D_{dis} scenario, 41.2 and 64.3% of the benzoate was consumed after 24 and 30 h, whereas in the D_{net} scenario, benzoate consumption accounted for 80.7 and 90.3% at the respective time points (**Figure 3A**, solid and dashed line). In both scenarios, the total amount of benzoate was degraded after 48 h.

CFU numbers in the D_{dif} scenario were roughly 23-fold lower compared to the D_{hom} scenario after 24 h of incubation ($1.05 \cdot 10^8$ and $2.39 \cdot 10^9$ CFU ml^{-1}, respectively, **Figure 3F**). With increasing incubation time, CFU numbers in the D_{dif} scenario steadily increased to $3.8 \cdot 10^8$ CFU ml^{-1} after 48 h, whereas in the D_{hom} scenario CFU numbers already showed a decline at 30 h (**Figure 3F**, dot-dashed and dotted line). At 48 h, D_{hom} and D_{dif} scenarios exhibited the same CFU number. In the D_{dis} and D_{net} scenarios, CFU numbers continuously increased to $1.35 \cdot 10^9$ and $1.97 \cdot 10^9$ CFU ml^{-1} at 48 h, respectively. However, in the D_{net} scenario, we consistently observed a higher CFU number at the different time points (**Figure 3F**, dashed and solid line).

Impact of Different Spatial Degrader Distributions on the Response of Biodegradation and Population Growth to Varying Osmotic Potentials

Apparently, in the D_{hom} scenario, a reduction of $\Delta\Psi_o$ down to −0.5 MPa had no effect on biodegradation efficiency. After 24 h, the entire benzoate added to the microcosms was degraded also at $\Delta\Psi_o = -0.25$ MPa and $\Delta\Psi_o = -0.5$ MPa (**Figures 3A–C**, dotted line). However, lower $\Delta\Psi_o$ of −1 MPa and −1.5 MPa led to decelerated benzoate biodegradation in the D_{hom} scenario (**Figures 3D,E**, dotted lines). At $\Delta\Psi_o = -1$ MPa, the benzoate in the D_{hom} scenarios was almost completely degraded after 30 h (97.4%), whereas at $\Delta\Psi_o = -1.5$ MPa still 18.9% remained in the microcosms after 48 h (**Figures 3D,E**). In the D_{dif} scenario, we observed only minor changes in biodegradation with decreasing osmotic potentials (**Figures 3A–E**, dot-dashed line). After 48 h, between 26.1% at $\Delta\Psi_o = 0$ MPa and 13.6% at $\Delta\Psi_o = -1.5$ MPa of the total benzoate was degraded.

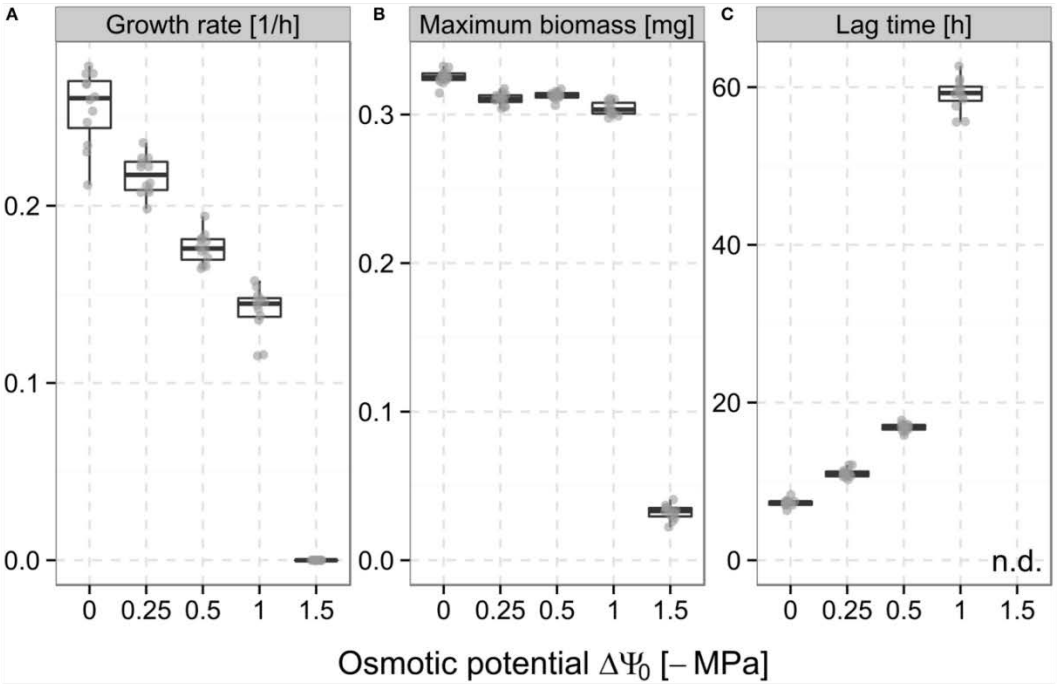

FIGURE 2 | Maximum specific growth rate (A), maximum biomass (B), and lag time (C) of *Pseudomonas putida* KT2440 during growth in liquid culture at the different osmotic potentials ($\Delta\Psi_O$) adjusted with sodium chloride (cf. Section Bacterial Population Growth Kinetics under Osmotic Stress). Boxes show the medians and the interquartile range of values (between 25th and 75th percentile). Whiskers extend to values not more than 1.5-fold out of this range. Points represent the values of individual replicates (12 in total for each $\Delta\Psi_O$).

Biodegradation in the D_{dis} scenario was markedly decelerated at lowered $\Delta\Psi_o$. While at $\Delta\Psi_o = -0.25$ MPa, 24.8% of the benzoate was consumed, we did not observe any biodegradation at $\Delta\Psi_o = -1.5$ MPa after 24 h. At the end of the experiments (after 48 h), almost the whole amount of benzoate was degraded at $\Delta\Psi_o$ ranging from 0 to −1 MPa, whereas at $\Delta\Psi_o = -1.5$ MPa still 97.2% of the benzoate was left in the microcosms. Also in the D_{net} scenario, benzoate biodegradation was decelerated at lowered $\Delta\Psi_o$. However, the amount of degraded benzoate always exceeded the values obtained for the D_{dis} scenario and still accounted for 57.2% at $\Delta\Psi_o = -1.5$ MPa.

Generally, bacterial population dynamics in the D_{hom} scenario exposed to osmotic stress reflected the degradation patterns. Also here, drastic reductions in CFU numbers were observed at $\Delta\Psi_o = -1$ MPa with a 6.7-fold lower value compared to $\Delta\Psi_o = 0$ MPa after 24 h (**Figures 3F,I**, dotted lines). In the D_{dif} scenario, CFU numbers remained very low for all osmotic potentials (**Figures 3F–J**, dash-dotted lines). Moreover, the final values (after 48 h) also gradually decreased from $3.8 \cdot 10^8$ CFU ml^{-1} for $\Delta\Psi_o = 0$ MPa to $2.43 \cdot 10^8$ for $\Delta\Psi_o = -1.5$ MPa. For the D_{dis} and D_{net} scenarios, we observed decelerations similarly to the biodegradation patterns at decreasing $\Delta\Psi_o$. Also final CFU numbers steadily decreased for both scenarios between $\Delta\Psi_o$ of −0.25 and −1.5 MPa ($1.35 \cdot 10^9$ to $2.52 \cdot 10^8$ CFU ml^{-1} for D_{dis} and $3 \cdot 10^9$ to $3.35 \cdot 10^8$ CFU ml^{-1} for D_{net}).

Relative Counteracting Effects of Dispersal and Diffusion at Different Osmotic Potentials

AUC values of D_{hom}, D_{dis}, and D_{net} scenarios decreased with decreasing osmotic potentials, but remained almost stable at a low level in the D_{dif} scenario (24.1 ± 2.9, **Figure 4A**; cf. **Figures 3A–E**). Relative counteracting effects (RCE) were calculated by dividing the AUC values for the D_{dif}, D_{dis}, and D_{net} scenarios by the AUC value for the D_{hom} scenario at each osmotic potential. In the D_{dif} scenario, only 18.3% of the biodegradation performance relative to the D_{hom} scenario was achieved at $\Delta\Psi_o = 0$ MPa. At lowered $\Delta\Psi_o$, RCE varied between 13.8% ($\Delta\Psi_o = -0.25$ MPa) and 21.3% ($\Delta\Psi_o = -1$ MPa), but increased to 48% at $\Delta\Psi_o = -1.5$ MPa (**Figure 4B**, dot-dashed line). Autonomous bacterial dispersal (D_{dis}) could maintain biodegradation to 68.6% at $\Delta\Psi_o = 0$ MPa and decreased only slightly at $\Delta\Psi_o = -0.25$ MPa. At $\Delta\Psi_o = -0.5$ and −1 MPa,

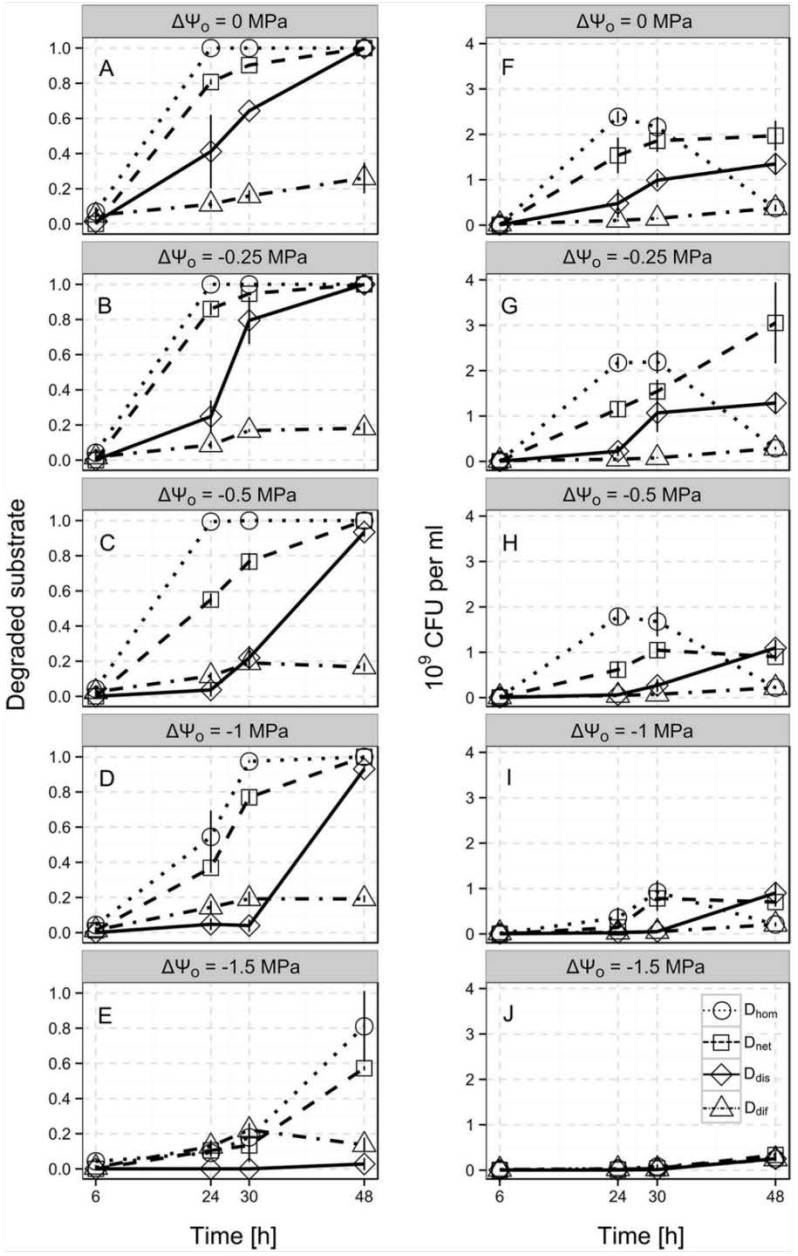

FIGURE 3 | Time-resolved benzoate biodegradation performance (A–E) and bacterial population size (F–J) of *Pseudomonas putida* KT2440 for the different scenarios Di at the different osmotic potentials ($\Delta\Psi_O$, indicated by the subplot labels). Single lines show three heterogeneous scenarios allowing for either solely substrate diffusion (D_{dif}; dot-dashed lines), substrate diffusion and autonomous bacterial dispersal (D_{dis}; solid lines), and substrate diffusion, autonomous bacterial dispersal and network-mediated bacterial dispersal (D_{net}; dashed lines) and the reference scenario with a homogeneous cell distribution (D_{hom}; dotted line). Benzoate biodegradation performance and population dynamics were assessed by the relative amounts of benzoate degraded and the CFU numbers after 6, 24, 30, and 48 h. Error bars represent the standard deviation from 4 biological replicates.

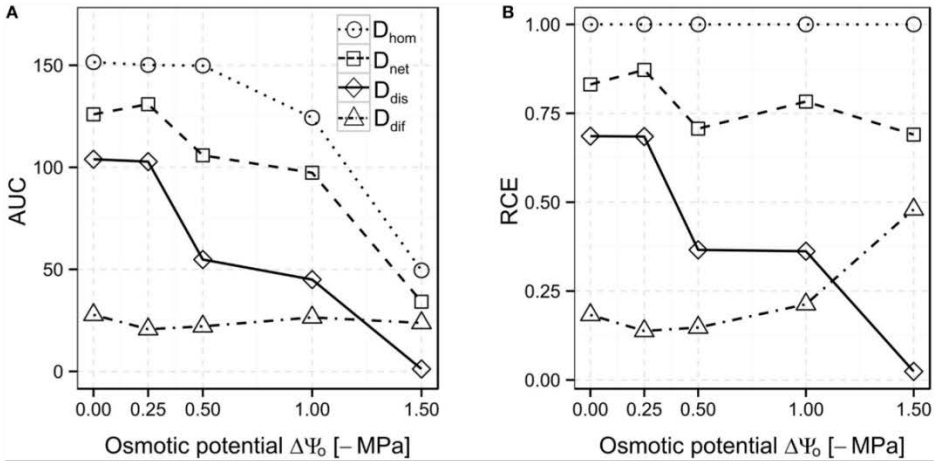

FIGURE 4 | Area under the curve (AUC; A) and Relative Counteracting Effects (RCE; B) calculated for the different scenarios *Di* at different $\Delta\Psi_o$. For RCE calculation, AUC values were divided by the AUC value of the reference scenario D_{hom} (cf. Equation 1).

RCE dropped to 36.6 and 36.2%, respectively (**Figure 4B**, solid line). However, we consistently obtained a higher RCE in the D_{dis} compared to the D_{dif} scenario at $\Delta\Psi_o$ from 0 to −1 MPa. The only exception occurred at $\Delta\Psi_o = -1.5$ MPa, where bacterial dispersal exhibited a lower RCE compared to D_{dis} (RCE of 2.4 and 48%, respectively, **Figure 4B**). In the D_{net} scenario, RCE was always the highest compared to the other scenarios and varied between 87.2 and 69% over the range of osmotic potentials (**Figure 4B**, dashed line).

DISCUSSION

Importance of the Spatial Degrader Distribution

In this study, we tested how different spatial processes can counteract a heterogeneous degrader distribution. We compared biodegradation in scenarios allowing for different spatial processes to a scenario in which degraders were homogeneously distributed to mimic optimal distribution conditions. Salinity is known to affect bacterial dispersal and thus we analyzed how the counteracting effects change with decreasing osmotic potentials and if there is some kind of threshold at which not the spatial processes but rather the physiological limitations control biodegradation efficiency. The soil bacterium *P. putida* KT2440 was chosen because of its well-characterized motility behavior (Dechesne et al., 2010b), which recently was investigated also under osmotic stress conditions (Worrich et al., 2016). In our study, sodium benzoate served as a representative of polar, aromatic contaminants (e.g., pesticides) as its physicochemical properties are similar to those of 2,4-D, dicamba or fluroxypyr (Dechesne et al., 2010a).

A heterogeneous distribution of the cells concentrated at the inoculation point clearly limited bacterial population growth and biodegradation efficiency compared to the situation with a homogeneous distribution of bacterial cells in liquid cultures. This is in line with studies on 2,4-D biodegradation in soil columns showing that biodegradation is most efficient if degraders are uniformly dispersed (Pallud et al., 2004; Pinheiro et al., 2015). By systematically decreasing the heterogeneity of the spatial degrader distribution, Dechesne et al. (2010a) also found a clear improvement of benzoate mineralization. Here, we developed an approach to estimate the impact of spatial heterogeneity on biodegradation by assessing also the performance for a quasi-optimal distribution of degraders. Thus, we found in our experiments that biodegradation performance was at less than 19% of the quasi-optimal performance if diffusion was the sole spatial process that could potentially counteract the disadvantages caused by the spatial separation between bacteria and substrate. This observation emphasizes the big impact of small-scale spatial heterogeneities for contaminant biodegradation.

Effects of the Spatial Degrader Distribution on the Response to Varying Osmotic Potentials

We induced osmotic stress down to $\Delta\Psi_o = -1.5$ MPa by adding different concentrations of sodium chloride to our experimental system, comparable to other studies (Holden et al., 1997; Chang et al., 2007). The lowest value was chosen because of its environmental relevance in representing permanent wilting point conditions for many agronomic plants in soil (Harris, 1981). In the homogeneous liquid culture system, biodegradation was reduced at $\Delta\Psi_o = -1$ MPa probably as a result of the reduced population growth rates and extended lag times observed in the growth kinetic experiments

(**Figures 2A,B**). Furthermore, a homogeneous cell distribution causes a high effective exposure of the bacteria to the osmotic stress across the microcosm area. In order to resist exposure to high concentrations of salts, bacteria have evolved stress tolerance mechanisms like the accumulation of osmolytes at concentrations that are proportional to the osmolarity of the medium (Csonka, 1989; Oren, 2001). However, synthesizing osmolytes requires large amounts of energy and thus poses a significant metabolic burden for microorganisms (Oren, 1999). As a consequence, less energy is available for growth explaining the delayed growth as well as the lowered final biomass yields observed with decreasing $\Delta\Psi_o$.

In the scenario allowing only for substrate diffusion (D_{dif}), the changing osmotic potentials had only a minor impact on population growth and biodegradation probably because the diffusion limitation is the major controlling factor masking the physiological limitations imposed by the osmotic stress. However, we assume that the restricted dispersal of the cells also leads to an accumulation in the inoculation point and thus shields the bacteria from the osmotic stress. This is in line with different biofilm studies reporting an increased resistance of aggregated bacteria compared to planktonic states also for osmotic stress (Wai et al., 1998).

Several studies have demonstrated that osmotic stress may affect soil microorganisms by reducing their biomass (Tripathi et al., 2006), amino acid uptake and protein synthesis (Norbeck and Blomberg, 1998), and respiration (Gennari et al., 2007). In addition, also serious consequences for the provision of ecosystem services were reported (Stark and Firestone, 1995). Following up on this, we could show that not only the spatial heterogeneity of the degraders but also their response to osmotic stress (i.e., to a decrease of $\Delta\Psi_o$) has to be taken into account for the natural attenuation capacity of ecosystems.

Effects of Bacterial Dispersal at Different Osmotic Potentials

In the present study, bacterial movement through the agar matrix and along the dispersal networks was found to counteract the disadvantage due to spatial degrader heterogeneity at different osmotic potentials. The counteraction ability was higher in case of glass fiber networks, which had been shown to accelerate bacterial dispersal processes earlier (Banitz et al., 2011a; Worrich et al., 2016). Glass fibers were used to simulate hyphae surrounded by liquid films (Banitz et al., 2011b; Pion et al., 2013a) and to exclude effects of hyphal activities on bacterial growth and nutrition (Furuno et al., 2012; Banitz et al., 2013; Pion et al., 2013b; Schamfuß et al., 2013).

In this study we used the flagellated bacterium *P. putida* KT2440, which can disperse by swimming motility (Dechesne et al., 2010b). We used 0.3% agar in the experiments as this concentration is supposed to facilitate bacterial swimming through water filled channels in the agar matrix (Rashid and Kornberg, 2000). In addition, the movement of bacteria in the liquid films along fungal mycelia was shown to be enabled by flagella, as non-motile bacteria were not dispersed. It remains unclear whether this movement is associated to swimming or swarming motility (Kohlmeier et al., 2005). However, we never observed any cell movement on top of 0.5% agar plates probably because swarming in *P. putida* KT2440 relies on short pili, which are only expressed under specific conditions (Matilla et al., 2007). As the mechanisms underlying bacterial movement in our microcosms were not explicitly studied we referred to it more generally as dispersal.

Bacterial dispersal is considered a key factor for efficient biodegradation in soil (Harms and Wick, 2006; Banitz et al., 2011b) and the advantageous effects were reported several times (Wick et al., 2007; Dechesne et al., 2010a; Worrich et al., 2016). Here, we could show that bacterial dispersal is able to counteract the disadvantages caused by spatial degrader heterogeneity in our microcosm setup. However, at lowered $\Delta\Psi_o$, the benefit of bacterial dispersal in absence of dispersal networks vanished as a consequence of the osmotic stress which was shown to reduce dispersal of the bacterial population (cf. Supplementary Figure 3; Worrich et al., 2016). Bacterial dispersal is a result of growth, passive transport and motility. However, in previous experiments with a non-motile isogenic mutant of *P. putida* KT2440, we observed that growth and passive transport contributed only marginally to dispersal in the microcosms in absence of osmotic stress (Supplementary Videos 1, 2). Thus, it is likely that the reduced colony expansion observed in the experiments at lowered osmotic potentials is caused particularly by a restriction of bacterial motility. The high metabolic costs associated with the survival at low $\Delta\Psi_o$ probably led to a downregulation of motility genes to avoid further energetic disadvantages. Indeed, reduced expression of structural genes involved in flagellum synthesis has been observed for *Pseudomonas*, *Bacillus,* and *Enterobacter* strains under osmotic stress conditions (Soutourina et al., 2001; Kristoffersen et al., 2007). The minor effects shown for the scenario with the immobilized cells (D_{dif}) may thus further be associated with a higher energy status of the cells due to the repression of flagellum-synthesis under conditions leading to immobilization (high agar concentration in our experiments) similar to what was postulated for non-flagellated mutants of *P. putida* KT2440 showing a higher resistance to oxidative stress than the flagellated cells (Martinez-Garcia et al., 2014). At $\Delta\Psi_o = -1.5$ MPa, we observed bacterial dispersal exerting negative effects on benzoate biodegradation (**Figure 3E**). Recently, it was found that the colonization of stress-affected environments requires a critical population density in order to maintain activity in an antibiotic landscape (Hol et al., 2016). Possibly, this was the case also in our system for low $\Delta\Psi_o$ and the primary colonizers leaving the inoculation site were not able to establish, thus reducing the overall activity in the system.

