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Biotic Impacts on Ecosystem Services Provided by the Stream Interstitial Zone

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Preface

Anthropogenic impacts on the interstitial zone have the potential to affect its key ecosystem services and the resident biota. The interactions of biota from different taxonomic levels are crucial for ecosystem functioning. Effects of different biota on bacterial communities have neither been in the focus of studies about ecosystem engineers nor in that about invasive species. Considering the current gaps in knowledge about the role of organisms from different taxonomic levels and their interactions among each other in interstitial processes, this thesis closed the methodological gaps to study the effects of different ecosystem engineers on hyporheic zone properties under standardized laboratory conditions and elucidated their effects on physico-chemical and microbial properties in stream substrates.

Initially, native burrowing invertebrates were investigated with regard to their effects on physico-chemical habitat properties and bacterial community composition in the interstitial zone. The animals differed in their sediment reworking behavior (burrowing width and depth) as well as in their life style and were therefore expected to distinctly alter habitat properties. Thereby, the chosen test animals represent three functional different groups of ecosystem engineers: duck mussels (*Anodonta anatina*, Linnaeus 1774) act as regenerators creating open burrows by moving horizontally on the sediment and vertically in and out of it (Amyot & Downing 1998; Zieritz et al., 2014). The larvae of the mayfly (*Ephemera danica*, Müller 1764) are biodiffusors causing omnidirectional movements on the sediment surface (Winkelmann & Koop, 2007) and the tubificid worms (*Tubifex tubifex*, Müller 1774), which belong to the upward-conveyor group, feeding in the sediment depth and ejecting fecal pellets at the sediment water interface (Nogaro et al., 2009).

As the initial study on the three different organisms revealed the effects burrowing invertebrates have, a second experiment should elucidate how vertebrate ecosystem engineers influence substratum conditions and bacterial diversity in the interstitial zone. In the second experiment lampreys were chosen as study organisms. Due to their broad distributions and high densities especially in regions of the Northern Hemisphere, they are thought to have significant impact on freshwater substrates (Shirakawa et al., 2013). In particular, their burrowing behavior during the larval phase (Hardisty, 1979) qualifies them as potential ´ecosystem engineers´ (Jones et al., 1994). They cement their burrows with mucus and allow therefore an influx of subsurface water into the interstitial zone with potential effects on interstitial habitat properties. A native lamprey species *Eudontomyzon* sp*.* was chosen as test animal. Its effects on physico-chemical and microbial habitat properties in the interstitial zone were compared to a control without animals.

Invasive species have the potential to influence ecosystem services provided by the interstitial zone. Thus the aim of a third study was to evaluate the functional differences of an invasive and a native amphipod species to draw conclusions about the effects on ecosystem services due to a replacement of native by invasive amphipods. As test organisms, the native *Gammarus roeseli* (Gervais 1835) was chosen and compared to the invasive *Dikerogammarus villosus* (Sowinski 1894), which has mostly replaced *G. roesli* in the upper Danube River within a few years (Kley & Maier, 2006; Brandner et al., 2013a) with unknown consequences on ecosystem services. This study provides insight into the complex relationship of the presence and absence on invasive and native amphipods on leaf litter decomposition, abiotic habitat properties and microbiota.

The outcomes of the studies were discussed in the context of to-date literature on ecosystem engineers and invasive species. Finally, the findings on interstitial zone functioning and interactions between test animals and assessed physico-chemical and microbial data were linked to conservation planning, followed by a short outlook on possible next scientific steps and political goals.

Summary

Freshwaters are amongst the most threatened ecosystems worldwide. For the elaboration of conservation management concepts, an understanding of ecosystem functioning is crucial. In research of headwater stream ecosystems, one of the biggest knowledge gaps is the riverine interface between open water and groundwater – the interstitial zone. The latter is an important habitat for different species (invertebrates, vertebrates) and their life stages (egg development, larvae, adult organisms). Nevertheless, processes between free flowing and interstitial water as well as interactions between species of different taxonomic levels (from microbes to vertebrates) in dependence of abiotic habitat properties are understudied.

Therefore, this thesis elucidates processes and interactions taking place in the stream interstitial zone. Different organisms inhabiting the interstitial zone were investigated in regard to their effects on physical and chemical habitat properties (oxygen saturation and concentration, redox potential, pH, electric conductivity, concentration of Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}, F^-, Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) as well as bacterial community composition. In a first step, a standardized microcosm laboratory experiment was established. Therefore, substrate-filled plastic boxes which were equipped with redox electrodes and valves to take samples from interstitial water. These boxes were set under constant water flow and stocked with test animals. Functionally different burrowing invertebrates (mayfly nymphs, duck mussels and tubificid worms) were compared in regard to their effects on abiotic habitat properties and microbial community composition. The effects on physico-chemical properties and microbial community composition were also investigated for burrowing vertebrates (lamprey larvae) in comparison to a control without animals in a second step. Moreover, the complex relationship of the presence and absence of invasive and indigenous amphipods on leaf litter decomposition, abiotic habitat properties and microbiota was studied in a third step.