The network-mediated dispersal benefit also gradually decreased with decreasing $\Delta\Psi_o$. However, the presence of dispersal networks always led to improvements of bacterial population growth and biodegradation performance also at low $\Delta\Psi_o$. We hypothesize that the presence of dispersal networks creates a trade-off with a more even bacterial coverage leading to increased substrate access, but simultaneously causing a higher effective exposure to the osmotic stress. Nevertheless, it seems that the network-mediated benefits for bacteria could

compensate the energetic costs of dispersal and hence prevent the downregulation of motility genes. Probably, the potential accumulation of bacteria along the network may decrease the exposure to osmotic stress and helps to exceed the critical density threshold needed for establishment under lowered $\Delta\Psi_o$.

Importance of Spatial Processes at Different Osmotic Potentials

Bacterial dispersal ability was identified to be crucial for the fast removal of the benzoate in our system. Although diffusion could partially secure biodegradation under the compounded effects of spatial degrader heterogeneity and varying $\Delta\Psi_o$, bacterial motility and especially the network-mediated dispersal led to considerably higher performances (**Figure 4B**). At low $\Delta\Psi_o$, not the spatial arrangement of the degrader population but rather the insufficient growth caused by the salt is the bottleneck for biodegradation as shown by the growth kinetics experiment as well as by the analysis of the homogeneous reference scenario D_{hom} at the different $\Delta\Psi_o$. Growth itself was considered as a non-spatial process as it was shown not to account for any significant changes in bacterial spatial dynamics (Pallud et al., 2004).

The influence of many soil physicochemical parameters has been measured in order to assess controlling factors determining biodegradation efficiency (Dechesne et al., 2014). However, only a few covariates have been found which represent good predictors for biodegradation activity including pH (Rodriguez-Cruz et al., 2006; Hussain et al., 2013) and moisture (Cruz et al., 2008; Monard et al., 2012). Soil pH was shown to particularly affect bacterial growth (Bååth and Arnebrant, 1994) whereas soil moisture was shown to particularly affect bacterial motility (Dechesne et al., 2010b). Therefore, as the osmotic potential influences both bacterial growth and dispersal its potential role in determining the spatial distributions and activity should not be neglected.

Relevance for Field Conditions

Land degradation by salts is a major threat in arid and semi-arid regions of the world, which is mainly caused by low rainfall and high evapotranspiration (Rengasamy, 2006). Furthermore, this problem is anticipated to worsen in future due to irrigation and clearing of the native vegetation especially in agricultural lands (Pannell and Ewing, 2006; Rengasamy, 2006). However, the steadily increasing global population necessitates land use changes toward agriculture, which will at the same time also increase the amount of contaminants (primarily pesticides) applied to the soil. Given the fact that soil is characterized by a high spatial heterogeneity of degrading microorganisms, it is necessary to assess how this heterogeneity in combination with increasing salinization will affect contaminant biodegradation. Indeed, this is a rather complex question as the spatial distribution of degrading organisms in the field is not known a priori and, moreover, difficult to assess and control in replicated, comparative experiments (Dechesne et al., 2014). Therefore, we used microcosm experiments, which allow for defined changes of the relevant conditions but do not incorporate the full complexity of the soil environment. These simplifications, however, constitute a prerequisite to better understand the importance of single aspects in natural systems (Drake et al., 1996). Nonetheless, transferring our findings to natural soil systems, one needs to acknowledge that other factors may also influence the processes elucidated in this study. Especially the matric potential, which is the second major determinant of the water potential in terrestrial habitats, is known to adversely affect bacterial dispersal and substrate diffusion processes in soil (Harris, 1981; Dechesne et al., 2008). This was addressed by several studies without consideration of the osmotic potential. However, future studies should focus on the combined effects of osmotic and matric potential, in particular if considering that decreasing water amounts in soils always result in higher salt concentrations (Chowdhury et al., 2011).

Our work demonstrates that it is important to consider the spatial distribution of degraders as a driving factor for biodegradation. Hence, bacterial dispersal and especially bacterial dispersal along fungal mycelia should be facilitated by avoiding (i) desiccation and high evapotranspiration causing low osmotic potentials, and (ii) the energy-intensive mechanical mixing of soil. The latter prevents the mycelium development of fungi (Lamar et al., 1994), which we demonstrated to counteract the disadvantages of a heterogeneous bacterial distribution. Taking into account a length of up to 10^4 m of hyphae per g of soil (Ritz and Young, 2004) and a high tolerance toward low water activities and matric potentials, fungi seem to be a suitable and energy-efficient but yet unexploited alternative to conventional remediation approaches (Harms et al., 2011).

AUTHOR CONTRIBUTIONS

AW designed the research and performed the laboratory work. AW, SK, TB, FC, and MT analyzed the data. SK, TB, FC, KF, MT, HH, AM, LW, and MK provided consultation for the work. AW wrote the manuscript. All authors contributed significantly to the preparation of the manuscript and approve its submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01214

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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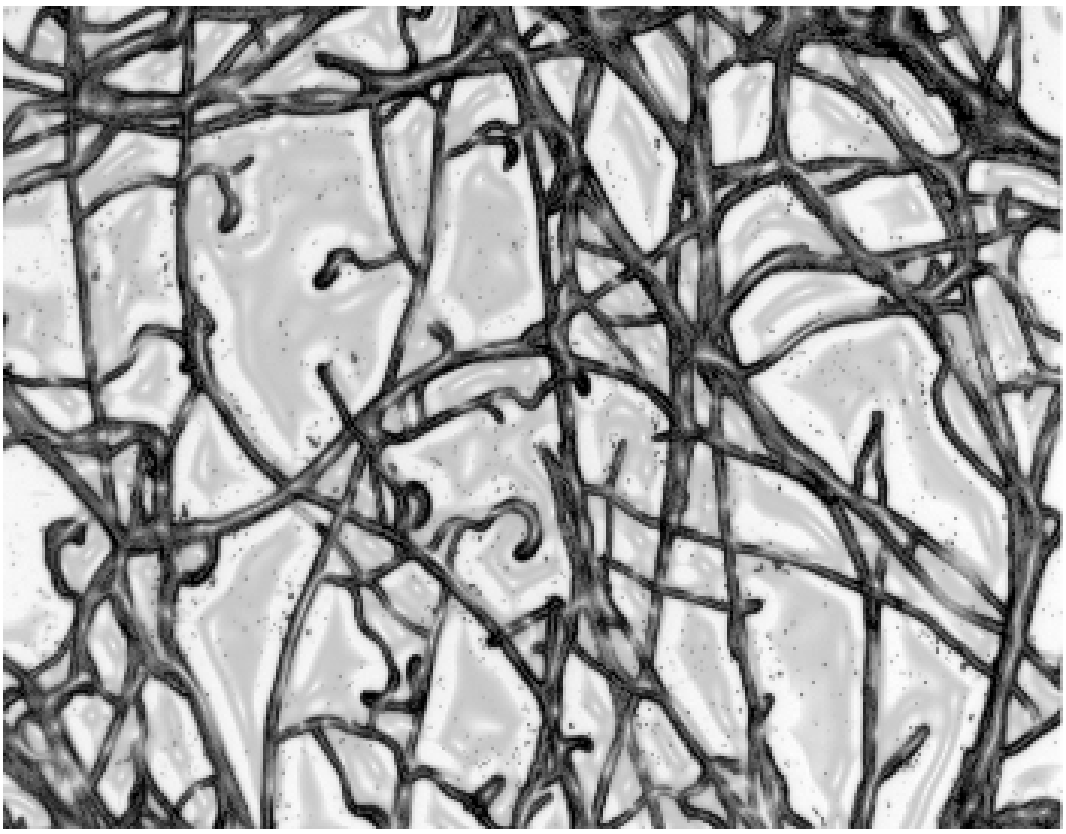
Chapter 5

RESOURCE PROVISIONING BY MYCELIA



Spores of Bacillus subtilis and hyphae of Pythium ultimum

5 RESSOURCE PROVISIONING BY MYCELIA

5.1 Mycelium-mediated Transfer of Water and Nutrients Stimulates Bacterial Activity in Dry and Oligotrophic Environments

Worrich, A., Stryhanyuk, H., Musat, N., König, S., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Richnow, HH., Miltner, A., Kästner, M., Wick, L.Y. Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments. Submitted

The manuscript in its current version is a invited revision for Nature Communications.

Significance: Although forming a major part of the soil microbial biomass interactions of bacteria and fungi under environmental stress scenarios still remain elusive. In the present study, we constructed a synthetic microbial ecosystem using the germination of *Bacillus subtilis* spores to examine whether mycelia reduce bacterial water and nutrient stress in an otherwise dry and oligotrophic microhabitat. We found that mycelia enabled germination of bacterial spores and promoted bacterial activity in dry and oligotrophic environments by providing nitrogen, carbon and water to bacteria. Considering the ubiquity of fungi and their interactions with bacteria, our finding emphasizes the importance of mycelial structures for bacterial adaptation and ecosystem functions and services in disturbed habitats.

Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments

Anja Worrich[1,3] , Hryhoriy Stryhanyuk[2], Niculina Musat[2,*], Sara König[3,4], Thomas Banitz[4], Florian Centler[3], Karin Frank[4,5,6], Martin Thullner[3], Hauke Harms[3,5], Hans-Hermann Richnow[2], Anja Miltner[1], Matthias Kästner[1,§], Lukas Y Wick[3,§]

[1]UFZ - Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany, Department of Environmental Biotechnology; [2]UFZ - Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany, Department of Isotope Biogeochemistry; [3]UFZ - Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany, Department of Environmental Microbiology; [4]UFZ - Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany, Department of Ecological Modelling; [5]German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, D-04103 Leipzig, Germany; [6]University of Osnabrück, Institute for Environmental Systems Research, Barbarastaße 12, 49076 Osnabrück, Germany

*Address correspondence to Niculina Musat, niculina.musat@ufz.de

§ contributed equally

Abstract

Fungal-bacterial interactions are highly diverse and contribute to many ecosystem processes. Their emergence under common environmental stress scenarios however, remains elusive. Here we use a synthetic microbial ecosystem based on the germination of *Bacillus subtilis* spores to examine whether mycelia reduce bacterial water and nutrient stress in an otherwise dry and oligotrophic microhabitat. We find that the presence of mycelia enables the germination and subsequent growth of bacterial spores near the hyphae. Using a unique combination of Time of Flight- and nanoscale Secondary Ion Mass Spectrometry (ToF- and nanoSIMS) coupled with stable isotope labeling, we link spore germination to hyphal transfer of water, carbon and nitrogen. Our study provides for the first time direct experimental evidence for the stimulation of bacterial activity by mycelial supply of scarce resources in dry and nutrient-free environments. We propose that mycelia may stimulate bacterial activity and thus contribute to sustain ecosystem functioning in stressed habitats.

Fungi and bacteria co-inhabit a wide variety of environments[1,2] and in their interactions are significant drivers of important ecosystem functions and services[3]. In nature, they live in habitats exposed to fluctuating environmental conditions and frequently underlie strong selection pressure by limiting resources or drought[4]. To survive periods of stress bacteria enter reversible states of low metabolic activity or dormancy[10]. Dormancy transiently disables bacterial functions while generating a seed bank of resting bacteria with the potential to be resuscitated in response to favorable environmental changes[5,10]. Fungi, by contrast, have been shown to possess higher resistance to drought and nutrient limitation due to efficient resource translocation between spatially separated source and sink regions in their mycelia[6,11-13]. Moreover, the redistribution of water in fungal mycelia was shown to enhance the ecosystemic resistance by preserving fungal carbon mineralization during drought[14]. While recent data suggest that mycelia create hospitable microhabitats for bacteria due to the exudation of carbonaceous compounds[2,15,16] and a moistening of the surrounding substrate[14], experimental evidence for a for a stimulation of bacterial activity in harsh environments is lacking.

During the past decade, powerful methods have been developed for the investigation of microbial interactions down to the single-cell scale[17,18]. Numerous studies revealed that resource exchange is a successful strategy in microbial communities to cope with unfavorable environmental conditions[19-22]. However, the role of resource transfer processes from fungi to bacteria for the maintenance of bacterial activity in dry and oligotrophic habitats remains to be elucidated. In the present study, we use a synthetic microbial ecosystem to examine if mycelia of fungi and oomycetes reduce water and nutrient stress for bacteria and thus enable bacterial activity in otherwise dry and oligotrophic environments. We introduced spores of the soil-bacterium *Bacillus subtilis*

into a water- and nutrient-free area and several hyphal model organisms, growing on a physically separated water and nutrient-rich agar piece, were allowed to overgrow the spore-bearing region. We find that the presence of mycelia enables both the germination of bacterial spores as well as vegetative growth near the hyphae. Spatially resolved secondary ion mass spectrometry in combination with stable isotope labeling reveals a long distance transport of labeled compounds in mycelia and a supply of water, carbon and nitrogen to the cells of *B. subtilis* located close to the hyphae. Spores more distant from the mycelium remain dormant and show no enrichment of the labeled substrates. Our results demonstrate that mycelium-forming fungi and oomycetes facilitate bacterial activity in dry and oligotrophic environments by providing nitrogen, carbon and water to bacteria and thus improving their habitat conditions.

Results

Effects of mycelia on spore germination and growth of *B. subtilis*. We developed a synthetic microbial ecosystem to assess whether fungal-bacterial interactions will emerge in presence of drought and nutrient limitation stress conditions. Specifically, we tested to what degree the presence of mycelia may evoke beneficial habitat conditions for bacterial activity and growth. To this end, we placed spores of *B. subtilis* on silicon wafers (Fig. 1a) and analyzed their germination and growth as an indicator for access to water and nutrients. The ascomycete *Fusarium oxysporum*, the basidiomycete *Lyophyllum* sp. Karsten, and the mycelium-forming oomycete *Pythium ultimum* were inoculated to a water and nutrient-rich agar patch physically separated from the spore-bearing silicon wafer (Fig. 1a). The mycelium overgrew the wafer within two (*P. ultimum*), four (*F. oxysporum*) and five days (*Lyophyllum* sp. Karsten) in direction of a second nutrient and water reservoir at the opposite side of the wafer. Subsequently, all biomass was detached from the wafer and the abundance of *B. subtilis* cells in presence and absence of mycelia determined by counting total colony forming units (CFU; Fig. 2a). The number of total CFU detached from mycelia-free controls ($(1.0 \pm 0.5) \cdot 10^5$ CFU) corresponded to the number of spores applied ($(1.0 \pm 0.3) \cdot 10^5$ CFU) and excluded harmful wafer effects on spore germination (Fig. 2b). When hyphae overgrew the spore region, however, the total average CFU numbers increased (Fig. 2b), indicating that mycelia supported growth of bacteria in dry and nutrient-free regions on the wafer. The highest CFU numbers of *B. subtilis* were observed in presence of the oomycete *P. ultimum* ($(3.5 \pm 1.8)\ 10^5$ CFU), followed by *Lyophyllum* sp. Karsten ($(2.8 \pm 0.6) \cdot 10^5$ CFU) and *F. oxysporum* ($(2.7 \pm 0.7) \cdot 10^5$ CFU). Furthermore, we analyzed the number of spores to obtain information on the

fraction of sporulated and germinated cells relative to total cell numbers (Fig. 2a). In the control, we found that 100 ± 6 % of the total CFU remained in form of spores on the wafers. However, on *P. ultimum* wafers only 10 ± 2 % of the total cells were dormant (Fig. 2c). For *F. oxysporum* and *Lyophyllum* sp. Karsten the fraction of spores was higher (34 ± 3 % and 24 ± 3 %, respectively), but vegetative cells still accounted for the majority of the total CFU (Fig. 2c). Microscopic analyses revealed the presence of vegetative *B. subtilis* cells always in close vicinity to the hyphae of *P. ultimum* (Fig.1b, blue arrows). At locations more distant to hyphae however, *B. subtilis* persisted in the spore form (Fig.1b, red arrow).

Chemical mapping of *B. subtilis* and *P. ultimum* using ToF-SIMS. ToF-SIMS analyses without prior isotope labeling were used to map the structural arrangement and elemental composition of the mycelium, vegetative bacterial cells and bacterial spores directly on the silicon wafer without previous isolation. This prevented us from losing spatial and chemical information typically occurring during sample preparation in SIMS experiments[23]. The mass peaks corresponding to O^-, OH^-, PO_2^-, CH^-, CN^- and S^- secondary ions were selected to represent the variance in composition of the biomass and its extracellular environment. In order to reduce the intensity modulation due to topography and effects related to the density of the sample, we normalized the intensity in respective ion yield distribution maps to total ion counts. As in microscopy the ToF-SIMS analysis showed that vegetative cells of *B. subtilis* were located in close vicinity to the hyphae, whereas the spores of *B. subtilis* were randomly distributed regardless of the presence of hyphae (Fig. 3). The presence of *B. subtilis* spores, which are known to be rich in sulfur compounds[24], were observed by bright spots in the normalized intensity maps of O^- and S^-

96 species (Fig. 3 a,f) and vegetative cells were detected by their enhanced intensity of OH^-
97 ions (Fig. 3b), respectively. The PO_x^- and CH^- ion signal intensities however, could not be
98 used for detailed biomass analysis, as a diffuse, strong PO_2^- (Fig. 3c) and CH^- (Fig. 3d) ion
99 yield, distributed over the whole field of analysis, precluded clear discrimination between
100 hyphae, spores and vegetative cells and point at the presence of extracellular material,
101 possibly due to excretion, lysis of hyphae or damaged bacterial cells. By contrast, high
102 secondary ion yields for CN^- (Fig. 3e) reflected locally distinct protein contents and likely
103 revealed locations of vegetative bacterial cells (white arrows) and residual hyphal
104 fragments.

105 **NanoSIMS analysis of water and nutrient transfer.** Building on the ToF-SIMS chemical
106 mapping, we used nanoSIMS and full isotope labeling approaches to obtain quantitative
107 information on the nutrient and water transfer from the hyphae of *P. ultimum* to spores and
108 vegetative cells of *B. subtilis*, respectively. The distribution of ^{18}O, ^{13}C and ^{15}N in hyphae,
109 and vegetative cells and spores was quantified after the mycelium of *P. ultimum* had
110 overgrown the spore-bearing wafer (Figs. 4 and 5). As joint full replacement of water and
111 nutrients with isotopically labeled homologues retarded growth of *P. ultimum*, we carried
112 out parallel experiments either with ^{18}O-labeled water or with a combination of ^{13}C-
113 glucose and ^{15}N-ammonium sulfate, respectively. Thereby, growth delay of the mycelium
114 could be prevented and experiments could be sampled after a similar incubation time as in
115 the experiments without isotope replacement. The localization of the biomass and its
116 integrity was assessed by $^{12}C^{14}N^-$ detection in two different fields of analysis (Fig. 4a,c).
117 Both, vegetative cells and spores consistently provided strong $^{12}C^{14}N^-$ signals, whereas
118 hyphae of *P. ultimum* were found to be intact only in one of the two observation fields

studied (Fig. 4a,c). As for microscopy and ToF-SIMS vegetative cells of *B. subtilis* were either observed in close distance to the intact hyphae or lysed hyphal biomass. Strikingly, vegetative cells were consistently positioned longitudinally along the hyphae, forming a regular stratification typical for *B. subtilis* biofilms[25] (Fig. 4c). Due to entire ^{12}C and ^{14}N replacement by their corresponding stable isotopes neither the hyphae nor the hyphae-attached vegetative cells were detected by the $^{12}C^{14}N^-$ signal in ^{13}C and ^{15}N labeling experiments. High $^{12}C^{14}N^-$ signals, however, were seen in spores distant from the hyphae as was further confirmed by the secondary electron image (Fig. 5a).

Fungal biomass was found to be uniformly enriched in ^{18}O except for small holes probably arising from ongoing hyphal disintegration (Fig. 4b). Vegetative cells in close vicinity to the hyphae showed higher enrichment in ^{18}O ($2.8 - 8.9$ %) than spores located at longer distance to hyphae ($1. - 3.1$ %; Fig. 6a) suggesting ^{18}O transfer from the hyphae to vegetative cells. Compared to intact hyphae ($APE = 12.1 \pm 0.0$ %), ^{18}O enrichment of *B. subtilis* cells however, was lower. This is presumably due to the dilution of the signal attributed to the spores' own oxygen-containing molecules or the core water (which can account for up to 27 – 55 % of the core wet weight[26]). Enrichment levels of ^{18}O in spores was higher in the ^{18}O labeling experiments compared to non-labeled samples (Figure 6a; Supplementary Fig. 1). Thus, some transport of water must have occurred via the gas phase. The transfer of carbon and nitrogen containing nutrients from hyphae to the bacterial cells was underpinned by significant ^{13}C and ^{15}N enrichment in hyphae and vegetative cells vicinal to hyphae yet not in spores more distant to hyphae (Fig. 5b,c). Relative to the ^{15}N and ^{13}C enrichment of the hyphae (^{15}N: 37.2 ± 0.0 % and ^{13}C: 34.6 ± 0.0 %) , vegetative bacterial cells were more enriched in ^{15}N ($38.3 - 70.1$ %) than in ^{13}C

(20.1 – 47.1 %) indicating both a lower dilution by spore-inherent nitrogen compounds and a higher nitrogen demand by germinating spores and growing cells of *B. subtilis*, respectively. By contrast, spores were ^{13}C and ^{15}N enriched by 1.2 – 4.8 % and 0.6 – 5.1 %, respectively, and showed C and N isotope abundances comparable to non-labeled samples (Fig. 6b,c; Supplementary Fig. 2). The ^{18}O and the joint ^{13}C and ^{15}N enrichments, hence, provided clear evidence of transfer of water and nutrients from hyphae to bacterial cells and the importance of the relative spatial organization of spores and hyphae on the wafer surface, respectively. The low levels of stable isotope incorporation into bacterial spores distant to the hyphae and the observation that vegetative cells were solely found in close proximity to hyphae indicate the negligible role of air-borne transfer of the isotope labels (e.g. by humidity or fungal volatiles) to the spores.

Discussion

By verifying hyphal uptake, translocation and transfer of ^{13}C, ^{15}N and ^{18}O labels to subsequently germinating *Bacillus* spores, our results demonstrate that mycelia enable bacterial activity in regions devoid of water and nutrients. Utilizing a synthetic microbial ecosystem and a novel combination of ToF- and nanoSIMS quantitative imaging of single-cell trophic interactions we showed that i) the presence of fungi and oomycetes in an initially dry and nutrient-free environment induced the germination of metabolically inactive spores of *B. subtilis* to actively growing cells, ii) only spores in close vicinity to the hyphae could germinate, and iii) a transfer of nutrients and water from hyphae to the bacterial cells underlay this activation. Thus, our study provides for the first time direct experimental evidence for stimulation of bacterial activity by mycelial supply of scarce resources in dry and nutrient-free environments.

Within microbial communities competitive and cooperative interactions are often related to the excretion and uptake of metabolites from involved partners[20,27,28]. It is widely assumed that fungal exudates are a source of nutrients for bacteria in the mycosphere including organic acids[29], sugars, polyols[30], and amino acids[30]. In addition to known germinants such as amino acids and sugars[31], the availability of water is required for spore germination. Water uptake by the spore enables the hydration of the spore core and represents a crucial step in the germination process[31]. Recently, fungal hyphae were shown to redistribute water and thereby compensate differences in the soil matric potential[14]. Although the authors provided evidence that such water redistribution drastically increased the functional ecosystemic stability under drought, the consequences of this underrated pathway for other soil organisms remained unexplored. Our results suggest that hyphal water translocation may not only provide fungi with a higher resistance to drought, but also facilitates bacterial activity in close proximity to the hyphae. Especially at low soil water potentials, the mycosphere may thus form a hotspot of microbial activity and significantly foster the maintenance of important ecosystem functions and services.

Three different strategies are described by which bacteria may derive nutrition from fungi: necrotrophy, extracellular biotrophy and endocellular biotrophy. In necrotrophic interactions, bacteria secrete molecules that kill the fungus and thus induce a release of nutrients. Extracellular biotrophs live in close proximity and use nutrients exuded from the hyphae, whereas endocellular biotrophs live inside the fungus and take up nutrients from the cytoplasm[32]. In accordance with existing literature, we never observed any endocellular occurance of *B. subtilis.* Hence, we consider endocellular biotrophy to be rather unlikely. Indeed, vegetative cells were always located tightly arranged in a longitudinal orientation along the hyphae resulting in maximum contact surface between the bacterial cell and the

189 hyphae. This strongly indicates extracellular biotrophy based on the consumption of fungal
190 exudates[32]. The regular cell chains observed along the hyphae (Fig. 5a) most probably
191 arose from subsequent growth of the cells leading to a colonization of the hyphae as
192 usually observed for the interaction of *B. subtilis* with plant roots[33]. In agriculture *B.*
193 *subtilis* strains are routinely applied to soil because their biocontrol activity was shown to
194 protect plants against fungal infestation[34]. Active killing of fungal hyphae in necrotrophic
195 interactions was described for different *Bacillus* strains[35,36] and most likely induced the
196 disintegration of the hyphae observed in the course of our SIMS experiments. However,
197 before germination the metabolically inactive spores cannot have evoked killing of the
198 hyphae. Moreover, we observed vegetative cells along intact hyphae of *P. ultimum* still
199 containing intact nuclei (Fig. 1c). This demonstrates that neither necrotrophy nor nutrients
200 leaking from dead hyphal cells can be solely responsible for the stimulation of bacterial
201 activity in the vicinity of mycelia. The dry conditions on the wafer strongly restricted the
202 diffusion of the exudates and thus only spores in close contact to the mycelium germinated.
203 Indeed, already at distances of more than 1.7 µm spores did not germinate (cf. Fig. 5a).
204 Thus, close proximity between the mycelium and the bacterial cells constituted a
205 prerequisite for diffusion-based transfer of resources as observed in different studies on
206 nutrient exchange[37] or chemical communication[38] in microbial communities. However,
207 under more humid conditions in soil, exudate diffusion probably extends beyond the direct
208 vicinity of the hyphae.

209 Our observation that fungi support the growth of bacteria is in accordance with
210 studies that showed an increased number of culturable bacteria in the vicinity of different
211 fungal hyphae in soil microcosms[15]. Given the evidence that bacteria are attracted to the
212 mycosphere[39], it is tempting to speculate that the local conditions in these regions are often

favorable for bacterial activity. Fungal mycelia were shown to change local habitat conditions and thereby affect bacteria by changing the pH, the soil structure or by secreting inhibitory or stimulatory compounds[40]. Thereby they create, modify or destroy habitats and thus pursue 'ecosystem engineering', which may influence the ecological success of other species[41]. In our experiments, representatives of different fungal phyla as well as a mycelium-forming oomycete could induce the germination and growth of *B. subtilis* by the supply of sufficient amounts of water and nutrients. The differences observed for total CFU and the amount of germinated cells may be explained by the differences in cell wall composition of oomycetes and fungi. The lack of chitin in *P. ultimum* might increase the leakiness of the mycelium. Furthermore, the tested mycelial organisms will likely exhibit differential growth on the wafers thus evoking differences in the relative coverage of the wafer with mycelium. Although the combination of the selected organisms may be artificial, we observed similar net effects for all three mycelial organisms likely indicating widespread nutrient and water transfer from mycelia to bacterial cells and a mechanism that may also expand to other types of biological material exuding compounds into the environment (e.g. plant roots). Moreover, the emergence of the interaction under the extremely harsh environmental conditions prevailing in the synthetic microcosms strongly suggests that the observed phenomenon could also arise under a wide range of less extreme conditions found in natural systems. The finding that mycelia facilitate bacterial activity by multiple resource supply may help to further understand the small-scale dynamics of bacterial activity in heterogeneous ecosystems exposed to fluctuating environmental conditions. The ability of mycelia to redistribute and transfer resources allows bacteria to occupy ecological niches which would otherwise be uninhabitable to them. For assessing the dynamics and stability of microbial ecosystem functioning, fungi and bacteria need to

237 be considered as interconnected entities rather than autonomously operating components of
238 the ecosystem.

Materials and Methods

Microcosm setup. Experiments were carried out in laboratory microcosms mimicking water and nutrient-rich (w/) and depleted (w/o) zones (Fig. 1a). To create w/ zones, two agar plugs (Ø 1 cm) were cut out from an FAB-medium agar plate (medium composition adapted from [42]) supplemented with 5 mM glucose as sole carbon source. These two plugs were placed in a petri dish (Ø 6 cm) at a distance of 1.4 cm from each other. Between the two plugs, a silicon wafer (Plano; Wetzlar, Germany) was placed representing the w/o zone. This wafer served as a carrier for spores of *Bacillus subtilis*, which were used as an indicator of changes in water and nutrient availability. Prior to its use, the wafer was cleaned with acetone, 70 % ethanol and finally with autoclaved water and dried under a laminar flow box. Spore suspensions were bought from Merck (Darmstadt, Germany) and controlled microscopically for the absence of vegetative cells before use. Spores were washed three times with sterile-filtered, double-distilled water and in each washing step the supernatant was separated from the spores by centrifugation at 10000 g for 10 min. Subsequently, spores were resuspended in bidistilled water at a final concentration of $2.5 \cdot 10^6$ spores ml^{-1} and 40 µl of the suspension were applied the wafer. Wafers were dried in an oven for 30 min at 70 °C.

Quantification of total cells and spores. The effects of mycelia in dry and oligotrophic environments on the overall cell number and the abundance of spores were quantified using the fungi *Fusarium oxysporum* or *Lyophyllum* sp. Karsten, or the oomycete *Pythium ultimum*. Therefore, a small amount of agar covered with mycelium from freshly overgrown FAB-glucose agar plates was cut out and transferred onto one of the agar plugs in the microcosms (Fig. 1a). Microcosms were sealed with parafilm, placed in a plastic

container and incubated at 25 °C in the dark. The experiments were run in triplicates and control wafers were included. The control wafers were also inoculated with the spore suspension and placed within the microcosms but close to the edge of each petri dish to avoid contact with the mycelium. This should guarantee that all samples were exposed to the same water vapor pressure in the microcosm atmosphere. Mycelium-overgrown wafers and controls were excised from the microcosms after 2 (*P. ultimum*), 4 (*F. oxysporum*) and 5 (*Lyophyllum* sp. Karsten) days and transferred to glass tubes containing 0.5 ml 1x PBS buffer (10 mM, pH 7). Cell detachment was carried out by vortexing the tubes for 1 min. Immediately, 400 µl were pipetted into new glass tubes and heated to 80 °C for 20 min to kill the vegetative cells[43]. Colony forming units in the heat-treated and untreated suspensions were counted using the drop plate method as described earlier[44]. Briefly, 10-fold dilution series of the supernatant were prepared directly in 96-well microtiter plates and five microliters of consecutive dilutions were dropped on FAB-glucose agar plates containing 0.2 % cycloheximid to suppress growth of *P. ultimum*. Plates were incubated at 25 °C for 48 h. Droplets giving rise to 5 - 30 single colonies were used to determine CFU numbers.

Visualization of spore germination in presence of mycelia. Setups with *P. ultimum* were used to visualize germination of spores of *B. subtilis* in water and nutrient-free regions of the wafer. After the mycelium had overgrown the wafers, hyphae around the wafers were cut and the wafers were directly observed under the microscope (Axio Imager Z2, Carl Zeiss) using bright field illumination. Pictures were aquired with a Zeiss Axiocam 506 color camera. To prove hyphae disintegration, samples were also stained with 4-6-diamidino-2-phenylindole (DAPI)–amended mountant containing 9 parts Citifluor

mountant (Citifluor, Leicester, United Kingdom), 1 part PBS (1x), and 1 µg ml^{-1} DAPI. Epifluorescence images were aquired using DAPI filter settings.

ToF-SIMS analysis. In preparation for the envisaged nanoSIMS analysis a boron-doped silicon wafer overgrown with *P. ultimum* was analyzed via Time of Flight Secondary Ion Mass Spectrometry technique (ToF-SIMS) employing a ToF-SIMS.5 (ION-TOF GmbH, Münster) instrument. The conditions of the ToF-SIMS experiment allowed for a detection of atomic and molecular secondary ions with m/z (mass to charge) ratio from 1 to about 500. The broad-range mass spectrum (1-500 amu) acquired for each raster point enabled us to compare the yield of different secondary ion species extracted from the sample. Detailed conditions of the analysis are specified in Supplementary Methods 1. The lateral distribution of ion yield was analyzed using the proprietary ION-TOF SurfaceLab 6.5 software. The accumulation of acquired scans was done after lateral drift correction and the resulted total stack was analyzed for the lateral distribution of the ion yield. The ToF-SIMS images produced were normalized by the total ion image.

Analysis of water and nutrient transfer. ^{13}C-labeled glucose (99 atom %; Euriso-Top, Saarbrücken, Germany), ^{15}N-labeled ammonium sulfate (99 atom %, Sigma, Munich, Germany) and ^{18}O-labeled water (97 atom %; Campro Scientific, Berlin, Germany) were used for the labeling experiment. For the experiments with the labeled water, FAB-agar plates with 5 mM glucose were dried completely in an oven at 60 °C until only an agar foil was left. Thirty mg of this foil were transferred into an 5 ml Eppendorf tube and 2 ml of the labeled water were added. For the ^{13}C and ^{15}N labeling experiments FAB-agar plates without glucose and ammonium sulfate were prepared, dried and again 30 mg were transferred to a 5 ml Eppendorff tube. Subsequently, 2 mg of ^{13}C labeled glucose, 4 mg of

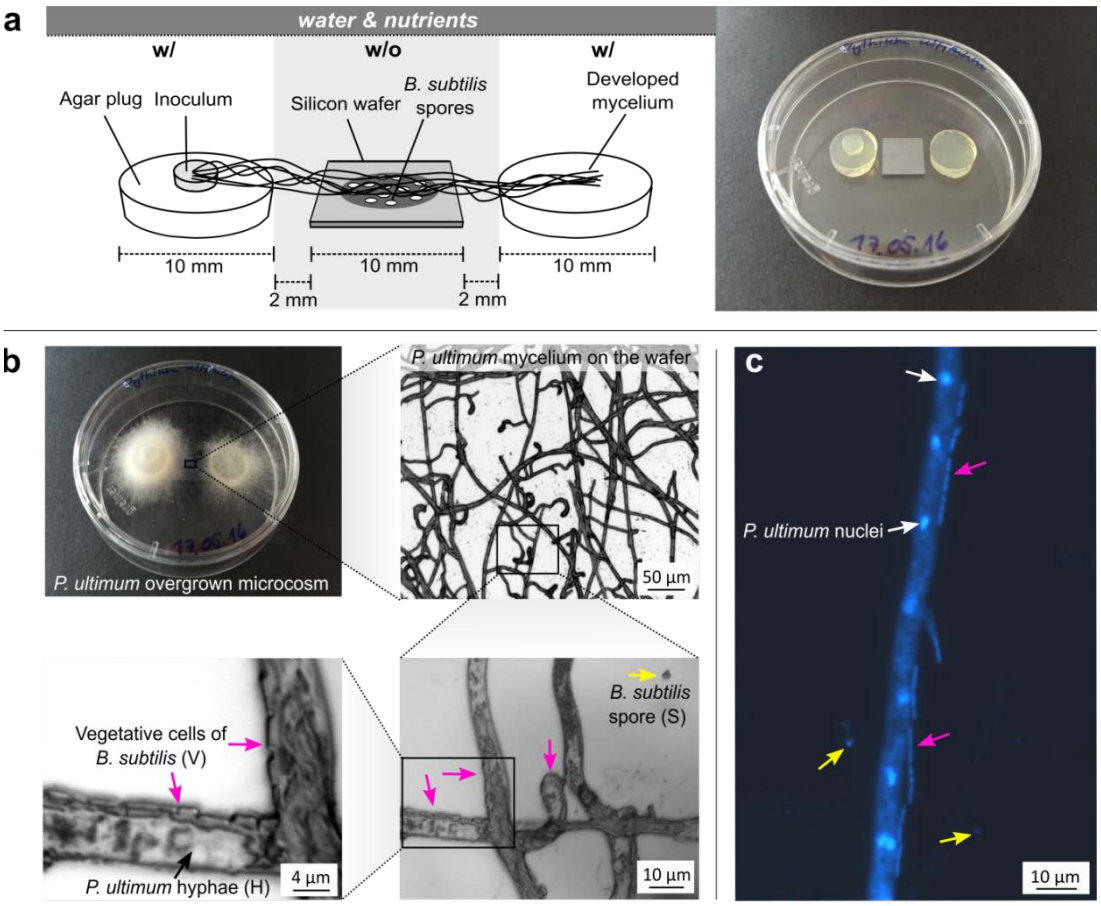
^{15}N labeled ammoniumsulfate and 2 ml autoclaved bidistilled water were added. The tube contents were incubated at 95 °C with continuous shaking at 700 rpm until the agar was completely dissolved. Subsequently, two droplets of 400 µl were introduced into the petri dishes at a distance of 1.4 cm from each other and dried for 3 min under the laminar flow box until it formed hard agar blocks. The spores to serve as inoculum were prepared as described above. For the nanoSIMS experiment a conductive chromium-coated, round-shaped silicon wafer (10 mm diameter) was used as a spore carrier. Experiments were carried out using *P. ultimum* and inoculation was conducted as described above. Each labeling experiment was performed in duplicates. After 48 h, the wafers were removed from the microcosms and the biomass was fixed in an atmosphere over a solution of a 10 % paraformaldehyde and 37 % ethanol at 30 °C for 2 h. Wafers were stored in a vacuum box until analysis. Parallel control experiments (n=2) were performed with non-labeled compounds to assess the natural isotopic composition of the samples.

NanoSIMS analysis. The wafers were analyzed with a nanoSIMS-50L instrument (Cameca, Gennevilliers Cedex, France) to assess the transfer of the stable isotopes from the hyphae to the bacterial cells. Depending on the arrangement of hyphae, vegetative cells and spores, fields of 20 x 20, 30 x 30 and 40 x 40 µm were selected. Measurements were performed in negative extraction mode employing a DC source of primary Cs^+ ions. Detailed information on the analytical conditions for the measurements are described in Supplementary Methods 2.. The $^{12}C^-$, $^{13}C^-$, $^{16}O^-$, $^{18}O^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, $^{13}C^{14}N^-$ and $^{32}S^-$ secondary ion species were collected at a mass resolution sufficient to separate the $^{12}C^{15}N^-$ from the $^{13}C^{14}N^-$. Images and data were processed using the Look@NanoSIMS software (*see SI*). Quantification of the atomic percent enrichment in ^{18}O, ^{13}C and ^{15}N for hyphae,

331 vegetative cells and spores was carried out by defining regions of interest around
332 individual cells using the secondary electron images. The number of analysed fields and
333 replicate wafers for each labeling experiment as well as the numbers of individual
334 vegetative cells and spores used to quantify APE is specified in Supplementary Table 1.

335 **Data availability.** The data that support the findings of this study are available from the
336 corresponding author upon reasonable request.

337 # Figures



338

339 **Figure 1: Synthetic microbial ecosystem revealed spore germination in presence of**
340 **mycelia in dry and oligotrophic environments.** a) Scheme and photographs of the setup
341 employed to carry out the germination, growth and labeling experiments. The synthetic
342 ecosystem is comprised of two agar plugs serving as water and nutrient sources (w/) for the
343 fungi or the oomycete inoculated on top of one of the agar plugs. A silicon wafer free of
344 water and nutrients (w/o) placed in the middle between the two w/ zones served as carrier
345 for spores of *B. subtilis*. An air gap between the w/ and w/o zone prevented the diffusion of
346 water or substrates to the spore region. b) Gradual enlargement of bright-field micrographs
347 of the silicon wafer overgrown by mycelium of *P. ultimum*. In close vicinity to the hyphae

348 (black arrow) rod-shaped, vegetative bacterial cells (magenta arrows) were found, whereas
349 smaller, round-shaped spores (yellow arrows) were located more distantly. c) Fluorescence
350 micrograph of the 4′,6-diamidine-2-phenylindol (DAPI)-stained wafer showing *P. ultimum*
351 hyphae containing nuclei (white arrows) and vegetative cells as well as spores of *B.*
352 *subtilis.*

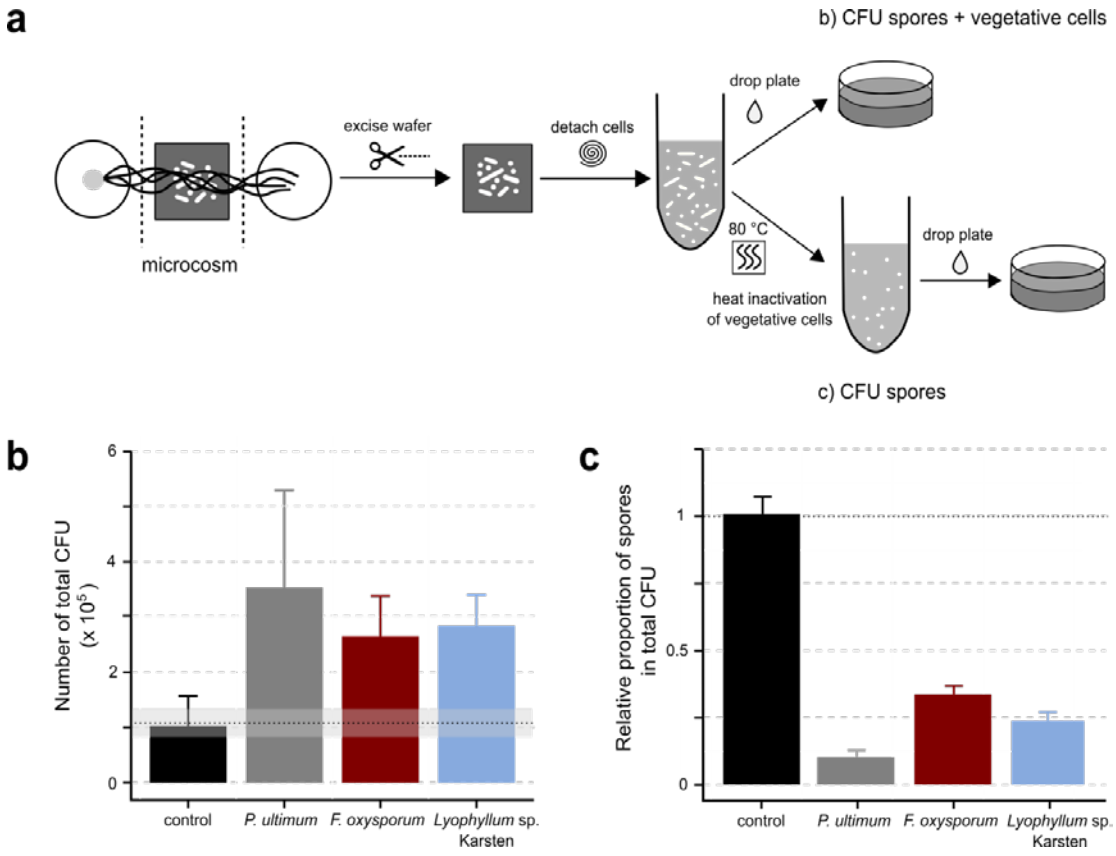


Figure 2: The presence of mycelium enables bacterial growth and spore germination. a) Scheme of the experimental procedure providing information about vegetative growth and germination in presence of mycelium. Total cell number was determined as CFU after cell detachment from the wafer and plating on agar. Spores were obtained by counting CFU after heat-inactivation of the vegetative cells. b) CFU of *B. subtilis* after detachment from the control wafer (no contact to mycelium) and wafers overgrown by *P. ultimum, F. oxysporum* or *Lyophyllum* sp. Karsten. The dashed line shows the number of CFU applied to the wafer with the inoculum. In presence of mycelium, the number of total CFU increased compared to the control. Bars show the average number of CFU and error bars indicate the standard deviation. c) Respective proportions of *B. subtilis* spores, calculated by dividing the number of spore CFU by the total cell CFU determined in b). The columns

366 show the average of the quotients for the three replicates and the standard deviation. The
367 number of spores was different from the number of total cells for the respective strain but
368 did not differ for the control.