Invertebrate bioturbators from variable functional groups caused microhabitats with distinct physico-chemical properties (e. g. high oxygen availability in presence of mussels, low oxygen availability in presence of tubificid worms) and therefore differences in bacterial community composition (many aerobe bacteria in the mussel treatment, many anaerobe bacteria in the tubificid worm treatment). Lamprey larvae as vertebrate bioturbators caused big burrows and consequently a strong increase in interstitial oxygen availability followed by a shift in microbial community composition dominated by aerobe bacteria. Pronounced effects in feeding rate (much lower) and a shift in bacterial community composition at the substratum surface were detected in the invasive amphipod treatment compared to the native amphipods. The replacement of indigenous by invasive species caused a shift in organic matter processing with potential alterations in nutrient cycling and therefore in interstitial food web structure.

Vertebrate and invertebrate bioturbators caused strong effects, partly in different directions as revealed by shifts in bacterial community composition. In further studies the interactions of different species in the same microcosms should be investigated. As a loss or replacement of functionally indispensable species potentially results in a deterioration of ecosystem services provided by the interstitial zone (e.g., organic matter processing, nutrient cycling, filter function) it is also important to research effects of other invasive species that inhabit the interstitial zone or have an impact on it in another way (i.e. spawning behaviour). The findings of the present study highlighted the importance of ecosystem services provided by the interstitial zone, which should be taken into account in conservation concepts and hydraulic engineering plans.

Zusammenfassung

Süßgewässer gehören zu den meist gefährdetsten Ökosystemen weltweit. Die Kenntnis der funktionalen Zusammenhänge eines Ökosystems ist unabdingbar um Schutzkonzepte entwickeln zu können. Bei der Erforschung von Flussoberläufen stellt der Übergangsbereich zwischen frei fließendem Wasser und Grundwasser – das Kieslückensystem – die größte Wissenslücke dar. Die interstitielle Zone fungiert als wichtiges Habitat für verschiedene Arten von Invertebraten und Vertebraten, sowie für deren verschiedene Entwicklungsstadien (Eientwicklung, Larvalstadium, Adultstadium). Trotz dieser bekannten, wichtigen Funktionalitäten sind sowohl die Prozesse zwischen frei fließendem und interstitiellem Wasser, als auch die Interaktionen zwischen verschiedenen taxonomischen Ebenen (von Mikroben bis hin zu Vertebraten) in Abhängigkeit der abiotischen Habitatbedingungen noch unzureichend erforscht.

Diese Arbeit soll daher Prozesse und Interaktionen beleuchten, die in der interstitiellen Zone von Fließgewässern stattfinden. Hierzu wurden verschiedene Organismen untersucht, die das Kieslückensystem als Habitat nutzen. Das Hauptaugenmerk lag dabei auf dem Einfluss der untersuchten Organismen auf pysikalische und chemische Habitatbedingungen (Sauerstoffsättigung und –konzentration, Redoxpotenzial, pH-Wert, elektrische Leitfähigkeit und Ionenkonzetrationen von Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}, F^-, Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) sowie die Zusammensetzung der bakteriellen Gemeinschaft. In einem ersten Schritt wurde ein standardisiertes Laborexperiment etabliert. Dazu wurden in sedimentgefüllte Plastikboxen Redoxelektroden für direkte Messungen und Hähne zur Entnahme von Wasserproben eingebaut. Die Mikrokosmen wurden konstant mit Wasser versorgt und mit Versuchsieren besetzt. Funktional unterschiedliche grabende Invertebraten (Eintagsfliegenlarven, Teichmuscheln und Tubifexwürmer) wurden hinsichtlich ihrer Einflüsse auf abiotische Habitatbedingungen und die mikrobielle Gemeinschaft untersucht. Diese Einflüsse wurden in einem zweiten Schritt auch für grabende Vertebraten (Neunaugenlarven) im Vergleich zu einer Kontrolle ohne Tiere untersucht. In einem dritten Schritt wurden darüber hinaus die komplexen Einflüsse von An- und Abwesenheit invasiver und heimischer Amphipoden auf den Abbau von Blattabfällen, abiotische Habitatbedingungen und die mikrobielle Gemeinschaft untersucht.

Wirbellose Bioturbatoren aus verschiedenen funktionellen Gruppen bewirkten die Entstehung von Mikrohabitaten mit unterschiedlichen physiko-chemischen Bedingungen (z.B. hohe Sauerstoffverfügbarkeit in Anwesenheit von Muscheln, geringe Sauerstoffverfügbarkeit in Anwesenheit von Tubifexwürmern) und damit einhergehend Unterschiede in der bakteriellen Gemeinschaft (vermehrt aerobe Bakterien in Mikrokosmen mit Muscheln, vermehrt anaerobe Bakterien in solchen mit Tubifexwürmern). Neunaugenlarven als vertebrate Bioturbatoren riefen einen starken Anstieg des im Interstitialraum verfügbaren Sauerstoffs hervor, was zu einer von aeroben Bakterien dominierten mikrobiellen Gemeinschaft geführt hat. Starke Effekte bezüglich der Fraßrate (wesentlich geringer) und eine Verschiebung der Zusammensetzung der mikrobiellen Gemeinschaft an der Substratoberfläche wurden in Anwesenheit von invasiven Amphipoden im Vergleich zu heimischer Amphipoden festgestellt.