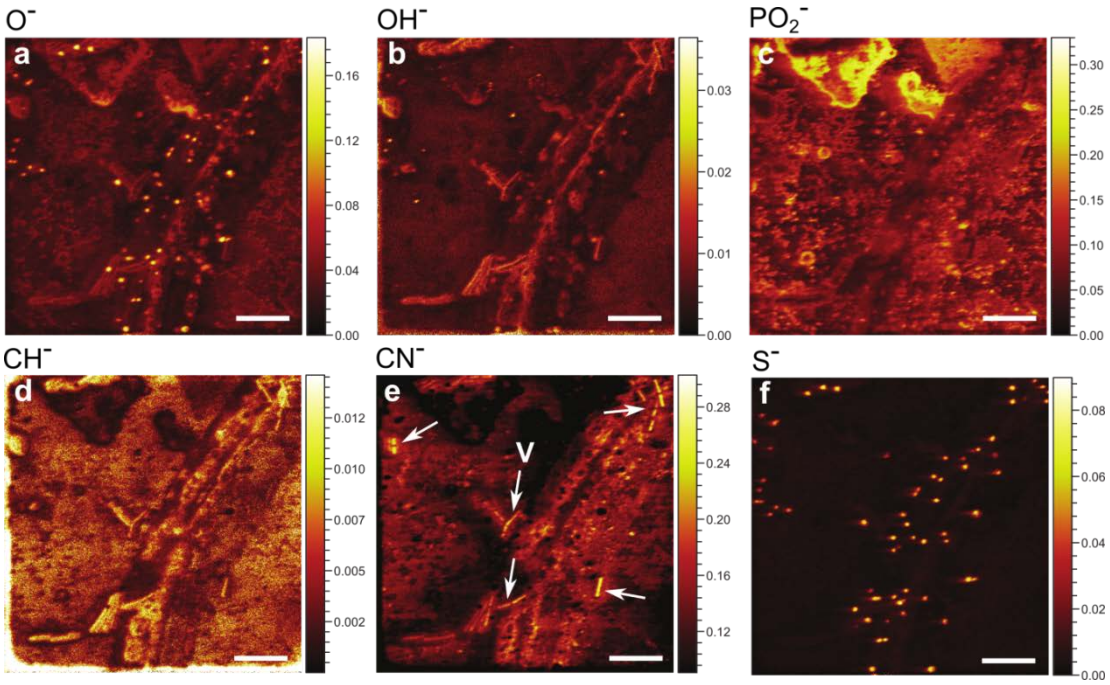
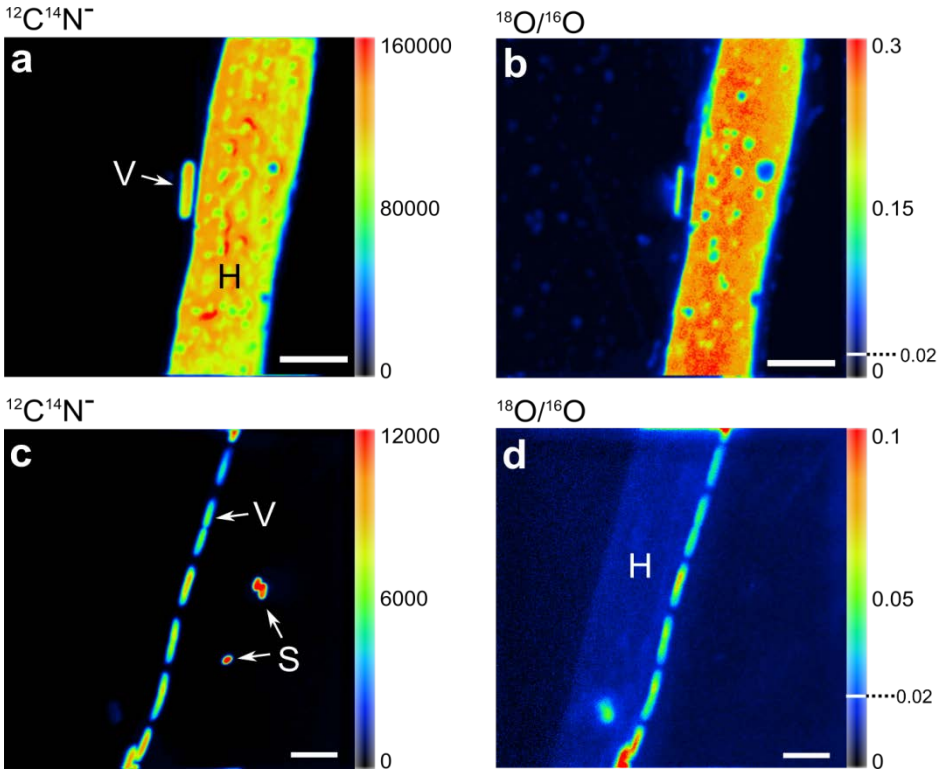


Figure 3: ToF-SIMS reveals sample composition via yield of secondary ion species. Mass-resolved chemical map of *P. ultimum* hyphae as well as vegetative cells and spores of *B. subtilis* on top of the silicon wafer. Six negative secondary ion species were detected (a – f) in a 56×56 µm sample area. ToF-SIMS images produced were normalized by the total ion counts. Legends indicate the relative ion counts for each secondary ion species. Arrows in e) point to vegetative cells (V). Scale bars, 10 µm.



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377 **Figure 4: Water is transferred from hyphae to bacterial cells in dependence of the**
378 **spatial organization.** NanoSIMS images of *P. ultimum* hyphae (H), *B. subtilis* spores (S)
379 and vegetative cells (V) identified in the total biomass ($^{12}C^{14}N$) images (a, c) of ^{18}O-
380 enriched samples. The ratio images of $^{18}O/^{16}O$ show the incorporation of label from water
381 from the w/ zones into the biomass of *P. ultimum* and *B. subtilis* (b, d). Dashed lines
382 indicate natural abundance of ^{18}O. Images represent different fields of analysis
383 corresponding to sample areas of 20×20 (a,b) and 30×30 µm (c,d). Scale bars, 4 µm.

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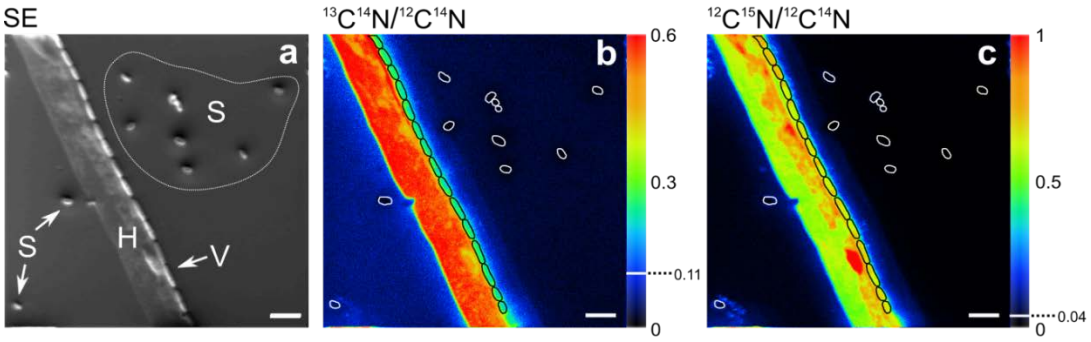

Figure 5: Bacterial cells in close proximity to the hyphae receive carbon and nitrogen. NanoSIMS images of *P. ultimum* hyphae (H), *B. subtilis* spores (S) and vegetative cells (V) identified in the secondary electron (SE) image (a) of ^{13}C and ^{15}N -enriched samples. The ratio images of $^{13}C^{14}N/^{12}C^{14}N$ (b) and $^{12}C^{15}N/^{12}C^{14}N$ (c) show the incorporation of label into the biomass of *P. ultimum* and *B. subtilis*. Images represent a field of analysis corresponding to a sample area of 40×40 µm. Vegetative cells and spores are outlined in black and white, respectively. Scale bars, 4 µm.

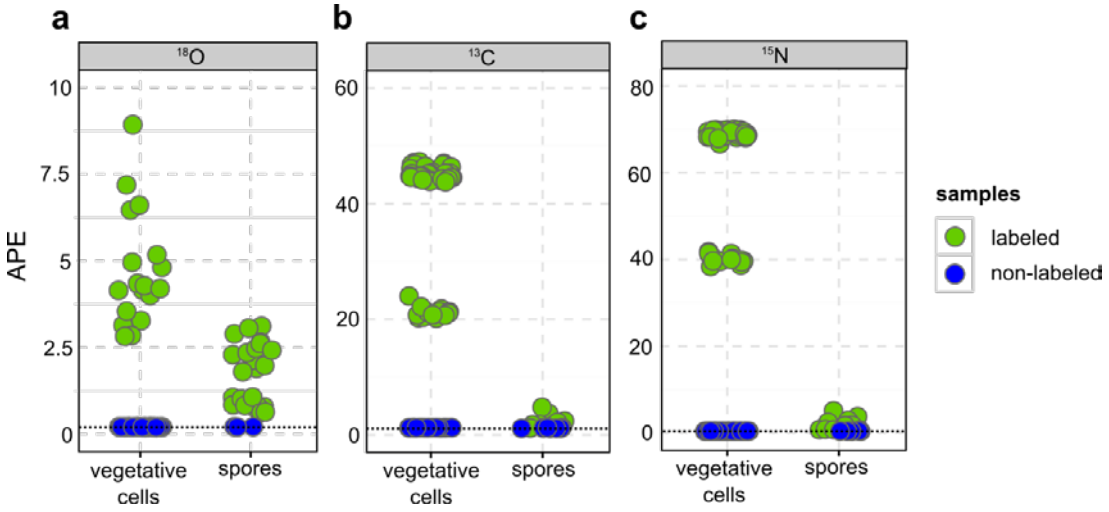

Figure 6: Vegetative cells are higher enriched in ^{13}C, ^{15}N and ^{18}O compared to spores of *B. subtilis*. Atom Percent Enrichment (APE) for (a) ^{18}O, (b) ^{13}C and (c) ^{15}N by single cells and spores of *B. subtilis* measured with NanoSIMS in labeled (green) and non-labeled (blue) samples. Data are derived from different fields of analysis and from replicate wafers (for details see Supplementary Table 1). Dashed lines represent literature values for the stable isotopes' natural abundance.

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Author contributions

All authors conceived the study framework and discussed the theoretical background. A.W., N.M., M.K. and L.Y.W. planned the study. A.W. and H.S. conducted the experiments and compiled the data. L.Y.W and M.K. supervised the experiments. The manuscript was written by A.W. with extensive input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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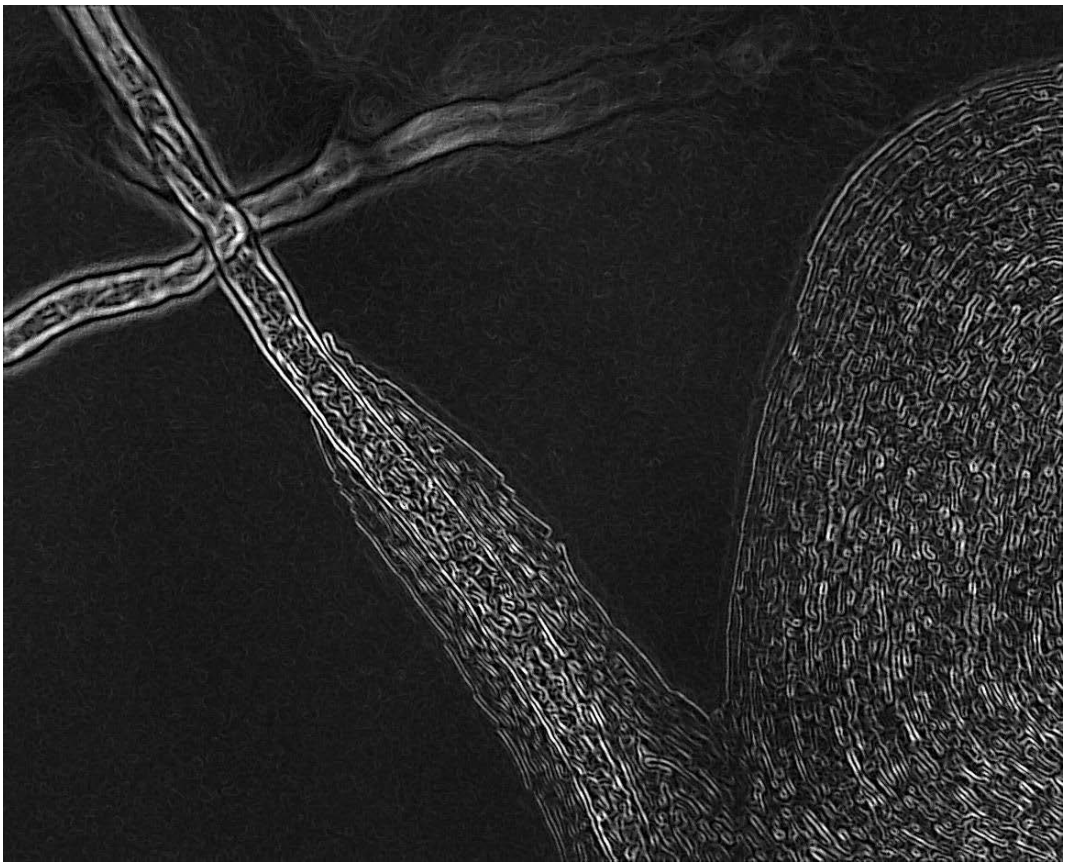
Chapter 6

DISCUSSION



Mycelium of Pythium ultimum colonized by Bacillus subtilis

6 DISCUSSION

'To know how to wonder and question is the first step of the mind toward discovery.'

- Louis Pasteur -

Although the effects of global climate change have gained increasing interest in the scientific community, we still lack a mechanistic understanding on how soil microbes respond to environmental change and how those responses affect ecosystem functioning and the delivery of ecosystem services. Drought (*i.e.* reduced soil water potentials) is the most common environmental stress for soil microbes and the frequency of drought periods is predicted to increase under climate change[171,172]. Soil drying directly influences bacteria via a restricted motility and imposed physiological costs for acclimation causing a reallocation from growth to survival pathways (*e.g.* osmolyte synthesis; cf. 2.3.2). Moreover, as soils dry, substrate diffusion becomes limited and microbes may experience resource limitation that might decelerate bacterial activity or even induce dormancy[173] (cf. 2.3). Two fungus-mediated transport mechanisms have been discovered in the past that i) facilitate bacterial dispersal processes ('fungal highways') and ii) provide bacteria with resources ('fungal pipelines'; cf. 2.5). The overall aim of this thesis was to contribute to an advanced understanding on the role of these fungus-mediated transport mechanisms under environmental stress conditions that commonly restrict bacterial activity (cf. Fig. 12 for a schematic overview).

To study whether 'fungal highways' support bacterial dispersal at low osmotic and matric potentials we developed a multiple microcosm setup with the motile, soil-dwelling bacterium *Pseudomonas putida* and glass fibers as an abiotic model for fungal hyphae. Structural and functional effects of changed dispersal capabilities were examined by following changes in bacterial abundance and substrate degradation in the microcosms (chapter 3, P1). Based on these experiments, we further investigated the relative contribution of network-based bacterial dispersal, autonomous bacterial dispersal and substrate diffusion to efficient biodegradation under varying osmotic stress levels by comparison with a quasi-optimal scenario exhibiting a homogenous distribution of bacterial cells (chapter 4, P2).

Whether the 'fungal pipeline' also serves as a path for the transport and transfer of water and nutrients was studied by means of a tailored synthetic microbial ecosystem. Spores of *Bacillus subtilis* were exposed to dry and oligotrophic conditions and subsequently, different fungi and an oomycete were tested for their potential to induce bacterial spore germination taken as a proxy for the stimulation of bacterial activity. Moreover, the extent of spore germination and vegetative growth served as a measure to quantify the impact of resource translocation processes by mycelia in dry and oligotrophic environments. Using stable isotope labeling in combination with secondary ion mass spectrometry, direct evidence was provided for a transfer of scarce resources from water and nutrient rich domains by the mycelium to bacterial cells (chapter 5, P3).

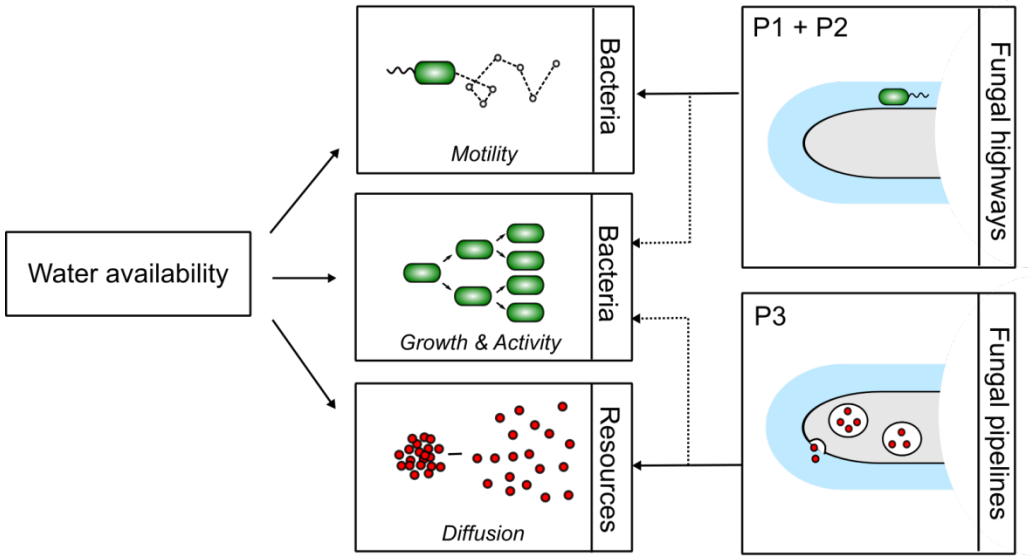


Figure 12 Scheme showing the different aspects studied in the thesis. The influence of the osmotic and matric potential on bacterial motility and the consequences for bacterial growth and activity (*i.e.* substrate degradation) in presence and absence of 'fungal highways' mimicked by glass fiber networks (results in publications (P) 1 & 2; cf. chapter 3 & 4). The translocation of resources in the 'fungal pipeline' and the provision of the transported resources to bacteria located in dry and oligotrophic environments with the subsequent effects on bacterial activity (results in P3, cf. chapter 5).

6.1 Role of 'Fungal Highways' for Biodegradation at Different Water Potentials

6.1.1 Bacterial Dispersal at Different Water Potentials

In this thesis, the impact of various water potentials on bacterial dispersal processes was studied for a broad range of environmentally relevant osmotic and matric potentials. This influence has been extensively studied in the past, albeit for a comparably narrow range of Ψ_m (– 1 up to -50 kPa) corresponding to relatively wet conditions in soil[170]. In this work, we therefore decreased the water potential in a range extending down to -1.5 MPa, which corresponds to permanent wilting point conditions for plants in soil. Furthermore, we directly compared the impact of the two most important components contributing to the overall water potential in soil: the osmotic (Ψ_o) and the matric (Ψ_m) potential. In earlier studies, this has been studied only with respect to bacterial community composition and activity[59,63] but not to bacterial dispersal. Our results demonstrate the significant impact of the water potential on bacterial motility. Both, the osmotic and matric potentials restricted colony expansion of *P. putida* KT2440. However, different thresholds were observed for Ψ_w adjusted by either the addition of NaCl (Ψ_o) or PEG-8000 (Ψ_m). With decreasing Ψ_o, dispersal decreased gradually and was almost completely inhibited at -1.5 MPa. In contrast, dispersal stopped already at -0.25 MPa when Ψ_m was reduced. The different observations can likely be explained by the different effects of NaCl and PEG-8000. NaCl (used to adjust Ψ_o) primarily evokes

physiological costs in bacterial cells for acclimation to increased osmotic pressure. Although this obviously could be compensated at intermediate stress levels (< -0.5 MPa), lower Ψ_o probably caused a repression of flagellum rotation or even flagellum synthesis as observed for example in different *Bacillus* and *Pseudomonas* strains[85,86]. In contrast, the overlay technique with PEG directly leads to a removal of water from the agar, which restricted bacterial motility already at -0.25 MPa. Indeed, flagellar motility has been reported to play a negligible role for colony expansion of *P. putida* already at Ψ_m of – 2kPa[83]. However, given the fact that motility is a highly conserved trait among bacteria and that two-thirds of sequenced bacteria are motile[174], other pathways enabling bacterial dispersal at low water potentials commonly found in soil are likely to exist. This might involve other forms of bacterial motility such as gliding and twitching[79,80] or the dispersal and hitchhiking on vectors.

6.1.2 *Bacterial Dispersal in Presence of Dispersal Networks at Different Water Potentials*

Different vectors have been proven to facilitate the movement of bacteria, including plant roots[175,176], zooplankton[177], protists[178] and earthworms[179]. Already in the 1930s, Cholodny suggested that fungal hyphae could influence the distribution of bacteria in soil by providing pathways for movement[180,181]. However, the mobilization was shown to strongly depend on the specific combination of fungi and bacteria as well as hyphae properties (*e.g.* the hydrophobicity of the hyphal surface)[126]. To avoid effects of specific fungal properties on bacterial dispersal, but also to exclude specific BFI and the effects of fungal metabolic activity, glass fiber networks were used in the present study as an abiotic model for fungal hyphae. Thus, we focused on the biophysical interaction of bacteria with dispersal networks at different Ψ_w and the potential benefit obtained by the provision of a continuous dispersal path in otherwise motility-restricting environments.