Vertebrate und invertebrate Bioturbatoren haben einen zum Teil starken Einfluss auf das Kieslückensystem. Die Effekte und deren Stärke unterscheiden sich jedoch zwischen den untersuchten Organismen wie die Veränderungen der Gruppierung mikrobieller Gemeinschaften gezeigt haben. In weiterführenden Untersuchungen sollten zusätzlich Interaktionen verschiedener Organismen innerhalb eines Mikrokosmos erforscht werden. Da der Austausch von heimischen durch invasive Arten Änderungen im Abbau organischer Materie mit potentiellen Folgen für den Nährstoffkreislauf und damit für das Nahrungsnetz des Interstitialraums bewirkt, ist es wichtig die Einflüsse weiterer invasiver Arten, die das Kieslückensystem bewohnen oder temporär nutzen (z.B. Laichen), zu untersuchen. Die Ergebnisse dieser Studie verdeutlichten wie wichtig die Ökosystemdienstleistungen sind, die im Kieslückensystem ablaufen (z. B. Abbau organischer Materie, Nährstoffkreislauf, Filterfunktion). Dies sollte bei der Erarbeitung von Schutzmaßnahmen sowie wasserbaulichen Konzepten berücksichtigt werden.

1. The interstitial zone in context of ecosystem services and contemporary impacts

1.1 Ecosystem services

Freshwater ecosystems provide numerous resources and services to humans (Baron et al., 2002), which are mostly provided directly or indirectly by biota (Covich et al., 2004; Hooper et al., 2005; Loreau, 2010). The Millennium Ecosystem Assessment (2005) defined four different categories of ecosystem services: provisioning, regulating, cultural and supporting services.

Provisioning services are those that provide resources such as food, for example fish, mussels, and in many Asian and sub-Saharan African countries as well as in India also freshwater insects (Chen et al., 2009; van Huis, 2003). Particularly for women the latter are a source of income in these countries (Srivastava et al., 2009). Other provisioning ecosystem services are the storage and retention of fresh water for drinking and irrigation (production of food and other resources like fodder and fuelwood), the provision of biochemical products that are mostly extracted from biotic materials including genetic materials useful for pharmaceutical and agro-industrial products (Millennium Ecosystem Assessment, 2005). Additionally to provisioning ecosystem services, regulating ecosystem services provided by rivers and streams are crucial for human well-being: the regulation of climate (e.g., temperature, greenhouse gases) and hydrology (groundwater storage), pollutant control (detoxification, removal of excess nutrients) and the protection from erosion and natural hazards (flood control, retention of soils) are most important (Millennium Ecosystem Assessment, 2005). Cultural services are a bit more difficult to value, because spiritual, recreational, aesthetic and educational aspects are very individual. Nevertheless, freshwaters provide a lot of these services (boating, swimming, religious significance, appreciation of natural features).

Finally, supporting ecosystem services are indispensable for the global metabolism of freshwaters and adjacent ecosystems. The provision of biodiversity (by a variety of macro- and micro habitats) and the soil formation, including the accumulation of organic matter, are important requirements for nutrient cycling (Millennium Ecosystem

Assessment, 2005). Organic matter processing and nutrient recycling are primarily performed by invertebrates and microbes. Aquatic insects (especially their larvae) and amphipods inhabiting the interstitial zone break down organic matter (plant detritus, carcasses) into dissolved organic matter (DOM) and fine particulate organic matter (FPOM). So, the shredding activity increases the surface area of organic detritus and thus prepares it for microbial processing (Cuffney et al., 1990). In addition to shredders, there are further ecosystem engineers that bioturbate and bioirrigate the interstitial zone (Macadam & Stockan, 2015; Pozo et al., 2011; Dafoe et al., 2011, Schwalb & Pusch, 2007). These bioturbators enhance the flux of materials and overlying water across the sediment-water interface and increase the penetration of surface particles and electron acceptors into the sediment (Mermillod-Blondin et al., 2003, Pelegrí & Blackburn, 1996). This in turn stimulates biogeochemical and microbial processes (Wang et al., 2001; Stief et al., 2005; Nogaro et al., 2008). Microbes such as bacteria mainly operate the recycling of nutrients in the stream interstitial zone by catalytic chemical reactions via their metabolic pathways. Key processes are nitrogen recycling (ammonification, nitrification and denitrification) (Lefebvre et al., 2004; 2006) and the carbon cycle (Febria et al., 2010, Nogaro et al., 2013) as well as the phosphor cycle (Mulholland et al., 1997; Vervier et al., 2009). Recycled macro-nutrients (calcium, magnesium, potassium, sodium, chlorine) and trace elements (iron, zinc) are essential for any other organism in freshwater ecosystems such as different algae and macrophytes (Vilmi et al., 2016; Licursi et al., 2016), which in turn are important food resources for organisms of higher trophic levels (Losey & Vaughan, 2006; Brandner et al., 2013a), which partly are food resources for humans (e.g., fish). According to de Groot et al. (2002), the ecosystem services provided by the interstitial zone –nutrient recycling being the most important - are amongst the most valued ones (range between 87 to 21000 US $/ ha per year). With the exchange between open, interstitial and groundwater, happening in the interstitial zone, the latter contributes to the supply of water for human consumptive use (drinking, irrigation, industrial use). Within the flux of open and interstitial water towards groundwater aquifers, the surface water gets purified from high nutrient loads and pollutants (Caschetto et al., 2014) – an important supporting ecosystem service. Another supporting ecosystem service is the provision of a variety of macro- and microhabitats giving refuge to many different species and life stages. The interstices are habitat for lamprey larvae (Beamish & Jebbink, 1994), freshwater insects (Macadam & Stockan, 2015), oligochaetes (Jablonska, 2014), and microbes. Particularly important is the interstitial zone as spawning ground for diverse species such as trout, salmon, and greyling (Sternecker et al., 2013a; 2013b; Kondolf, 2000; Crisp, 1996) and their fry (Sternecker et al., 2013a; 2013b). Furthermore, it serves as important habitat for mussels (Geist & Auerswald, 2007, Denic et al., 2014b) and especially their sensitive life stage as young mussels during their critical post-parasitical phase (Geist & Auerswald, 2007; Geist, 2010).