The presence of dispersal networks facilitated bacterial dispersal in the microcosm system down to Ψ_w of -0.5 MPa for both Ψ_m and Ψ_o. This constitutes a clear dispersal improvement especially for the systems exhibiting decreased Ψ_m. In such treatments, most likely the water films surrounding the fibers facilitated bacterial swimming motility. In the Ψ_o treatment, the beneficial effect of the glass fibers first seemed contradictory as the adjustment of Ψ_o does not induce water extraction from the agar as it is the case for PEG. However, the network-mediated benefits (*i.e.* improved substrate accessibility), which were already observed in the systems without osmotic stress, may compensate for the energetic costs of flagellar maintenance in *P. putida* KT2440 and hence prevent the downregulation of motility genes (cf. 6.1.1). This hypothesis could be explicitly tested by a reisolation of the cells from the microcosms with and without dispersal networks and a subsequent observation of differences in the motility behavior in microscopy chambers or on motility agar plates.

Earlier studies indicated that the movement of *B. subtilis* along killed hyphae of the oomycete *Pythium ultimum* was restricted to soil matric potentials of >-15 kPa[182]. However, this might be due to a disappearance of the water film when the hyphae lysed or a change of the surface properties after hyphal death, making them less suitable vectors for bacterial dispersal.

Moreover, it is conceivable that resources leaking from dead hyphae may cause reduced bacterial dispersal according to the ecological concept of conditional dispersal confirmed for bacterial colony growth[183]. Consequently, living mycelia may facilitate bacterial dispersal also at water potentials below >-15 kPa.

Overall, it could be demonstrated that bacterial dispersal in soil in the presence of dispersal-enabling mycelia is probably more dynamic than previously assumed and may not be restricted to the usually reported high Ψ_w. Moreover, a key message of the thesis is that the effects of decreasing Ψ_o should not be neglected as a factor controlling the dispersal of bacteria in soil. This is particularly true as soil salinization is a major threat in arid and semiarid regions of the world and anticipated to further increase due to irrigation and clearing of the native vegetation especially in agricultural lands[184,185].

6.1.3 Consequences for Benzoate Biodegradation

In the microcosm experiments, sodium benzoate was used as an analogue of polar, aromatic contaminants (*e.g.* pesticides) as its physicochemical properties are similar to those of 2,4-D, dicamba or fluroxypyr[170]. Especially in agriculture, pesticide application is a common practice to protect plants from pests and weeds. Without bacterial degradation these pollutants would persist and probably contaminate water resources[40]. Therefore, biodegradation is considered an important regulating ecosystem service of high economic benefit (cf. 2.2).

Restricted bacterial dispersal in the microcosms was accompanied by reduced sodium benzoate biodegradation. This is in line with different studies stressing that bacterial dispersal is a key process for efficient biodegradation in soil[150,186–188]. Dechesne et al., for example, found that the mineralization of sodium benzoate in glass bead filled columns was strongly affected by limited degrader dispersal as a consequence of changes in the matric potential[170]. In our experiments, the addition of dispersal networks enhanced biodegradation by up to 52 and 119% for Ψ_o and Ψ_m, respectively. Such positive effects on biodegradation were also observed with a simulation model testing the influence of dispersal networks under heterogeneous bacterial dispersal conditions and heterogeneous initial resource distributions[150]. In summary, these results point to a key role of network-mediated dispersal for biodegradation at low water potentials in soil.

6.1.4 Network-mediated Dispersal as a Key Process under Osmotic Stress

Based on the results of our first study, the importance of network-mediated dispersal for biodegradation at various Ψ_o was assessed by modifying the applied microcosm setup. Different scenarios were created, which allowed for different spatial processes, all of which could contribute to overcome spatial separation between degrading bacteria and pollutants in soil: i) substrate diffusion, network-mediated and autonomous bacterial dispersal, ii) substrate diffusion and autonomous bacterial dispersal and iii) substrate diffusion only. In this study, a special focus was laid on Ψ_o due to its often neglected impact on bacterial dispersal and biodegradation. Sodium benzoate biodegradation was analyzed at different Ψ_o in the different

scenarios and evaluated by comparing it to a quasi-optimal scenario, in which the bacterial cells were homogenously distributed. This enabled the differentiation of effects arising due to physiological stress of the NaCl (*i.e.* restricted growth) from the effects owing to differences in the spatial distribution of bacterial cells. Indeed, Ψ_o reduced biodegradation irrespective of the spatial distribution of the degrading cells by restricting the growth of *P. putida* KT2440. However, if bacterial cells were additionally heterogeneously distributed the effects were even more pronounced. While substrate diffusion and autonomous bacterial dispersal could only partially maintain biodegradation in the microcosms, network-mediated bacterial dispersal kept biodegradation almost consistently high regardless of the strength of Ψ_o. These findings strongly indicate that 'fungal highways' in soil may play an important role for the maintenance of ecosystem service delivery under environmental stress conditions.

6.1.5 Implications for Natural Systems

Although we obtained the presented results from artificial microcosm systems exhibiting a reduced complexity compared to natural systems, they contain important findings with a potential relevance for natural soil systems.

From an ecological perspective, the 'fungal highway' mechanism may significantly contribute to the functional stability of ecosystems towards environmental stress. Fungal hyphae may lift the barrier to bacterial dispersal in soil by providing continuous water pathways, which are expected to be rather rare even in moderately wet soils[188]. 'Fungal highways' thus may ensure that bacteria can still explore the environment for nutrients, escape from predator grazing or move away from detrimental physicochemical conditions[189], and thus remain active and carry out ecosystem functions. Moreover, the role of bacterial motility in water-unsaturated soils still constitutes an evolutionary puzzle as the normally prevailing motility restricting conditions are anticipated to favor a sessile lifestyle of bacteria[16]. Potential explanations were almost exclusively based on rare flooding events that temporarily allow for bacterial motility[190]. The results obtained in the thesis provide a potential explanation for the maintenance of energetically costly flagella by bacteria in soil as these flagella may increase bacterial fitness in presence of 'fungal highways'[190].

With respect to successful bioremediation of contaminated soils, the obtained results suggest that fungal networks have the potential to improve biodegradation even under adverse abiotic conditions hampering the dispersal of degrading bacteria. The use of microbes for bioremediation approaches is considered a 'green technology'. It has several advantages over conventional remediation techniques such as land filling and incineration, *e.g.* it is less disruptive, often less expensive and it eliminates the pollutant permanently[191]. However, successful bioremediation has to overcome many challenges such as i) the limited bioavailability of the compound due to sorption or physical heterogeneity in the environment, ii) fluctuating environmental conditions restricting the activity of microbes, and iii) difficulties to guarantee an establishment if the microorganisms are specifically introduced to the contaminated site. The latter is primarily a problem in bioaugmentation, because fine-grained porous media (*i.e.* sand or silt) act as filters for microbial cells, and therefore pregrown

cultures can hardly be transported to deeper horizons of the vadose zone[44] (cf. 2.2.2). Our results suggest that fungi in combination with bacteria may be better suited to overcome these challenges. Indeed, it was demonstrated that this dispersal mechanism promotes the degradation of PAH in heterogeneous soil microcosms[147]. Similarly, accelerated bacterial degradation in the presence of fungal hyphae has been shown for several fungal–bacterial consortia and the pesticide diuron as well as the even more persistent dichlobenil metabolite BAM[a,145,146]. Thus, the often applied homogenization of the soil matrix via tilling should be weighed against the possibilities of natural attenuation[b] involving fungi and bacteria, because mechanical treatment is known to destroy the mycelial infrastructure and prevent mycelium development[192].

[a] 2,6-dichlorobenzamide; a metabolite of the herbicide dichlobenil

[b] Variety of physical, chemical, or biological processes that act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil or groundwater

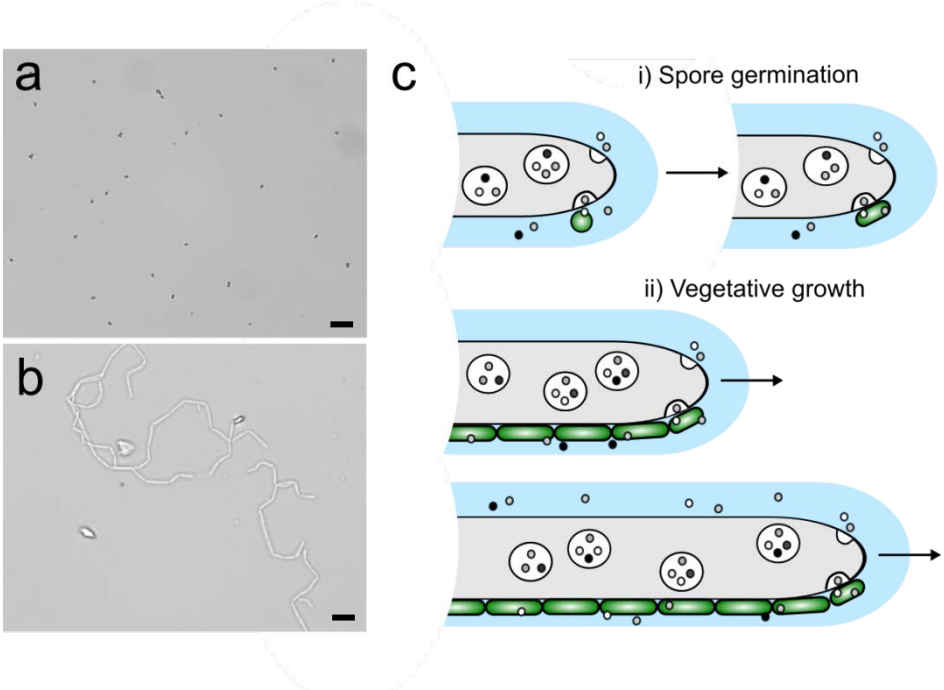


Figure 13: Morphology and growth of *Bacillus subtilis*: a) Spores in Aqua bidest., b) Vegetative cells in LB medium, c) Proposed mechanism of spore germination and vegetative growth in presence of (fungal) hyphae. Scale bars = 10 µm.

6.2 Role of 'Fungal Pipelines' Under Water and Nutrient Deprivation

6.2.1 Stimulation of Bacterial Activity

A second key topic of the thesis was to study the transfer of water and nutrients in fungal mycelia and the supply of bacteria in dry and oligotrophic environments. To this end, a synthetic microbial ecosystem was developed consisting of an agar patch serving as nutrient and water reservoir for either one of the different fungi or an oomycete. Adjacent to this reservoir, but separated by an air gap, endospores of *B. subtilis* were placed on a nutrient-free wafer and dried completely. Such endospores allow *B. subtilis* to resist harsh environmental conditions for prolonged periods[43]. In the spore, the bacterial genome is sequestered until environmental conditions improve, upon which the spore quickly germinates and returns to the vegetative state[193]. Stimulation of bacterial activity was analyzed via a cultivation-based quantification of vegetative cells and spores, which were discriminated based on the differences in their heat resistance (cf. chapter 5).

The tested fungi (selected from different taxonomic groups) as well as the oomycete supported spore germination and vegetative growth of *B. subtilis* on top of the wafer. Moreover, compared to controls without mycelium, the relative proportion of spores was always lower in presence of mycelia. This is clear evidence that the presence of mycelium led to a favorable shift in the local environmental conditions thus inducing spore germination and vegetative growth.

To capture spatial patterns, wafers were also observed under a microscope. Spores and vegetative cells can easily be distinguished based on their morphology. While spores are small

(≈ 1 µm), round-shaped and refractive, vegetative cells are rod-shaped, translucent and could become comparably long (> 10 µm; cf. Fig. 13a,b). Vegetative cells of *B. subtilis* were observed solely in close vicinity to the hyphae of *P. ultimum*, whereas further away spores did not germinate. Thus, close proximity between the mycelium and the bacterial cells obviously is a prerequisite for transfer of resources as observed in different studies on nutrient exchange[161] or chemical communication[194] in microbial communities. Moreover, vegetative cells were arranged mostly as single-layer cell chains along the hyphae thus forming a regular stratification typical for *B. subtilis* biofilms[195]. Based on these observations, we propose a germination and growth mechanism as illustrated in Fig. 13c. When mycelium overgrows the wafer, spores and hyphae come into contact and exudates released from the hyphae trigger spore germination. As the release of exudates occurs primarily at the apical zone, bacterial cells divide towards the extending hyphal tips. Released compounds diffuse in the liquid film surrounding the hyphae and are subsequently taken up by the vegetative cells located right behind the tip, which prevents their resporulation.

6.2.2 *Water and Nutrient Transfer*

To elucidate the mechanism behind the observed spore germination in presence of mycelia, we applied a combination of *time of flight* and *nanoscale secondary ion mass spectrometry* (ToF- and nanoSIMS) with *stable isotope labeling* in the synthetic microbial ecosystem. From previous studies there is experimental evidence that mycelia act as vectors for the transport of water and hydrophobic organic contaminants[154,196]. Our aim was to test whether resources translocated in the mycelium are provided to bacteria in dry and oligotrophic environments thus stimulating their activity (cf. chapter 5). Active and diffusive longitudinal resource translocation comprises a universal transport mechanism in mycelial organisms (*i.e.* fungi and oomycetes) independent of their physiological, functional and phylogenetical traits and occurs over distances of centimeters[92,94,154]. This mechanism is known to enable the maintenance of spatially extensive mycelial networks in spatially heterogeneous environments. The obtained results provide direct evidence that carbon, nitrogen and water are translocated in hyphae of *P. ultimum* and subsequently transferred to bacterial cells located in the vicinity of the hyphae. Pion et al. could show that ^{13}C-labeled carbon was transferred from fungi to bacteria taking the 'fungal highway'[41]. However, this transfer occurred under rather optimal environmental conditions on nutrient-rich agar plates and under high-moisture conditions. Our results demonstrate for the first time that carbon transfer also takes place under harsh environmental conditions and, moreover, is accompanied by a transfer of nitrogen and water. Scarce resources thus become available to bacteria in an otherwise dry and oligotrophic environment (cf. chapter 5).

6.2.3 *Implications for Natural Systems*

The findings of this study indicate that under environmental stress conditions in soil the mycosphere constitutes a hotspot of bacterial activity by providing refugia from abiotic stress.

This is enabled by the 'ecosystem engineering' activity of fungal mycelia, which actively change the abiotic environment and thereby create, alter or destroy habitats. Fungi affect soil structure by their ability to explore and fill pores with their hyphae[95], they modify the local pH or affect water infiltration by the secretion of hydrophobic compounds[33,99,197]. Moreover, they modify the resource availability to other species due to the exudation of compounds from the mycelium[198]. These compounds could serve as growth substrates for bacteria and were postulated several times to be responsible for the increased bacterial abundance observed beneath fungal fruiting bodies[96,97]. By demonstrating that mycelia also enable bacterial activity under harsh environmental conditions (*i.e.* complete absence of water and nutrients in the spore locations) we revealed a mechanism by which fungi might help to maintain ecosystem functions and services in soil systems exposed to extreme drought. Recently, Guhr et al. could show that hydraulic redistribution[c] within fungal mycelia can compensate for water deficiency in desiccated soils. This significantly enhanced carbon mineralization and enzymatic activity in these dry soil compartments[196]. The authors hypothesized that hydraulic redistribution likely leads to transfer of water to hotspots of fungal activity in dry soils with preferential wetting of the surrounding substrate. Our results indicate that this wetting is not restricted to hotspots of fungal activity as the wafers in our experiments contained no utilizable nutrients for the fungus. Instead, the wetting enabled bacterial activity irrespective of whether the local conditions were favorable for mycelial activity. Together with the provision of nutrients for bacteria via exudation, these findings suggest a potentially often prevalent and robust phenomenon of 'ecosystem engineering' by mycelia.

Indeed, it is not yet clear if the fungus obtains any advantage from the excretion of resources and the stimulation of bacterial activity or if the effort to avoid this leakage is simply larger than the 'value' of the lost resources . Maybe the excretion of water is a mechanism to keep the surface of the hyphae moist in order to preserve an intact cell envelope and to maintain resource translocation processes in the mycelium. Another explanation might be that the fungus stimulates bacterial activity as it somehow profits from the presence of the bacteria as shown in a study on bacterial farming by the fungus *Morchella crassipes*[41].

Although not explicitly tested, we assume that the resource transfer to bacteria also occurs in soils at intermediate Ψ_w, where diffusion is less restricted. This probably expands the active zones beyond the direct vicinity of the hyphae and presents a convincing argument for the consideration of a mycosphere (similar to the rhizosphere) as a narrow and heterogeneous subregion of soil that is directly influenced by the presence of fungal hyphae.

Fungi from different taxonomic groups (basidio- and ascomycetes) and an oomycete were shown to stimulate bacterial activity in the microcosms. Fungi and oomycetes differ considerably from each other regarding *e.g.* cell wall composition and septation. Thus, the observed nutrient and water transfer from mycelia to bacterial cells obviously occurred independent of these characteristics and probably constitutes a widespread mechanism that may also expand to other types of organisms exuding compounds into the environment (e.g. plant roots). Overall, the results might help to understand and explain the distribution and

[c] Passive transport of water in soils through organisms along a gradient in soil water potential[196]

abundance of bacteria in soils a well as their spatial dynamics under fluctuating environmental conditions.

6.3 Role of Fungus-mediated Transport Mechanisms for Functional Ecosystem Stability

Ecosystem stability is the ability to resist and recover from disturbances[199]. The term distinguishes itself from the previously used term 'stress' as the latter already describes a physiological or functional effect caused by a disturbance. A disturbance, in turn, is a physical force, agent, or process, either abiotic or biotic that can vary in duration, intensity and frequency[200]. If the system stays essentially unchanged despite the presence of a disturbance it is considered to be resistant. Recovery, in turn, describes the return of the system to a reference state after the occurrence of a disturbance[11]. Several key factors are described in literature mostly with respect to microbial community composition, which determine the stability of ecosystems[11]. However, only few studies focused on factors which play a role for functional stability, which is the maintenance and recovery of ecosystem functions. De Vries et al. demonstrated that fungal-based food webs in extensively managed soils are more resistant to drought than the bacterial-based food webs in intensively managed soils, which resulted in mitigation of C and N loss[172]. Recently, also changing interspecific bacterial interactions in response to different environmental conditions have been reported to be essential for ecosystem functioning[201]. However, the role of BFI, and especially fungus-mediated transport mechanisms, for functional ecosystem stability remained unknown. The results of this thesis indicate that both, 'fungal highways' and 'fungal pipelines' might significantly contribute to the functional resistance of ecosystems towards drought by i) maintaining bacterial dispersal in the system and ii) stimulating bacterial activity via resource provision. Although dispersal is generally assumed to play an important role for the recovery of microbial communities and their associated functions in soil[11], we could show that under low water potentials bacterial dispersal along fungal mycelia may also be important to maintain ecosystem functioning in presence of disturbances. Dispersal along mycelia does not only increase the contact probability of bacteria and contaminants leading to improved biodegradation, it also enables bacteria to escape from adverse environmental conditions, escape predation or acquire new genes through horizontal gene transfer and thus to adapt to new environmental conditions or access and exploit new food sources[127]. All these aspects might contribute to the overall capacity of soil ecosystems to buffer the negative effects of low water potentials. The provision of resources by fungal mycelia may constitute another mechanism by which the negative effects of drought on bacterial activity and ecosystem functioning in soil are mitigated. Water translocation in fungal mycelia has been proven to increase the functional resistance of ecosystems with respect to carbon mineralization under drought. Our results provided evidence that this translocation also feeds back to other soil organisms. While bacteria in bulk soil enter dormancy or die due to ongoing starvation or desiccation, the mycosphere might still enable bacterial activity by supporting growth, dispersal and resource supply, which at least partially maintains ecosystem functioning.

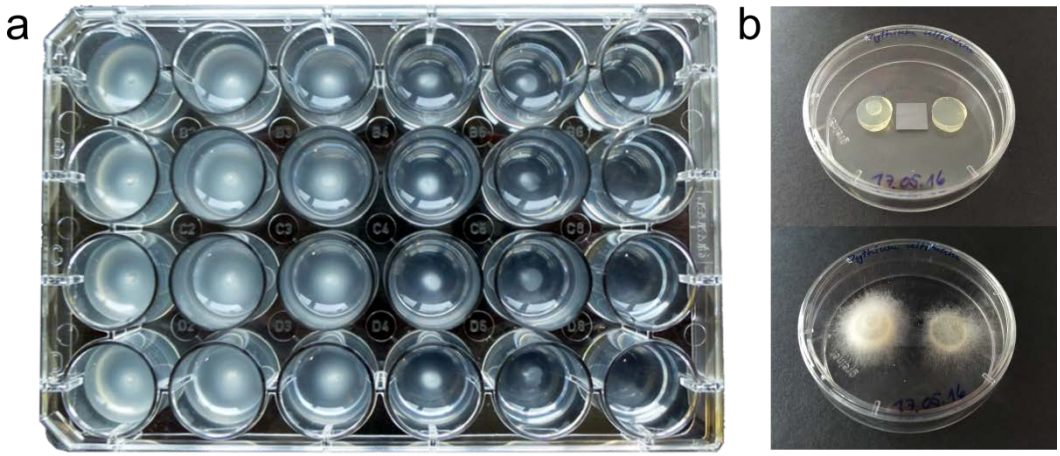


Figure 14 Synthetic microbial ecosystems developed in the thesis: a) Multiwell microcosms used to study the effects of 'fungal highways' at different water potentials, b) Agar patch microcosms to investigate resource transfer processes in the 'fungal pipeline' and the subsequent activation of bacteria in dry and oligotrophic environments.

6.4 Suitability of the Developed Synthetic Microbial Ecosystems

In this thesis, two different synthetic microbial ecosystems were developed to study the role of fungi as transportation networks (cf. 2.5). The multiwell microcosms (Fig. 14a) were applied to study the role of 'fungal highways' under different osmotic and matric potentials. The agar patch microcosms (Fig. 14b) were used to investigate resource transfer processes in the 'fungal pipeline' and the subsequent transfer to bacteria.