Finally, some biota inhabiting the interstitial zone even provide cultural services. Freshwater insects for example are model organisms for monitoring the quality of fresh waters (Orwin & Glazaczow, 2009) and some of them are imitated for catching fish (artificial flies) (Macadam & Stockan, 2015). Additionally, literature, art, music, traditional medicine, and witchcraft are inspired by freshwater insects such as dragonflies. Especially children gain educational benefits from inhabitants of the interstitial zone while they are ´pond-dipping´ or ´creeking´ (as it is known in the U.S.) and thus get to know different species hiding in the interstitial zone and get educated about freshwater biodiversity (Macadam & Stockan, 2015).

1.2 The interstitial zone and contemporary impacts

In 1959, the interstitial zone (also known as hyporheic zone) was defined for the first time as the interface between surface and ground water within river substrata (Orghidan, 1959), followed by a few decades of interstitial zone research dominated by characterizing communities of benthic and groundwater organisms (Marmonier et al., 2012). More than 50 years later, the importance and relevance of the interstitial zone for the global river diversity are well established (reviewed by Marmonier et al., 2012). Moreover, it is accepted that the processes taking place within the interstitial zone are of great importance for the metabolism and functioning of rivers and streams (Boulton et al., 1998; Krause et al., 2011). To date, the interstitial zone is frequently characterized as a very dynamic and fluctuating ecotone depending on variable abiotic and biotic factors (Brunke & Gonser, 1999; Fowler & Scarsbrook, 2002; Lautz & Fanelli, 2008; Krause et al., 2009).

After a long time of considering different zones within ecosystems as separate research units, it is of increasing importance to study them as functionally linked and dependent ecotones and to think across boundaries. The stream interstitial zone is influenced by a variety of other fluvial zones (Fig. 1.1) and parameters and exhibits therefore high habitat heterogeneity (Braun et al., 2012). Beginning with impacts on the catchment scale, geomorphology and geological processes in the headwater regions determine many physical and hydrological phenomena from the montane to the coastal plains, such as fine-scale granulometric features (Boulton et al., 1998 and cited references). Boulton et al. (1998) further state that size, shape and composition of river substrates depend on the nature of upstream rocks and are additionally a product of three-dimensional flow conditions (horizontal and vertical direction as well as velocity). Figure 1.1 gives an example of possible hydrological flow paths between different areas adjacent to the stream interstitial zone. The variety of water fluxes shown in Fig. 1.1 is enhanced with increasing geomorphological heterogeneity in the flood plain, the parafluvial zone, and the streambed. Additionally, the introduction of particles, influenced by the kind of land use in the catchment area, affects habitat properties in the interstitial zone. In case of intensive cropland use for the cultivation of corn, high loads of fine sediments are introduced into rivers and consequently clog the interstices. This affects physical as well as chemical processes (mainly mediated by microbes) in the reach scale of the interstitial zone since hydrological exchange rates are decreased (Schälchli, 1992). It is of great importance to understand the vertical changes in physico-chemical and hydrological characteristics (Marmonier et al., 2012) in order to be able to predict the long-term effects of anthropogenic influences.

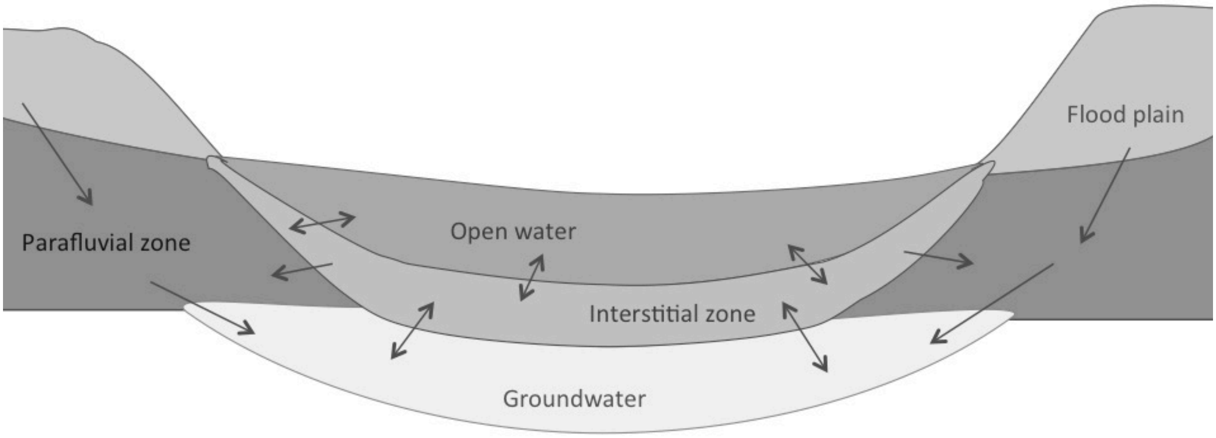

Fig. 1.1: The stream interstitial zone and its adjacent areas with possible hydrological flow paths. Arrows indicate different flow directions between water bodies and water-bearing formation.

The link between physico-chemistry, microbial communities and biotic activities in the interstitial zone is barely understood (Marmonier et al., 2012 and cited references). Biotic activities can be performed by ecosystem engineers, organisms that have the potential to alter habitat properties via their life style (e. g., burrowing of feeding behaviour). They are very common in marine and freshwater ecosystems, but their way of bioturbating substrata can be very diverse. Many bioturbators such as tubificid worms, many insect larvae, lamprey larvae and mussels inhabit at least for a certain time period of their life benthic habitats including the stream interstitial zone (Mermillod-Blondin & Rosenberg, 2006; Zieritz et al., 2014; Winkelmann & Koop, 2007; Shirakawa et al., 2013). Additionally, gravel-spawning fish species like trout and salmon are well-known examples that periodically (e.g., during spawning period) bioturbate the interstitial zone to increase hatching success of their fry by washing out fine sediments and consequently create more oxygen-rich conditions (Sternecker et al. 2013a; 2013b; Hassan et al., 2015). Moreover, species can alter habitat properties in the interstitial zone due to their feeding habits, such as amphipods, which are crucial for leaf litter degradation (MacNeil et al., 2011) and therefore might be linked to microbial assemblages. Consequently, microbial processes themselves are highly influenced by bottom up (nutrient availability) and top-down (consumer activity) factors (Cheever & Webster, 2014) – fluctuation and dynamics of the interstitial zone in a smaller dimension. In recent literature, the role of ecosystem engineers in interaction with bacterial communities has been underrepresented (Mermillod-Blondin et al., 2003;

Hunting et al., 2012). Most studies considered effects of ecosystem engineering on physical and chemical habitat properties (Fellows et al., 2001; Wang et al., 2001; Lewandowski et al., 2007; Gallon et al., 2008; Lowell et al., 2009; Febria et al., 2010; Shirakawa et al., 2013) or inter-species relationships (de Haas et al., 2005), but not the interaction with bacteria. Important functions of bacterial communities have often been studied in dependence of pollutants (Feris et al., 2003; Winderl et al., 2008; Rastogi et al., 2011; Stauffert et al., 2013; Johnston & Leff, 2015) and in context of nutrient cycling (Pelegri & Blackburn, 1995; Marshall & Hall, 2004; Maitra et al., 2015), but without considering top-down biotic impacts. The gap in knowledge for freshwater ecosystems is the link between both fields of study, which is in contrast to the situation in marine biology, where many studies deal with bioturbator-microbiota interactions (Laverock et al., 2010; Maryam et al., 2015; Wada et al., 2016) or with effects of artificially engineered sites on bacteria (Forth et al., 2015). Taken together, biotic and abiotic factors are closely linked and interdependent in the interstitial zone and the effects of single drivers cannot be studied isolated from each other (Shade et al., 2008).

To study different drivers of interstitial processes at the same time and compare them directly to each other, new methodological approaches for standardized data collection are necessary. In addition to the establishment of methods that allow a description of vertical gradients and temporal variations of physico-chemical characteristics in the interstitial zone with a possible upscale to reach and catchment processes (Marmonier et al., 2012), standardized (climate, light cycle) microcosm experiments are crucial to study interactions between biota of different trophic levels (bacteria, invertebrates, vertebrates) in combination with physico-chemical habitat properties. Moreover, microcosm experiments are beneficial for studying a high number of replicates at the same time in order to get statistically significant results. Marmonier et al. (2012) pointed out that there is a need for descriptors of stability and resilience. This field can be studied by comparing different functional groups of organisms, as well as indigenous and invasive species, and their effects on stream interstitial processes.

After achieving a basic understanding of interstitial processes such as interactions between vertebrates, invertebrates and bacterial communities in combination with abiotic habitat properties, a next step must be the evaluation of anthropogenic influences on interstitial assemblages and functions, like the effects of invasive species.

Introductions of invasive species are among the most important, least controlled, and least irreversible of human impacts on the world´s ecosystems, strongly affecting their biodiversity, biogeochemistry, and economic uses´ (reviewed in Strayer, 2010). Deliberately introduced species are such of high economic value, for example salmonids (aquaculture, sport fishing) that potentially affect native food webs (predation on macro invertebrates) (Baxter et al., 2004). Invasive species can be introduced undeliberately by anthropogenic vectors like ballast water tanks of globally operating ships, bait bucket escapes and aquarium releases and their dispersal is further facilitated by anthropogenic connections of different drainage systems (e.g., Rhine-Main-Danube channel). In invasive species research two main topics are discussed: first, the rapid changes the invader undergoes itself during and after the establishment process (Cerwenka et al., 2014a; 2014b; Elst et al., 2016) and second, the responses and dynamics of the invaded ecosystem. These include inter- (Britton, 2012; Brandner et al., 2013a; Makarewicz et al., 2016) and intra-species dynamics (Brandner et al., 2013b; Cerwenka et al., 2014a; 2014b) with alterations of food web structures (Brandner et al., 2013a; Riley et al., 2008).