The arrangement of 24 individual microcosms on an area of 12.8 x 8.6 cm in the multiwell microcosm system allowed for a simultaneous observation of bacterial colony expansion at 5 different water potentials (0, -0.25, -0.5, -1, and -1.5 MPa) plus an abiotic control in 4 replicates. Moreover, the system enabled us to apply different water potentials, while keeping all other parameters constant. This is almost impossible in natural systems, where changes in the matric potential simultaneously evoke changes in the osmotic potential, substrate diffusion, soil structure and so forth[16]. Thus, data obtained from field studies often represent the effects of several concurrently changed parameters making it extremely difficult to disentangle the influence of a single parameter. Another rationale for choosing agar-based microcosms is that bacterial dispersal processes can only be observed indirectly in porous media. Examples presented by Wolf et al.[163] describe the sampling of sand microcosms at different distances from an inoculation point and subsequent regrowth of bacteria on agar plates. However, the sampling of those microcosms and thus the acquisition of reliable data is extremely difficult as sampling from the water-film at the sand matrix-petri-dish interface must be avoided[163]. Our presented approach requires no sampling of the microcosms for assessing bacterial dispersal. Instead, it enables a semi-automatic and high-throughput analysis of bacterial colony expansion and can be easily adapted to questions on the influence of other factors such as temperature or the presence of chemicals. Moreover, it can be used to study the dispersal of other bacterial strains or even fungi and protists. Nevertheless, the transferability of the

obtained results to natural conditions needs to be verified. Although the use of gel media to study bacterial dispersal in soil is discussed controversially, we argue that soil surfaces, on top of which bacterial dispersal occurs, are not just bare mineral surfaces but rather covered by patchy materials most likely originating from broken cell envelopes[202]. These sponge-like structures are probably better represented by agar matrices than by ceramic or quartz surfaces.

The SME developed to test the effects of 'fungal pipelines' for bacterial activity under drought and nutrient limitation made it possible to observe bacterial activation microscopically and via an isolation-regrowth approach. In addition, the resource transfer processes could be observed directly on top of the included wafer using nanoSIMS. The incorporation of the wafer into the microcosm prevented us from losing spatial and chemical information typically occurring during sample preparation in SIMS experiments. In such experiments, cells typically need to be isolated and mounted on a conductive surface before measurement, as it is not yet possible to routinely analyze a microbial colony directly on agar-based media[203]. Thus, we could not apply existing microcosm setups to study the transport of substances in mycelia and the transfer to bacteria such as the 'whole cell bioreporter approach' presented by Schamfuß et al.[155,204], which relies on the embedding of cells in agar matrices. Moreover, the use of *B. subtilis* spores to study the activation of bacteria represents an integral part of the microcosm setup. Spores could resist the very harsh conditions prevailing on the wafer for the duration of the experiment and thus reliably reflect the ability of mycelia to induce favorable shifts in the environmental conditions by enabling a revival of spores. The use of non-sporulating bacteria would probably result in cell death and thus in an underestimation of the potential of fungal mycelia to stimulate bacterial activity.

Also for this experiment, resource transport and transfer to bacteria can hardly be captured directly in real soil systems. Soil microcosms would require a laborious preparation including sterilization, targeted introduction of stable isotopes and either cell detachment or intensive sample preparation methods if spatial information needs to be preserved for nanoSIMS analysis (soil slicing and resin embedding). Thus, the increased complexity of the microcosms would drastically increase the methodical challenges probably at the expense of a reduced number of replicates as well as a less clear demonstration of the resource transfer between bacteria and fungi and the role of the spatial proximity of the organisms.

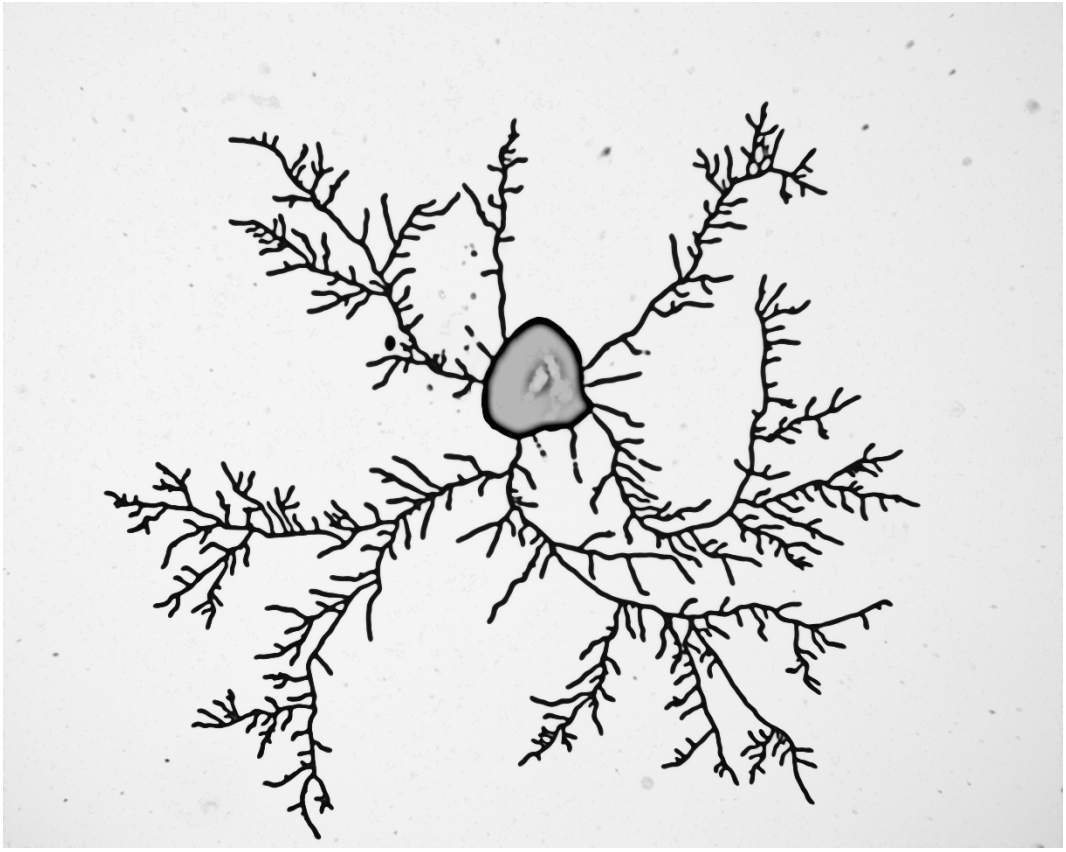
Although it seems that cultivation-based approaches are more and more replaced by cultivation-independent studies using high-throughput sequencing methodologies, one has to consider that microbial interactions are not directly revealed by these approaches. Instead, they rather provide an inventory description of diversity and community composition of natural communities making it possible to infer potential interactions between species by network analysis of significant taxon co-occurrence patterns[205]. However, this does not provide evidence that any type of interaction actually takes place *in-situ* nor what the nature of the interaction is. Thus, SME are necessary to investigate microbial interactions and to link them with ecosystem functioning. Hence, next-generation sequencing methods do not eliminate the need for SME, but rather should be regarded as highly complementary as the combination of both might help to explain the composition of microbial communities in their

natural habitats. The results of this thesis illustrate the potential of SME to test specific hypotheses on microbial interactions and to unravel underlying mechanisms. Moreover, we could demonstrate that they can be coupled with cutting-edge techniques in contemporary microbiology (e.g. ToF- and nanoSIMS), which surely will further increase the impact of SME in microbial ecology.

In this thesis, the application of SME enabled us to show that fungi are essential in maintaining ecosystem services provided by bacteria by facilitating bacterial dispersal and supplying them with essential resources.

Chapter 7

CONCLUSION AND OUTLOOK



Mycelium of Pythium ultimum growing on agar of reduced osmotic potential

7 CONCLUSION AND OUTLOOK

'The game of science is, in principle, without end.'

- Karl Popper -

Bacteria significantly contribute to vital ecosystem functions and the provision of ecosystem services. However, in soil they typically experience conditions, which are suboptimal for growth and reproduction and which diminish their activity. In this thesis, two fungus-mediated transport mechanisms were investigated to unravel their role under drought and nutrient limitation representing common and also interrelated environmental stress factors in terrestrial ecosystems. Using tailored synthetic microbial ecosystems, we were able to observe strong benefits for bacterial activity evoked by the presence of artificial or real mycelial networks under defined stressful environmental conditions. Hence, the thesis provides the following insights into potential mechanisms determining the stability ecosystem functioning:

- 'Fungal highways' mimicked by glass fiber networks significantly enhanced bacterial dispersal under lowered water potentials in the microcosms. This effect was observed for both, changes in the osmotic and matric potential. Moreover, facilitated bacterial dispersal along the networks increased the population growth of *Pseudomonas putida* KT2440 in the system as well as the degradation of benzoate.

- Network-mediated bacterial dispersal probably constitutes a key process for the stability of ecosystem functioning under osmotic stress. Over a range of different, environmentally relevant osmotic potentials substrate diffusion and autonomous bacterial dispersal were insufficient to compensate for the adverse effects on biodegradation evoked by spatial degrader heterogeneity. Network-mediated bacterial dispersal, however, kept biodegradation almost consistently high irrespective of the strength of the osmotic stress.

- In otherwise dry and oligotrophic environments, the presence of mycelia enabled bacterial activity, which was demonstrated by the germination of *Bacillus subtilis* spores along hyphae of different fungi and an oomycete. The underlying mechanism was shown to involve a translocation of scarce resources in the 'fungal pipeline' and a subsequent transfer to bacteria.

In summary, the findings of this thesis propose important ecological roles of the two investigated fungus-mediated transport mechanisms for bacterial activity and the functional ecosystem stability to water and nutrient limitations.

Bacterial motility in soil is probably more dynamic than previously assumed as 'fungal highways' could lift the barrier to bacterial dispersal under motility restricting conditions evoked by drought. Thus, the general assumption that bacterial motility only plays a role under rare flooding events is not certainly true as the role of fungal mycelia as continuous dispersal pathways under fluctuating hydration conditions was not taken into account. Indeed, this fungus-mediated transport mechanism might also explain the maintenance of energetically costly flagella in terrestrial environments, which so far remained poorly understood. In

addition to those ecological aspects, we also found that bacterial biodegradation performance was significantly enhanced in presence of dispersal networks even under adverse abiotic conditions. This clearly illustrates the importance of bacterial dispersal processes for ecosystem functioning. Future bioremediation approaches should therefore consider bacterial-fungal interactions to overcome bioavailability limitations and to achieve optimal results. This involves the preservations of fungal network integrity by avoiding the mechanical mixing of soil, which is often conducted in remediation schemes developed for bacteria.

Besides, also the 'fungal pipelines' may facilitate bacterial activity in soil under abiotic stress. Due to increased resource availability for bacteria, the mycosphere may provide bacterial refugia from water and nutrient limitations in bulk soil. This probably leads to the creation of bacterial activity hotspots in soil, which could still provide ecosystem functions and services and thus contribute to the functional resistance of the ecosystem. From an ecological point of view, bacteria and fungi should be considered as highly interconnected entities as many of their spatial and temporal dynamics in soil likely emerge from their interactions.

In the following sections we describe several additional experiments, which could help to complement the picture on the effects of fungus-mediated transport mechanisms in soil.
Firstly, we suggest further experiments, which are directly linked to the objective of this thesis. To this end, we distinguish between experiments complementing either the 'fungal highway' or the 'fungal pipeline' study and describe possible variations covering either bacteria, fungi or the environmental conditions. Secondly, we introduce a potential experimental design intended to test the combined effects of the two fungus-mediated transport mechanisms for ecosystem functioning under drought. Thirdly, we address scientific issues in other fields of microbial ecology in which 'fungal highways' and 'fungal pipelines' might have a substantial influence but are not yet considered.

7.1 Further Experiments Related to the Objective of this Thesis

7.1.1 Further Experiments Related to 'Fungal Highways'

Bacterial flagellum maintenance at different Ψ_w: The hypothesis that the presence of fungal mycelia prevents the downregulation of flagellum synthesis still needs experimental validation. As indicated in chapter 6 (section 6.1.2) this could be tested via a reisolation of bacteria from experiments with and without network presence and direct comparison of the motility behavior in motility assays or via tracking of individual cell motility in microscopy chambers. Moreover, one could analyze the dynamics in flagellar gene transcription[61] or directly observe potential loss of flagella with scanning electron microscopy.

Real fungal networks and their growth in response to different Ψ_w: The glass fiber networks applied in this study should be replaced by fungal mycelia. The choice of fungal test organisms for such experiment should be given careful consideration. Criteria for selection should be primarily the hydrophobicity of the mycelium as this property is considered to be the major determinant of bacterial dispersal[126]. To this end, also changes in the mycelium hydrophobicity in response to different water potentials should be investigated, since those might explain variations in mycelial facilitation of bacterial dispersal.

Although fungi are more resistant to lowered water potentials than bacteria and can even grow at low values (cf. 2.4.1) their colony expansions may decrease at lowered osmotic and matric potentials as well[206]. Hence, to complete the picture on 'fungal highway' effects under such stress conditions, the growth response of different mycelial organisms to low water potentials needs to be characterized. We already attempted this for the oomycete *Pythium ultimum* and the ascomycete *Fusarium oxysporum* to decreased water potentials. Preliminary experiments were carried out on agar of varying osmotic potentials with the microcosm setup described in chapter 3. In addition to the commonly measured colony diameter to assess fungal growth, we used two additional metrics to quantify the growth behavior of fungi, which are the fractal dimension[a] representing mycelium complexity and the lacunarity[b] reflecting the gappiness of the mycelium[207]. Both metrics have been used in the past, for example, to assess the effects of grazing on mycelium architecture[208]. First results looked promising (cf. Fig. 15) as differences in mycelium architecture evoked by changes in the osmotic potential could be quantified via this approach. In contrast, sole measurement of the colony diameter at different time points did not reveal differences and thus may not be an appropriate measure to assess fungal growth in response to changing abiotic conditions. Nevertheless, the two tested fungi showed a quite disparate behavior to changes in the osmotic potential, which indicates that the choice of the test organisms represents the critical step to finally obtain transferable results. There is a recent debate in microbial ecology, which proposes to apply trait-based approaches, commonly used in plant ecology, also to microbial ecology to better understand ecological phenomena[209]. So far, fungal traits have been used mainly in taxonomy for identification and classification issues but rarely in fungal ecology[210].

[a] Meters of complexity comparing how a pattern's detail changes with the scale at which it is considered[207]

[b] Measure of heterogeneity; in morphological analysis often defined as gappiness[207]

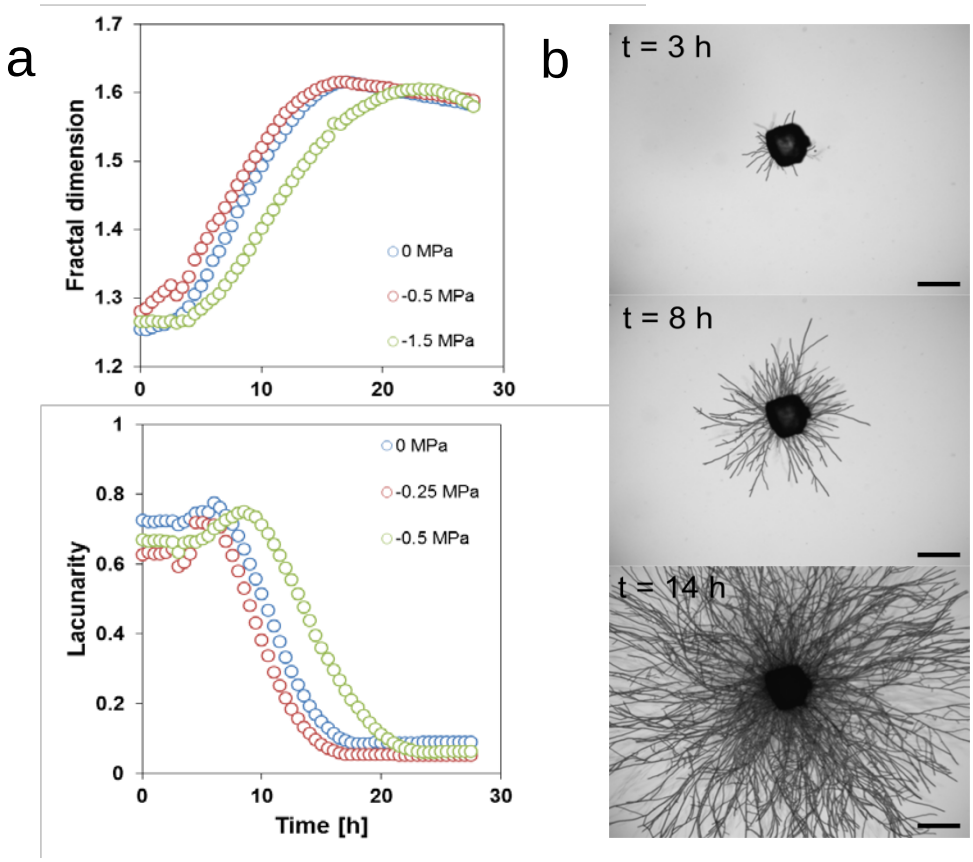


Figure 15 Quantification of the growth of *Fusarium oxysporum* at different osmotic potentials: a) Fractal dimension and lacunarity of the mycelial network obtained from automated image analysis using the ImageJ plugin *FracLac* b) Exemplary microscopic images used for the analysis. Images show the growth patterns of *F. oxysporum* at Ψ_o = -0.5 MPa at three different time points. Scale bar = 1 mm.

Nevertheless, the selection of fungi exhibiting differences in their traits to carry out experiments could help to understand their specific responses to changing environmental conditions and to identify properties that might be responsible for their resistance. Traits that should be considered for experiments of drought effects on fungi are the mycelial architecture, mycelial construction investments and aspects of life span as these are generally associated with stress tolerance[210].

Implementation of a porous structure: The 'fungal highway' study should also be complemented by implementing porous systems to better mimic soil conditions with discontinuous water pathways. The latter, for example, could be achieved with soil column approaches presented by Wick et al.[147] or Dechesne et al.[170], which would also allow to observe the spatial dynamics of degraders as well as the biodegradation performance in response to varying hydration conditions. However, care must be taken to differentiate effects on biodegradation evoked by limited dispersal from effects of restricted substrate diffusion as well as potential biodegradation benefits obtained by 'fungal highways' from overlapping effects of 'fungal pipelines'. While, changes in substrate diffusion can be theoretically calculated for different matric potentials, the extent to which substrate translocation in

mycelia could counteract restricted diffusion is not known and needs to be analyzed experimentally.

Degrading fungi: Throughout the thesis, mycelia were considered as transportation networks for bacteria or resources, which might finally result in enhanced bacterial biodegradation efficiency. However, fungi can also degrade contaminants by themselves (cf. 2.2.2). A bachelor thesis conducted in the framework of this thesis investigated whether fungal degradation of pyrene is affected by the matric potential. Although a reduced degradation was observed at lowered matric potentials on PEG agar plates (cf. chapter 3) for two different fungi (*Trametes versicolor* and *Botrytis cinerea*), we simultaneously observed a high loss of pyrene in abiotic controls most probably due to the volatility of pyrene. Thus, no significant difference could be detected though trends pointed to an influence of Ψ_m. Nevertheless, the experiments need to be repeated using a less volatile contaminant to obtain reliable results. Moreover, a potential correlation between biomass development at different Ψ_w and degradation should be tested. Again, this could be conducted via the already mentioned imaging techniques (*i.e.* fractal dimension and lacunarity) or by measuring the ergosterol content as a proxy for overall biomass[211]. In addition to fungal degradation of contaminants, it is not known so far if contaminant degrading fungi still serve as 'fungal highways' for contaminant degrading bacteria. By doing so, fungi would help to spread their own competitors, which seem highly disadvantageous under conditions of low substrate availability. But otherwise, fungi might also benefit from the presence of bacteria under certain environmental conditions. Faster fungal wood decomposition in presence of bacteria has been demonstrated and, furthermore, migratory bacteria were also shown to protect the fungus from the presence of fungicides[212]. However, if the spreading of bacteria on the 'fungal highway' can actively be influenced by the fungus depending on the environmental conditions remains to be tested. Our microcosms presented in chapter 3 would provide a useful tool to observe bacterial dispersal on degrading fungi under various environmental conditions (*e.g.* different substrate concentrations, presence of fungicides etc.) and also to assess if the presence of bacteria evokes beneficial effects on mycelium development compared to the presence of the fungus alone.

7.1.2 Further Experiments Related to 'Fungal Pipelines'

Differences between the tested fungi: In this study, the reasons why different fungi evoked different growth and germination efficiencies of *B. subtilis* remained open. Therefore, we suggest to relate the ability of different mycelia to induce germination to the relative coverage of the spore-bearing wafer surface by the mycelium. Probably, differences in hyphae density lead to different contact frequencies between spores and hyphae thus causing the discrepancies. However, this could be solved by the approach presented to study the growth of mycelia on top of agar plates. Taking microscopic images of the wafer as a whole would allow to assess differences in the relative surface coverage of the fungi by analysis of the lacunarity. The closer the value is to 0, the higher is the relative coverage with mycelium. The obtained values can then be tested for correlations with the amount of germinated cells and final cell abundances.

Resource transfer mechanism: Also the mechanism underlying resource transfer in mycelia remained untested. Although necrotrophy[c] as initial resource acquisition process could be excluded (due to metabolic inactivity of the spores), it still needs to be tested whether this mechanism played a role after germination of the spores. The cause for the observed hyphae disintegration should be uncovered to evaluate if it is a consequence of an antagonistic interaction between *B. subtilis* and mycelia or just an effect of fungal apoptosis[d] in response to other stimuli such as aging[213]. Indeed, we observed antagonistic interactions between *B. subtilis* and several fungal species leading to fungal growth inhibition on nutrient agar plates. However, we assume that under stress conditions interaction patterns may change, especially if the survival of the antagonistic species suddenly relies on the presence of the other species.

Resource transfer rates: The rates of resource translocation in mycelia might differ in response to changing environmental conditions. Thus, counteracting effects of 'fungal pipelines' under restricted substrate diffusion conditions need to be tested explicitly, for example, at different matric potentials using immotile or immobilized bacteria to exclude compensation evoked by bacterial dispersal. Moreover, analyzing the incorporation of labelled substrates into bacterial biomass at different time points could provide information on transfer rates. However, therefore the experimental setup presented in chapter 5 needs to be modified to be able to adjust different water potentials and to make sure that all bacterial cells had the same time of contact with the hyphae. In order to ensure this, spores could be applied on pre-established mycelium and regions of interest could be immediately defined.