The invasive Ponto-Caspian amphipod *D. villosus*, also referred to as ´killer shrimp´, is one of the worst 100 invasive species in Europe (DAISIE, 2009). Authors reporting on the ´killer shrimp´ focused on diverse topics, such as its diet and impacts on food webs, (Eckmann et al., 2008; Maazouzi et al., 2009; Piscart et al., 2011; Dodd et al., 2014), partly in interaction with climate change (Pellan et al., 2015). Additionally, the invaded regions (Rewicz et al., 2015; Koester et al., 2015), the distribution vectors (Baçela-Spychalska et al., 2013; MacNeil & Platvoet, 2013; Baçela-Spychalska, 2015) and the reasons for its invasive success, such as life history traits, reproductive capacity or robustness to exogenous factors are well studied (Devin et al., 2004; Poeckl et al., 2009; Sroda & Cossu-Leguille, 2011; Rewicz et al., 2014; Briffa et al., 2016). Finally, there are studies dealing with risk assessment and it's modelling (Gallardo et al., 2012; Gallardo & Aldridge, 2013; Boets et al., 2014). Although top-down effects in inter-species dynamics are an essential part of the puzzle how invasive species modify their environment, the effects of *D. villosus* on bacterial community composition have not been studied so far.

1.3 Objectives of the thesis

The overall objective of the present thesis is to elucidate biotic impacts on ecosystem functioning of the stream interstitial zone and to evaluate the role of organisms from variable taxonomic levels (Fig. 1.2). Standardized laboratory microcosm experiments were established to compare the effects of burrowing invertebrates and vertebrates, and indigenous versus invasive amphipods, using a multivariate statistical approach with a focus on the interactions between ecosystem engineers, substratum conditions and bacterial diversity in the stream interstitial zone (Figs. 1.2 and 1.3).

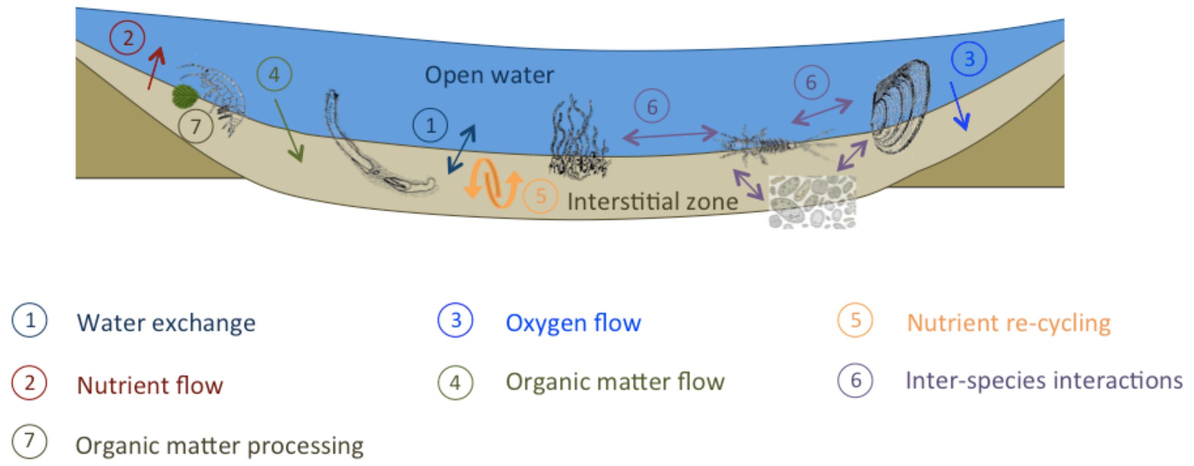

Fig 1.2: Interactions and functional traits in the stream interstitial zone studied and discussed in the present thesis.