Influence on ecosystem functioning: In this study, we could demonstrate that the 'fungal pipelines' stimulates bacterial activity, which we assume to be directly related to ecosystem functioning. Hence, a next step would involve to prove this hypothesis by directly measuring a certain ecosystem function such as carbon mineralization or plant litter decomposition. More specifically, we would suggest an experiment in sand microcosms, where spores are incubated under dry conditions and fungal mycelia are allowed to grow through the sand from a spatially separated water and nutrient reservoir (cf. Fig. 16). Three different treatments would be necessary to disentangle the effect of water translocation by mycelia on bacterial activity and ecosystem functioning in the microcosms: i) a setup containing only spores, ii) a setup containing only the fungus and iii) a setup where both, spores and the fungus are present (cf. Fig. 16). Crushed labelled plant litter should be mixed with the sand to achieve a nearly homogenous distribution of substrates. However, in a next step this litter could also be distributed heterogeneously, for example nearby the fungal inoculation zone to investigate both, the role of water and nutrient translocation with respect to ecosystem functioning. Decomposition activity could be directly followed by measuring the evolution of labelled CO_2 captured in a base trap containing KOH[170].

[c] Interaction involving killing of the host and feeding on dead matter
[d] Process of programmed cell death

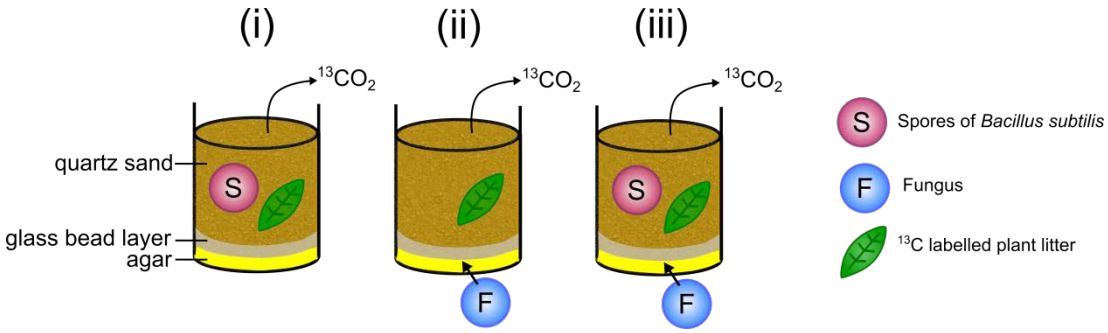


Figure 16 Scheme of the proposed microcosm setup. The fungus (F) is inoculated to the agar plug and covered by a thin layer of glass beads serving as a barrier between spores (S) and the nutrient and water reservoir. The sterile quartz-sand is mixed with the *B. subtilis* spore solution and labelled plant litter, and subsequently placed on top of the glass bead layer. Evolution of labelled CO2 is measured in a base trap. Three different treatments are necessary to unravel the beneficial effects of water translocation by fungal mycelia on bacterial activity and subsequent ecosystem functioning (i-iii).

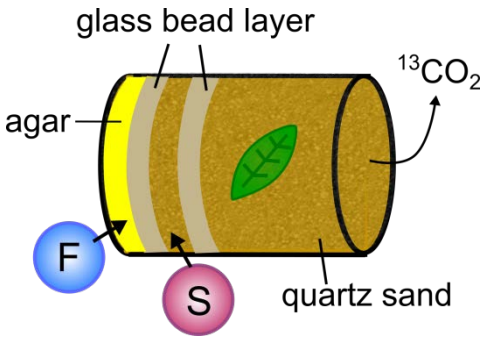


Figure 17 Proposed microcosm setup to prove the combined effects of 'fungal pipelines' and 'fungal highways' on ecosystem functioning. Spores (S) of Bacillus subtilis are introduced into a dry and nutrient-free quartz sand layer separated from the agar patch and the labelled plant litter compartment by two layers of sterile glass beads. We hypothesize that the fungus (F) grows through the different layers and thereby induces a germination of spores via resource delivery from the agar. Subsequently, vegetative cells disperse along the hyphae and thereby reach the quartz sand compartment containing the labelled litter. This experiment should be accompanied by the controls presented in Fig. 16 to assess decomposition by the fungus itself and to prove that ecosystems function is almost completely inhibited in absence of the fungus as bacterial cells are not able to access the labeled plant litter.

Combined Effects of 'Fungal Highways' and 'Fungal Pipelines': A further question arising in the course of this experiment was if the bacterial cells reactivated by the resource delivery could subsequently use the 'fungal highways' to escape from adverse environmental conditions. This can be tested in experiments exhibiting a spatial separation of the bacterial spores and resources (cf. Fig. 17). The occurrence of bacterial cells in the resource zone could then be analyzed, which would provide evidence for the activation (spores are not motile and could not disperse on fungal hyphae) and the subsequent dispersal of vegetative cells along the hyphae to the resource containing area. Moreover, the beneficial effects on ecosystem functioning could be assessed similar to the experiment presented in Fig. 16.

7.2 Further Experiments Related to Research Questions in Other Contexts of Microbial Ecology

7.2.1 Bacterial Coexistence

Identification of mechanisms that promote microbial diversity in soil is a central challenge for contemporary microbial ecology. Fragmentation of microbial habitats by changing hydration conditions has been proven to limit cell dispersal and growth thus promoting coexistence by reducing competition among populations[214]. However, our results suggest that 'fungal highways' still enable bacterial dispersal under lowered water potentials, which may lead to a higher connectivity between microbial habitats. Thus, the question arises if the increased connectivity evoked by bacterial dispersal along fungal mycelia reduces bacterial diversity or, on the contrary, helps them to escape from competition and access new niches[215]. Moreover, it would be conceivable that 'fungal pipelines' may favor microbial diversity via niche partitioning[e] between competing bacterial species. Indeed, it is known that environments with a high nutrient complexity, containing multiple resources or niches, can reduce competition and selection pressure[216]. The complex mixture of substrates in fungal exudates, which likely become available to bacteria in soils, may thus favor bacterial coexistence under nutrient poor conditions. Previous studies focused mainly on the influence of physicochemical soil properties on bacterial coexistence, while the role of fungus-mediated transport mechanisms remains completely unknown. With respect to our recent findings, BFI should be considered as factor with potential relevance to coexistence of bacterial species in soil.

7.2.2 Invasion Ecology

Invasion describes the transport of any organism into a new environment or community where it has never before existed[217]. Four fundamental processes are described in community ecology, which might control this establishment[f]: dispersal, selection, drift and diversification[218,219]. While selection has received attention in microbial community invasion research, the three other processes are often overlooked. Especially dispersal is not considered in the majority of microbial invasion studies, although it may be crucial to invasion success[219]. Indeed, an invader[g] will have more opportunities to immigrate into a community when dispersal to the resident community is strong. The abundance of an invaders population entering the resident community can be considered as the product of its dispersal rate by the duration of dispersal[219]. Thus, if dispersal is low the amount of invaders also becomes low, which decreases the probability of establishment, because a few immigrants are more prone to stochastic extinction[220]. As fungal mycelia were shown to increase bacterial dispersal rates

[e] Competing species use the environment differently

[f] Persistence of the alien type as a self-sustaining population in the new environment[221]

[g] potentially any species not currently part of the resident community[221]

and enable fast and directed movement they might also favor the immigration of invaders by simply increasing the number of cells that enter the resident community.
After an invader enters the community, selection[h] and drift[i] are the major factors determining its establishment. If the niche of the invader does not overlap with the niches occupied by the resident community, the invader will likely become easily established[219]. With respect to our results, the resources provided by fungal mycelia could represent an empty niche that potentially can be filled by an invader species entering the community via dispersal along fungal hyphae. Thus, both mechanisms, the 'fungal highways' and the 'fungal pipelines', should be considered in future with respect to their role in determining invasion success of bacteria in soil. Indeed, such an invasion study might be also relevant with respect to bioaugmentation techniques (cf. 2.2.2), which rely on the injection and invasion success of pregrown cultures to soil in order to enhance the degradation capacity.

[h] Changes in community composition caused by deterministic fitness differences between types[218]
[i] Changes in the relative abundance of types in a community over time caused by stochastic processes[218]

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9 APPENDIX

9.1 Declaration of Authorship

I herewith declare that

- have written this thesis autonomously incorporating my own ideas and judgments; I have made use of no other resources than stated and direct or indirect quotations from other work have been marked accordingly; full reference of their source has been provided in the proper way.
- all persons are listed that provided me with support for the selection and evaluation of the material for my thesis; nature and scope of my own contribution and the share of the co-authors is listed in 'Author contributions of published articles' (9.2).
- no other persons have provided support and thereby contributed to the thesis; in particular, no PhD consultants were used, and no third party has received direct or indirect financial benefits in goods and services for work that stands in relation to the work presented in the thesis.
- this thesis has not been submitted in an equal or similar form for examination for the degree of doctorate or any other degree at another academic institution, and has not been published.
- no further unsuccessful doctoral examination process has taken place.

Place Ort	Date Datum	Signature Unterschrift

9.2 Author Contributions of Published Articles

Author contributions for the three in this work enclosed publications are listed on the following pages:

Author contribution statement, Anja Worrich
Role of Fungal Transport Mechanisms for Bacterial Activity under Environmental Stress

Author contribution statement:

Title:	**Mycelium-Like Networks Increase Bacterial Dispersal, Growth, and Biodegradation in a Model Ecosystem at Various Water Potentials**
Journal:	Applied and Environmental Microbiology
Authors:	Anja Worrich, Sara König, Anja Miltner, Thomas Banitz, Florian Centler, Karin Frank, Martin Thullner, Hauke Harms, Matthias Kästner, Lukas Y. Wick

Anja Worrich	Study concept Microcosm development Cultivation, microscopy, sampling Analysis and interpretation of data Manuscript writing	Signature
Sara König	Study concept Analysis and interpretation of data Manuscript revision	Signature
Anja Miltner	Study concept Analysis and interpretation of data Manuscript revision	Signature
Thomas Banitz	Study concept Analysis and interpretation of data Manuscript revision	Signature
Florian Centler	Study concept Analysis and interpretation of data Manuscript revision	Signature
Karin Frank	Study concept Analysis and interpretation of data Manuscript revision	Signature
Martin Thullner	Study concept Analysis and interpretation of data Manuscript revision	Signature
Hauke Harms	Study concept Manuscript revision	Signature
Matthias Kästner	Study concept Analysis and interpretation of data Manuscript revision	Signature
Lukas Y. Wick	Study concept Analysis and interpretation of data Manuscript revision	Signature

Author contribution statement, Anja Worrich
Role of Fungal Transport Mechanisms for Bacterial Activity under Environmental Stress

Author contribution statement:

Title:	**Bacterial Dispersal Promotes Biodegradation in Heterogeneous Systems Exposed to Osmotic Stress**
Journal:	Frontiers in Microbiology
Authors:	Anja Worrich, Sara König, Thomas Banitz, Florian Centler, Karin Frank, Martin Thullner, Hauke Harms, Anja Miltner, Lukas Y. Wick, Matthias Kästner

Anja Worrich	Study concept Cultivation, microscopy, sampling Analysis and interpretation of data Manuscript writing	Signature
Sara König	Study concept Analysis and interpretation of data Manuscript revision	Signature
Thomas Banitz	Study concept Analysis and interpretation of data Manuscript revision	Signature
Florian Centler	Study concept Analysis and interpretation of data Manuscript revision	Signature
Karin Frank	Study concept Manuscript revision	Signature
Martin Thullner	Study concept Analysis and interpretation of data Manuscript revision	Signature
Hauke Harms	Study concept Manuscript revision	Signature
Anja Miltner	Study concept Manuscript revision	Signature
Lukas Y. Wick	Study concept Analysis and interpretation of data Manuscript revision	Signature
Matthias Kästner	Study concept Analysis and interpretation of data Manuscript revision	Signature

Author contribution statement, Anja Worrich
Role of Fungal Transport Mechanisms for Bacterial Activity under Environmental Stress

Author contribution statement:

Title:	**Mycelium-mediated Transfer of Water and Nutrients Stimulates Bacterial Activity in Dry and Oligotrophic Environments**
Journal:	submitted
Authors:	Anja Worrich, Hryhoriy Stryhanyuk, Niculina Musat, Sara König, Thomas Banitz, Florian Centler, Karin Frank, Martin Thullner, Hauke Harms, Hans-Hermann Richnow, Anja Miltner, Matthias Kästner, Lukas Y. Wick

Anja Worrich	Study concept Microcosm development Cultivation, microscopy, sampling Analysis and interpretation of data Manuscript writing	Signature
Hryhoriy Stryhanyuk	Study concept ToF- and nanoSIMS measurements Analysis and interpretation of data Manuscript revision	Signature
Niculina Musat	Study concept Analysis and interpretation of data Manuscript writing	Signature
Sara König	Study concept Manuscript revision	Signature
Thomas Banitz	Study concept Manuscript revision	Signature
Florian Centler	Study concept Manuscript revision	Signature
Karin Frank	Study concept Manuscript revision	Signature
Martin Thullner	Study concept Manuscript revision	Signature
Hauke Harms	Study concept Manuscript revision	Signature
Hans-Hermann Richnow	Study concept Manuscript revision	Signature
Anja Miltner	Study concept Manuscript revision	Signature

Matthias Kästner	Study concept Manuscript writing	Signature
Lukas Y. Wick	Study concept Manuscript writing	Signature

9.3 Curriculum Vitae

PERSONAL INFORMATION

Anja Worrich

Date of birth
22.07.1987

Address
Stöckelstraße 17
04347 Leipzig

Contact
+49 152 32 07 3519
anjaworrich@gmail.com

Nationality
German

EDUCATION

03/2013 – 10/2016	**PhD thesis** at the Helmholtz-Centre for Environmental Research - UFZ; Department of Environmental Microbiology; Working group Bioavailability
10/2010 – 02/2013	**Master of Science** Biotechnology and Applied Ecology, International Institute Zittau (central academic unit of TU Dresden) Grade: 1,1
03/2012 - 02/2013	**Master thesis** at the Helmholtz-Centre for Environmental Research – UFZ; Department of Environmental Microbiology; Working group Microbial Systems Ecology **Title:** Influence of Spatial Heterogeneity on Microbial Community Assembly – a Microcosm Study
10/2007 – 09/2010	**Bachelor of Science** Biotechnology at the University of Applied Sciences Zittau/Görlitz Grade: 1,4
05/2010 – 08/2010	**Bachelor thesis** at International Institute in Zittau **Title:** Production and Purification of Fungal Dye-decolorizing Peroxidases and Determination of Enzyme Substrate Specificity

07/2009 – 10/2009	**Practical semester** at International Institute in Zittau **Title:** Screening of White-rot Fungi for the Production of Dye-decolorizing Peroxidases and Development of a Purification Protocol

SKILLS AND COMPETENCES

Software	Programming: R (beg) Statistics: R (adv), SigmaPlot Office: Microsoft Office, Open Office Imaging: Inkscape, GIMP, ImageJ NanoSIMS: Look@NanoSIMS
Hands-on experiences	Microbiological techniques: Cultivation bacteria/fungi/protists, Design of microcosm experiments, Microscopy Molecular techniques: DNA/RNA isolation, PCR, T-RFLP, Sequencing, FISH Analytical techniques: HPLC, GC-MS, ToF-SIMS, NanoSIMS
Languages	German native speaker English fluent (Certificate UNIcert II) Russian basic communication skills Czech basic communication skills
Courses and Trainings	Approved Project Leader for Genetic Works and Responsible for Biological Safety (according to German laws § 14,15 GenTSV), Leipzig University, 2016

WORKING EXPERIENCES

11/2016 – 01/2017	Research assistant (UFZ Leipzig)
09/2011 – 03/2012	Research assistant (IHI Zittau)
01/2011 – 05/2011	Research assistant (IHI Zittau)
06/2010 – 11/2010	Research assistant (IHI Zittau)

AWARDS

12/2013	Ehrenfried-Walter-von-Tschirnhaus-Certificate awarded by TU Dresden for the best graduates of the Faculty of Mathematics and Natural Sciences

9.4 List of Publications and Conference Contributions

Publications

Liers, C., Pecyna, M.J., Kellner, H., **Worrich, A.**, Zorn, H., Steffen, K.T., Hofrichter, M., Ullrich, R. (2013). Substrate oxidation by dye-decolorizing peroxidases (DyPs) from wood-and litter-degrading agaricomycetes compared to other fungal and plant heme-peroxidases. *Applied microbiology and biotechnology* **97**, 5839-5849.

Worrich, A., König, S., Miltner, A., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Kästner, M., Wick, L.Y. (2016a). Mycelium-Like Networks Increase Bacterial Dispersal, Growth, and Biodegradation in a Model Ecosystem at Various Water Potentials. *Applied and Environmental Microbiology* **82**, 2902-2908.

Worrich, A., König, S., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Miltner, A., Wick, L.Y., Kästner, M. (2016b). Bacterial Dispersal Promotes Biodegradation in Heterogeneous Systems Exposed to Osmotic Stress. *Frontiers in Microbiology* 7, 1214.

König, S., **Worrich, A.**, Centler, F., Wick, L.Y., Miltner, A., Kästner, M., Thullner, M., Frank, K., Banitz, T. (2017). Modelling functional resilience of microbial ecosystems: Analysis of governing processes. *Environmental Modeling and Software* **89,** 31–39

Worrich, A., Stryhanyuk, H., Musat, N., König, S., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Richnow, HH., Miltner, A., Kästner, M., Wick, L.Y. Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments. Submitted

Conference Contributions

12.04 – 17.04, 2015	**EGU General Assembly**, Vienna, **presentation** 'Effects of dispersal networks on bacterial dispersal and biodegradation at varying water potentials'
12.04 – 17.04, 2015	**EGU General Assembly**, Vienna, **poster** 'Uncovering stability mechanisms in microbial ecosystems – combining microcosm experiments, computational modelling and ecological theory
07.06 – 11.06, 2015	**FEMS Congress**, Maastricht, **presentation** 'Effects of dispersal networks on bacterial dispersal and biodegradation at varying water potentials'
20.09 – 24.09, 2015	**ICCE**, Leipzig, **presentation** 'Impact of dispersal networks on contaminant biodegradation at varying water potentials'
29.11 – 03.12, 2015	**Ecology of Soil Microorganisms**, Prague, **presentation** 'Impact of mycelia-like dispersal networks on bacterial spatiotemporal dynamics linked to biodegradation at varying water potentials'

9.5 Acknowledgements

Zu guter Letzt möchte ich mich bei allen bedanken, die mich in den letzten Jahren bei der Anfertigung dieser Doktorarbeit unterstützt haben.

Mein Dank gilt meinem Doktorvater Prof. Hauke Harms für die Aufnahme in sein Department, die stetige Unterstützung während meiner Promotion, die zahlreichen Diskussionen und Anregungen in den Doktorandengesprächen und die schnelle und sorgfältige Überarbeitung von Manuskripten.

Meinem Betreuerteam Dr. Lukas Wick, Prof. Matthias Kästner und Dr. Anja Miltner danke ich für die zahlreichen Diskussionen, die vielen guten Ideen, den mir überlassenen Freiraum und das entgegengebrachte Vertrauen in meine Arbeit.

Dem Modellierer-Team des TWIN-Projekts Prof. Karin Frank, Dr. Martin Thullner, Dr. Florian Centler, Dr. Thomas Banitz und Dr. Sara König danke ich für die spannenden Einblicke in die Welt der Modellierung und die vielen hilfreichen Anregungen und Diskussionen bezüglich der korrekten Auswertung meiner Daten und der verständlichen Darstellung von Ergebnissen. Danke Sara für den ‚Growth Rate Generator' der mir die Arbeit so sehr erleichtert hat und für deine ständige Bereitschaft mir meine unendlich vielen Fragen zu R, Modellierung oder mathematischen Unklarheiten zu beantworten. Thomas, ich danke dir für deine unermüdliche Hilfe bei allen wissenschaftlichen Fragen und Problemen, die vielen inspirierenden Diskussionen und deine kontinuierliche Unterstützung beim Schreiben der Publikationen und dieser Dissertation.

Ich möchte mich außerdem bei der gesamten AG ‚Bioverfügbarkeit' an der UMB für die herzliche Aufnahme in die Gruppe bedanken und die freundliche Arbeitsatmosphäre. Danke Rita, Jana und Manuel für die ständige Hilfsbereitschaft im Labor und für das Filtrieren und Vermessen so unzählig vieler Proben. Ohne eure Hilfe hätte so manches Experiment in der Form gar nicht stattfinden können.

Meine Kollegen Sally, Nawras, Andreas, Jule und Tom danke ich für die vielen lustigen Momente ob auf Konferenzen oder im Labor. Für die Gespräche und die gegenseitige Unterstützung bei experimentellen Problemen.

Ein besonders großer Dank gilt meine lieben Bürokollegen aus ‚139', Sara und Benni. Danke dafür, dass ihr jede manchmal ärgerliche Situation in ein Lachen verwandelt habt. Ohne Euren Sinn für Humor hätte ich sicher manchmal den Kopf in den Sand gesteckt. Danke für den ganzen Spaß den wir hatten. Das wird mir wirklich sehr fehlen.

Danke Carola, dass du einfach immer alles möglich machst und uns in jeder administrativen Frage mit Rat und Tat zur Seite stehst. Danke auch an die restlichen aktuellen und auch ehemaligen Mitarbeiter der UMB, die dieses Department zu einem so angenehmen Arbeitsumfeld für mich gemacht haben.

Zuletzt danke ich natürlich auch meiner Familie und meinen Freunden für die Unterstützung während der letzten Jahre, für ihr Verständnis und dafür, dass sie immer für mich da sind.