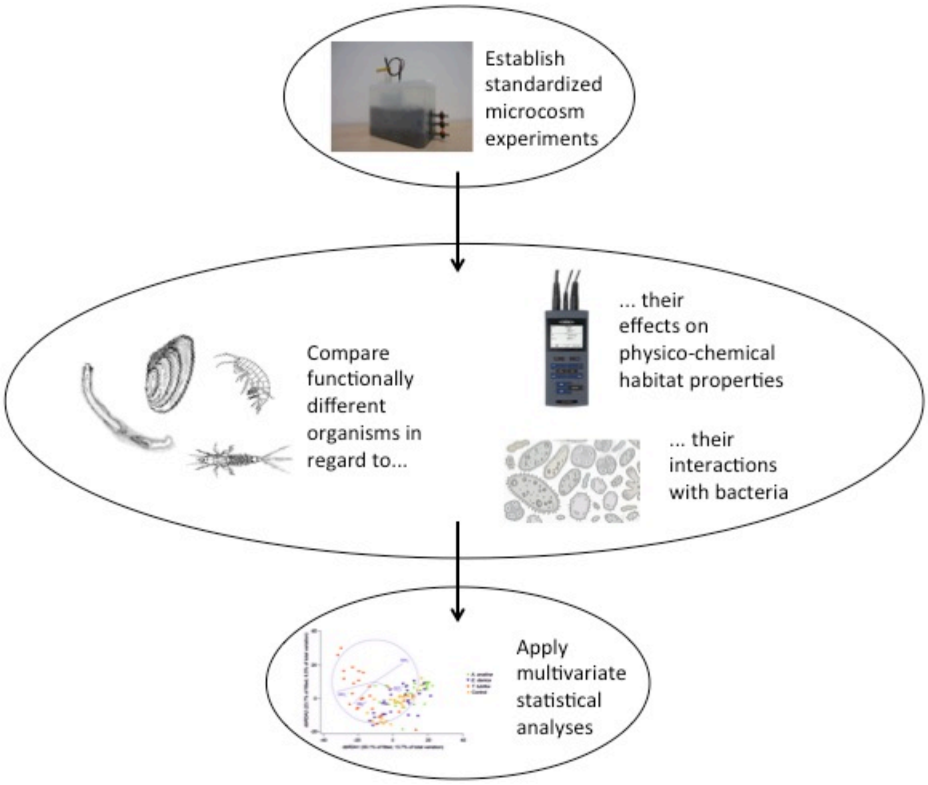

Fig 1.3: Methodological approach how to study interrelations of abiotic (physical and chemical habitat properties) and biotic factors (on a macroscopic and a microscopic level) in order to contribute to a better understanding of ecosystem functioning.

The aims of a first study were to evaluate the effects of three functionally different (size, burrowing depth, burrowing style) macroinvertebrates (*E. danica*, *T. tubifex* and *A. anatina*). It was hypothesized that (i) the analyzed organisms modulate physico-chemical conditions in the interstitial and open water distinctly which should be reflected in (ii) different microbial community composition in the presence of *E. danica*, *T. tubifex* and *A. anatina* versus the control, respectively.

Additionally to the effects of macroinvertebrates, vertebrate ecosystem engineers (larvae of *Eudontomyzon* sp.) were investigated in a second study. It was hypothesized that (i) physical and chemical habitat properties and (ii) microbial community composition between the controls and the lamprey treatments differ with regard to a more aerobic micro-environment in the lamprey treatment. Furthermore, alterations in

N-cycling as a consequence of a shift in microbial community composition after the introduction of lamprey larvae into the microcosms were expected.

To evaluate future impacts on the stream interstitial zone due to the replacement of indigenous by invasive species, a third study concentrated on the effects indigenous (*G. roeseli*) and invasive amphipods (*D. villosus*). It was assumed that the invasive *D. villosus* is functionally different compared to the indigenous *G. roeseli*. Therefore, the two species were compared for (i) differences in the feeding rate on alder leaves, (ii) the alteration of physico-chemical habitat properties in open and interstitial water, and consequently, (iii) differences in microbial community composition on the substrate surface and at five cm substrate depth.

2. Field and laboratory procedures

2.1 Study design

The studies included in this thesis were conducted using similar, standardized experimental setups. The impacts of different biota on pysico-chemical habitat properties and the bacterial community composition were evaluated in 35-day laboratory experiments under standardized conditions using microcosms (Boeker & Geist, 2015; Boeker & Geist 2016, Boeker et al., 2016).

For statistical purposes each experiment included nine biological replicates (nine microcosms) per treatment. Since data in ecological experiments often exhibit a high variability within the same group (Potvin & Roff, 1993), an appropriate number of replicates is required to detect statistically significant effects. The mathematical advantage of evaluating a high number of independent replicates over evaluating a low number of the latter is that the data set containing more replicates is more robust in terms of outliers. As a consequence, with an increasing number of independent replicates within a group of data, the within-group standard deviation decreases:

$$\sqrt{\frac{1}{n-1} \cdot \sum_{i=1}^{n} (x_i - \bar{x})}$$

A low within-group standard deviation increases the probability to detect slight between-group differences and to get a statistically significant result.

As described in Boeker & Geist (2015), Boeker & Geist (2016) as well as Boeker et al., (2016), the temporal process of the experiments was divided into two phases. After the beginning (day -15) of each experiment an initial phase of 15 days followed (day -15 until day 0) to determine a baseline (Fig. 2.1). During these days all microcosms were supplied with water but were not stocked with experimental animals. Physico-chemical parameters in open and interstitial water were measured on days -15, -10, -5 and 0. After that, the microcosms were stocked with the experimental animals and following 20 days with measurements of physico-chemical parameters in open and interstitial water on days 5, 10, 15, and 20. Additionally on day 0, 10 and 20, samples from the interstitial were taken for bacterial community composition analyses and ion chromatography (Fig. 2.1).