9.6 Supplementary Material for Publication 1

Supplementary material

Mycelia-like Networks Increase Bacterial Dispersal, Growth and Biodegradation in a Model Ecosystem at Varying Water Potentials

Anja Worrich,[a,b] Sara König,[a,c] Anja Miltner,[b] Thomas Banitz,[c] Florian Centler,[a] Karin Frank,[c,d,e] Martin Thullner,[a] Hauke Harms,[a,d] Matthias Kästner, [b]# and Lukas Y Wick[a]

UFZ- Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany, Department of Environmental Microbiology[a]; UFZ- Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany, Department of Environmental Biotechnology[b]; UFZ- Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany, Department of Ecological Modelling[c]; German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, D-04103 Leipzig, Germany[d]; University of Osnabrück, Institute for Environmental Systems Research, Barbarastaße. 12, 49076 Osnabrück, Germany[e]

#Address correspondence to Matthias Kästner, matthias.kaestner@ufz.de.

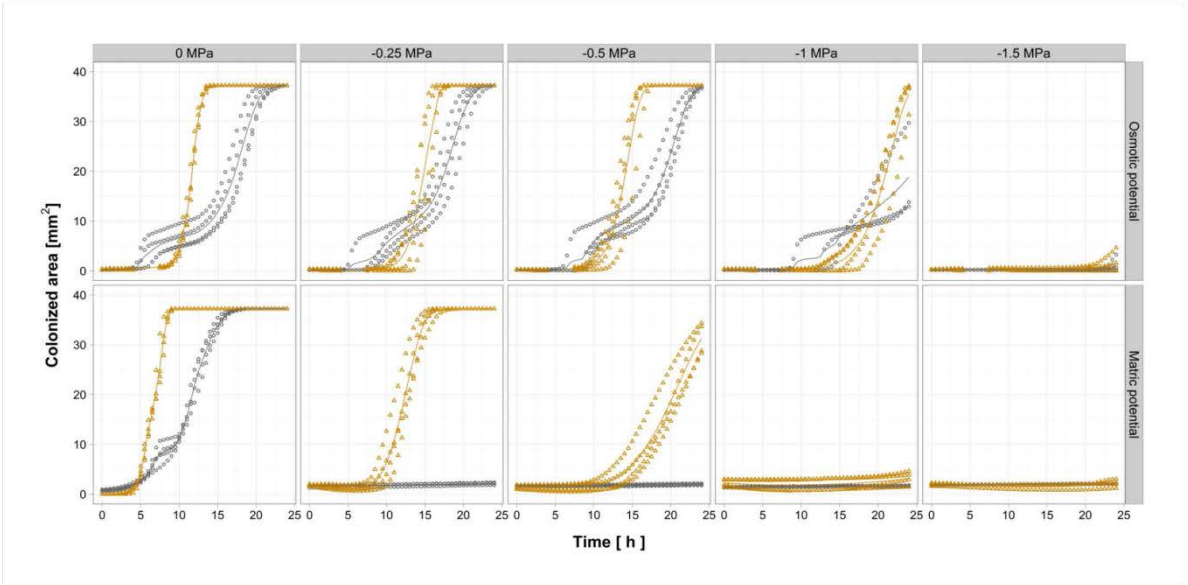


Figure S1: Dispersal of *P. putida* KT2440-gfp in absence (gray circles) and presence (orange triangles) of dispersal networks for the different ΔΨo and ΔΨm. Dispersal was analyzed microscopically by measuring the colonized area every 30 min for 24 h using. Individual points represent the values obtained from the replicate microcosms within the multiwell plate and the solid lines show the averages of replicates used for AUC calculation.

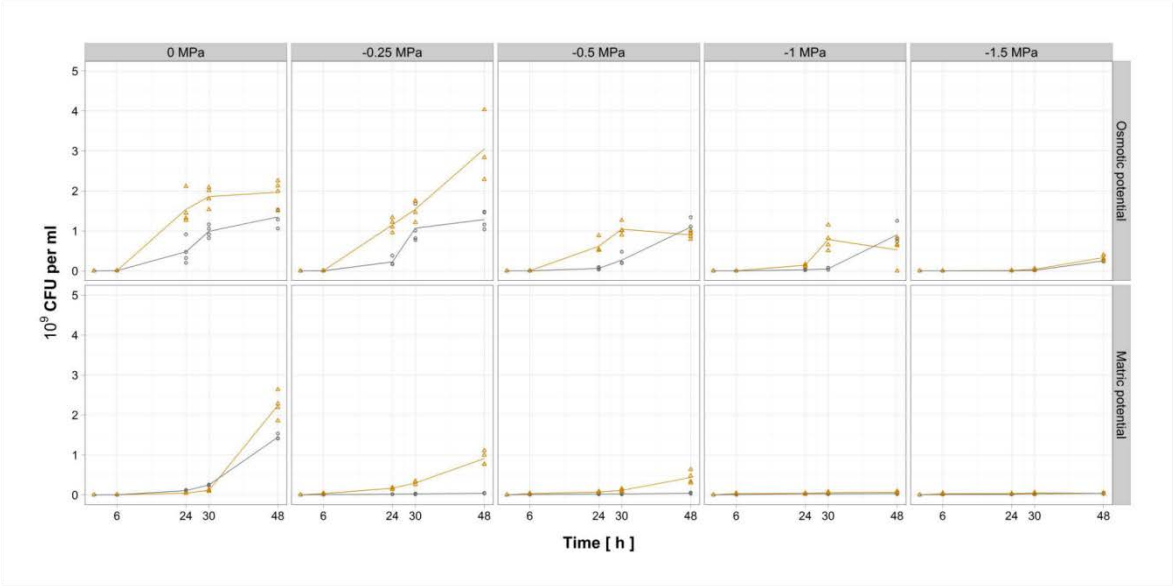


Figure S2: Growth of *P. putida* KT2440-gfp in absence (gray circles) and presence (orange triangles) of dispersal networks for the different ΔΨo and ΔΨm. Growth was analyzed by counting colony forming units (CFU) after 6, 24, 30 and 48 h. Individual points represent the values obtained from the replicate microcosms within the multiwell plate and the solid lines show the averages of replicates used for AUC calculation.

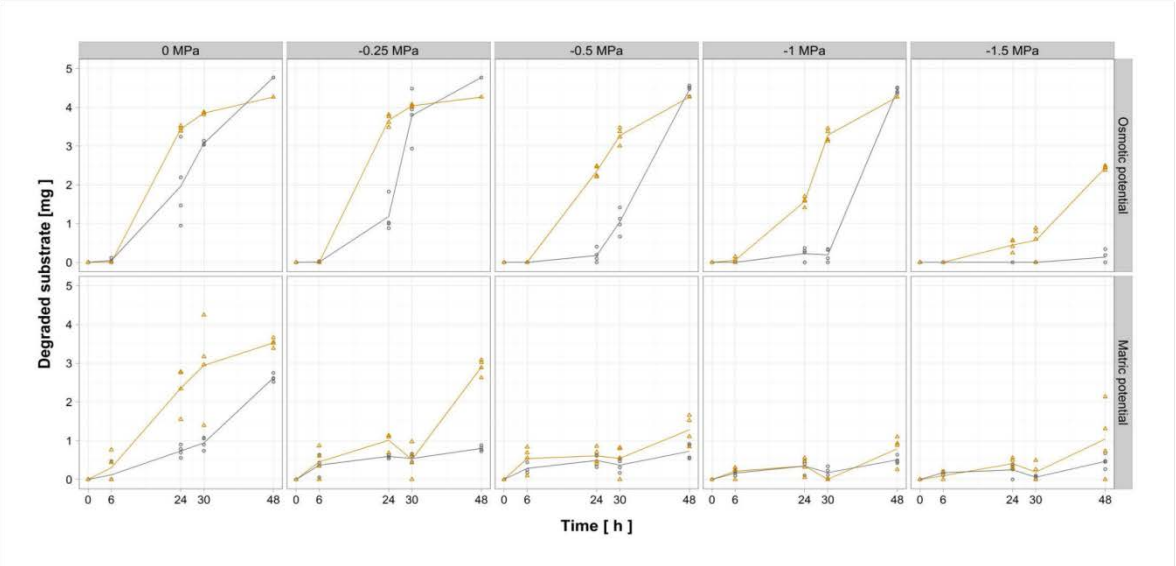


Figure S3: Benzoate degradation by *P. putida* KT2440-gfp in absence (gray circles) and presence (orange triangles) of dispersal networks for the different ΔΨo and ΔΨm. Degradation was analyzed by measuring the remaining benzoate concentration after 6, 24, 30 and 48 h. Individual points represent the values obtained from the replicate microcosms within the multiwell plate and the solid lines show the averages of replicates used for AUC calculation.

9.7 Supplementary Material for Publication 2

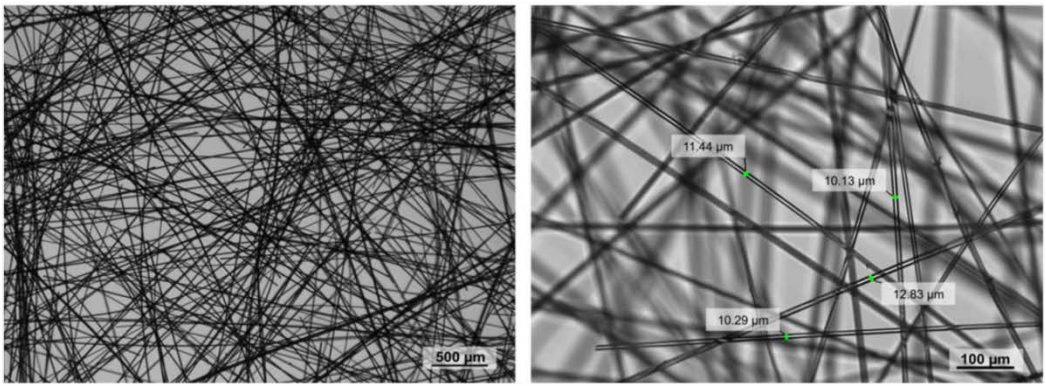
frontiers

Supplementary Material

Bacterial dispersal promotes biodegradation in heterogeneous systems exposed to osmotic stress

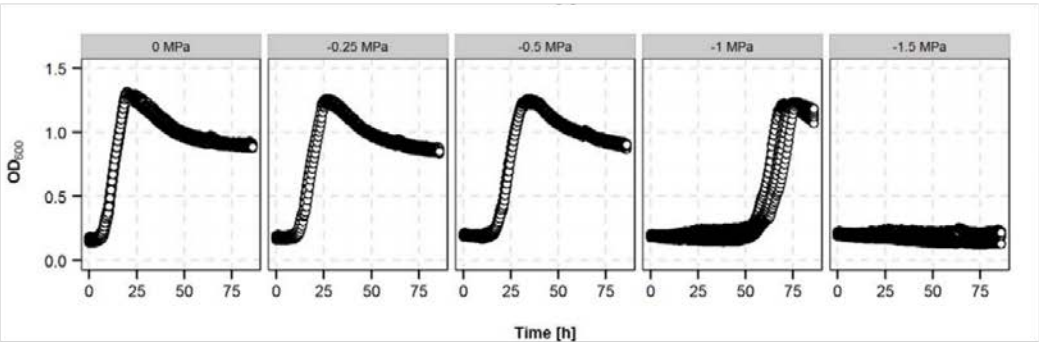
Anja Worrich[1,2], Sara König[1,3], Thomas Banitz[3], Florian Centler[1], Karin Frank[3,4,5], Martin Thullner[1], Hauke Harms[1,4], Anja Miltner[2], Lukas Y Wick[1*] and Matthias Kästner[2]

Correspondence: Lukas Y. Wick; lukas.wick@ufz.de

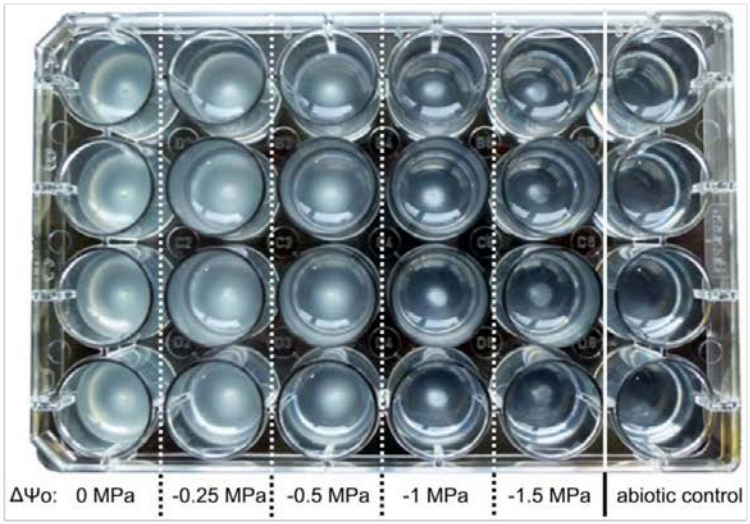


Supplementary Figure 1. Micrograph of the glass fiber network used to simulate fungal mycelia. Green lines indicate the position used to measure fiber diameters.

Supplementary Material



Supplementary Figure 2. Growth curves of *Pseudomonas putida* KT2440 on 50 mM sodium benzoate at different osmotic potentials (indicated by the subplot label) adjusted by the addition of sodium chloride. Growth was measured spectrophotometrically at a wavelength of 600 nm every 30 min. Obtained growth curves were used to derive maximum specific growth rates, lag times and maximum biomass.



Supplementary Figure 3. Microcosm colonization by *P.putida* KT2440 after 30 h of incubation in the D_{dis} scenario at different osmotic potentials.

Supplementary Material

Supplementary Video 1: Time-lapse video showing the population dispersal of the flagellated wild type *P.putida* KT2440 in D_{dis} scenario at $\Delta\Psi_o = 0$ MPa. The video was constructed from 49 images taken at 30 min intervals.

Supplementary Video 2: Time-lapse video showing the population dispersal of a nonflagellated isogenic mutant of *P.putida* KT2440 in D_{dis} scenario at $\Delta\Psi_o = 0$ MPa. The video was constructed from 49 images taken at 30 min intervals.

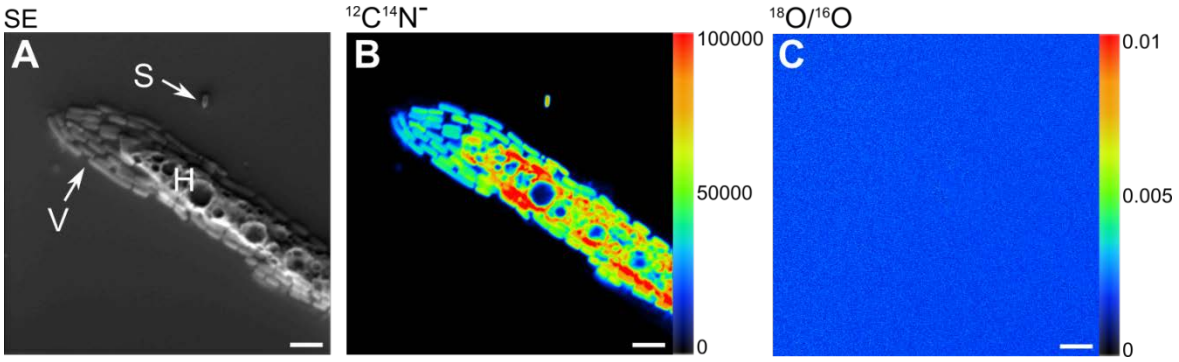
9.8 Supplementary Material for Publication 3

Supplementary information

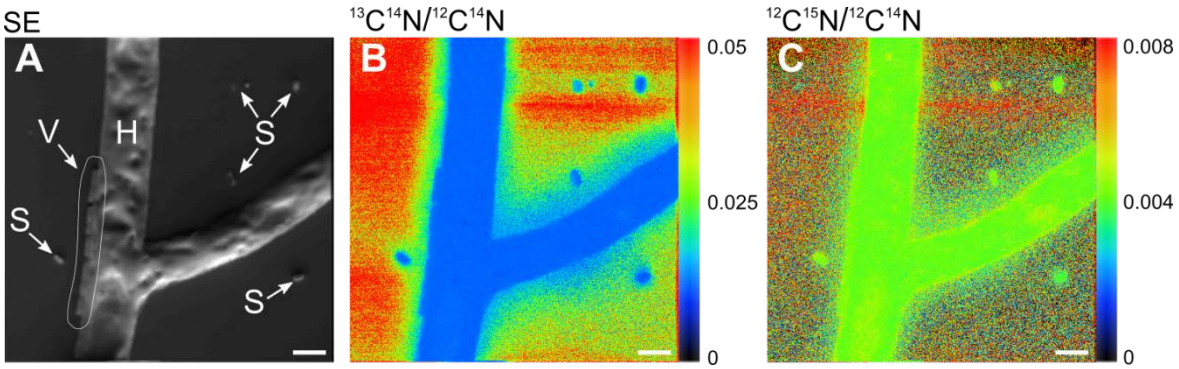
Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments

Anja Worrich[1,3] , Hryhoriy Stryhanyuk[2], Niculina Musat[2,*], Sara König[3,4], Thomas Banitz[4], Florian Centler[3], Karin Frank[4,5,6], Martin Thullner[3], Hauke Harms[3,5], Hans-Hermann Richnow[2], Anja Miltner[1], Matthias Kästner[1,§], Lukas Y Wick[3,§]

*Address correspondence to Niculina Musat, niculina.musat@ufz.de



Supplementary Figure 1: NanoSIMS images of *P. ultimum* hyphae (H), *B. subtilis* spores (S) and vegetative cells (V) identified in a secondary electron and total biomass ($^{12}C^{14}N^-$) image (A, B) of a non-labeled sample. The ratio images of $^{18}O/^{16}O$ show the natural abundance of ^{18}O in the biomass of *P. ultimum* and *B. subtilis* (C). Images represent a field of analysis corresponding to a sample area of 40×40 µm. Scale bars, 4 µm.



Supplementary Figure 2: NanoSIMS images of *P. ultimum* hyphae (H), *B. subtilis* spores (S) and vegetative cells (V) identified in a secondary electron image (A) of a non-labeled sample. The ratio images of $^{13}C^{14}N/^{12}C^{14}N$ (B) and $^{12}C^{15}N/^{12}C^{14}N$ (C) show the natural abundance of ^{13}C and ^{15}N in the biomass of *P. ultimum* and *B. subtilis*. Images represent a field of analysis corresponding to a sample area of 40×40 µm. Scale bars, 4 µm.

Supplementary Table 1: Number of individual fields and replicate wafers analysed with NanoSIMS in ^{18}O and $^{13}C^{15}N$ labeling experiments and ^{16}O and $^{12}C^{14}N$ control experiments as well as numbers of vegetative cells and spores used to calculate APE shown in Fig. 6.

Sample	**# Fields**	**# Wafers**	**# Vegetative cells**	**# Spores**
^{18}O	3	2	20	23
^{16}O	2	2	106	3
$^{13}C/^{15}N$	2	1	57	11
$^{12}C/^{14}N$	3	2	104	7

Supplementary Methods 1

Time of Flight Mass Spectrometry (ToF-SIMS). For a qualitative analysis of sample composition the yields of its secondary ion species was explored with the Time of Flight Secondary Ion Mass Spectrometry technique (ToF-SIMS) employing a ToF-SIMS.5 (ION-TOF GmbH, Münster) instrument. The ToF-SIMS experiment has been performed using imaging mode of ToF-SIMS.5 operation in combination with delayed extraction[1] of negative secondary ions providing the Mass Resolving Power (MRP) above 3000 and lateral resolution of about 130 nm. Under these experimental conditions, the 30 keV NanoProbe LMIG source was delivering 0.02 pA of primary Bi_3^{2+} cluster ions in 100 ns pulses with 200 µs repetition period. The analysis has been done in 400 scans/plains with 5 shots of Bi_3^{2+} primary cluster ions per pixel distributed randomly in 512×512 raster over 56×56 μm^2 sample area. The stacking of acquired scans was performed after lateral drift correction and the resulting total stack was analysed for lateral distribution of ion yields using SurfaceLab 6.5 software (ION-TOF GmbH).

Supplementary Methods 2

Nano-focused Secondary Ion Mass Spectrometry (nanoSIMS). For the quantitative analysis of water and nutrient transfer processes two replicate wafers labelled with ^{18}O, and ^{13}C/ ^{15}N isotopes as well as two control wafers with natural isotopic composition were studied with a NanoSIMS-50L instrument (CAMECA, AMETEK) in negative extraction mode employing a DC source of primary Cs^+ ions. The 2 pA beam of Cs^+ ions was focused into about 70 nm spot at the sample surface during the analysis. The energy of Cs^+ collision with the sample was set to 16 keV in the analysis mode. The sample was scanned in a 512×512 px matrix over areas of 20×20, 30×30 and 40×40 µm with 5 msec dwell time per pixel. Before the analysis with 16 keV Cs+ beam, the sample surface of 60×60 µm² area was treated with 10 nA of low-energy (50 eV) Cs^+ beam for 10 minutes. The low-energy implantation/deposition of cesium has been performed with the purpose to equilibrate the working function for negative secondary ions and to make the outermost layer of the sample available for the analysis avoiding its sputtering during high-energy implantation. The secondary ions were analyzed for their mass and charge ratio (m/z). Using the seven available collectors the following secondary ion species were detected: $^{12}C^-$ (collector-1), $^{13}C^-$ (collector-2), $^{16}O^-$ (collector-3), $^{18}O^-$ (collector-4), $^{12}C^{14}N^-$ and $^{12}C^{15}N^-$ (collector-5), $^{13}C^{14}N^-$ (collector-6), $^{32}S^-$ (collector-7). Two secondary ion species ($^{12}C^{14}N^-$ and $^{12}C^{15}N^-$) were detected using the collector-5 by switching the deflector voltage in combined analysis mode. The mass resolving power (MRP) was checked to be between 8000 and 12000 with the exit slit width of 40 µm, 20 µm wide entrance slit, 200 µm aperture slit and with the energy slit cutting 30% of secondary ions in high-energy tail of their energy distribution. It has been proven to get the sample sputtered completely within 35 scans upon the used

analysis conditions; scans 2-30 were thus considered for the analysis employing LANS software[2] allowing for stacking the scans with lateral drift correction and quantitative analysis of isotopic ratios ($^{18}O/^{16}O$, $^{13}C^{14}N/^{12}C^{14}N$ and $^{12}C^{15}N/^{12}C^{14}N$).