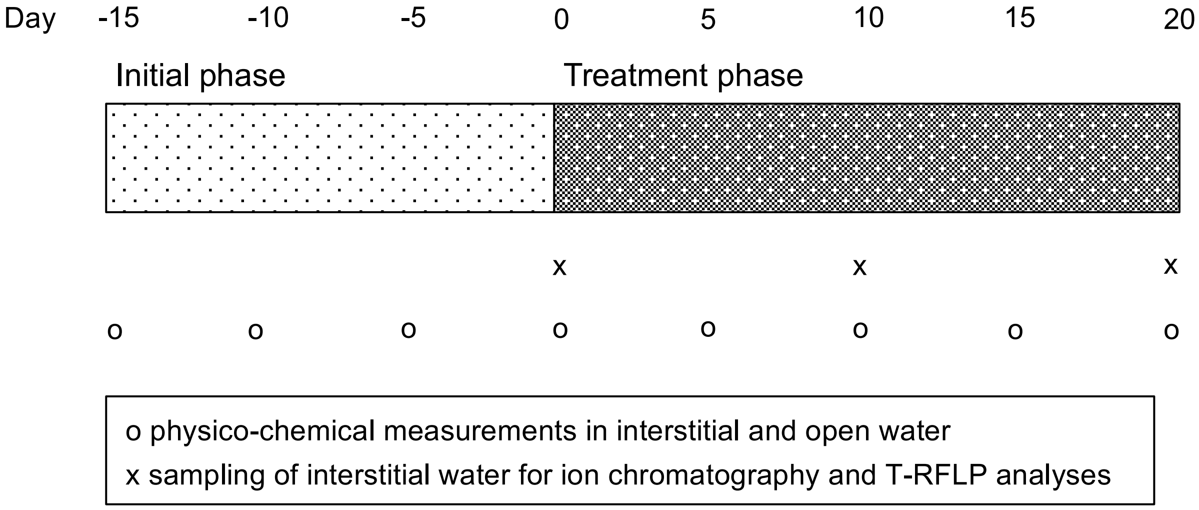

Fig. 2.1: Timetable for 35-day microcosm experiments divided into an initial phase without treatments and a treatment phase with treatments. On day -15, -10, -5, 0, 5, 10, 15 and 20 physico-chemical parameters were measured in open and interstitial water. Additionally, on day 0, 10 and 20 samples from interstitial wter were taken for ion chromatography and T-RFLP analyses.

The initial phase (day -15 until 0) was important to generate similar experimental conditions in all microcosms. Initial differences between microcosms in abiotic habitat properties and the microbial community composition decreased during the first 15 days. At the same time, characteristically interstitial habitat properties developed, such as low oxygen availability in the artificially generated interstitial zone. Both conditions, a low variability between microcosms, and characteristically habitat properties were indispensable for a correct data evaluation and interpretation of the results and for transferring the results to an ecological reasonable meaning.

To create natural conditions and to generate standardized laboratory conditions in order to exclude as many unknown variables as possible, the experiments were performed with a constant 12 hour light-dark cycle.

2.2 Experimental setup

The microcosms providing the artificial interstitial zone were placed randomly into three fiberglass reinforced plastic-channels (3.55 x 0.45 x 0.17 m, AGK Kronawitter GmbH, Wallersdorf, Germany) according to the balanced latin square scheme. Every microcosm was constantly supplied with water from the Moosach River (a calcareous river, 48 ° 23´ 39.22“ N; 11 ° 43´ 26.65“ E) by a dripping system with a flow rate of 3.5 – 4.0 l/ h per microcosm (Fig. 2.2). The overflowing water from the microcosms was collected in the plastic channels up to five cm below the height of the microcosms. Therefore, the temperature regime in each microcosm was very similar, which was required for standardized experimental conditions and comparable results. Moreover, the water from the Moosach River was pumped into two connected, FDA conform 1000 l tanks in the laboratory (Fig. 2.3) (Boeker & Geist, 2015; Boeker & Geist, 2016; Boeker et al., 2016).

Fig. 2.2: Overall experimental setup. On the left are two 1000 l tanks filled with water from the Moosach River that is pipelined to each of the microcosms, put randomly in the artificial flow channels (green).

Fig. 2.3: Two microcosms constantly supplied with water from the Moosach River by a dripping system via a pipeline system (dark grey) and therein installed control valves (red).

2.3 Microcosms

As microcosms, plastic boxes solid on five sides (24.2 x 15.5 x 18.2 cm, Rotho Kunststoff AG, Würenlingen Switzerland) were equipped with a lid of gauze (mesh width 500 µm, Aquacultur Fischtechnik GmbH, Nienburg, German) to prevent the experimental animals from escaping and to allow a constant top-down flow of water into the microcosms. All microcosms were filled with a 10.0 cm substratum layer. The substratum was taken from the Moosach River, sieved into defined grain size fractions (0.85, 0.63 and 0.063 mm in a proportion of 1.5:0.5:4.0) and dried at 90 °C. The substratum contained no animals when it was filled into the microcosms. Additionally, three perforated pipes (Volume 5.0 ml, Gardena GmbH, Ulm, Germany) were installed in 2.5, 5.0 and 7.5 cm depth of the 10 cm substratum layer. At one end, each pipe had a control valve for collecting samples of interstitial water without disturbing interstitial habitat properties and bacterial communities by penetrating the substratum with a pipe to suck interstitial water (Fig. 2.4). For the same reason (non-invasive measurements), a Platinum electrode (ELANA, Boden und Wasser Monitoring, Arendsee, Germany) was installed fix into the box (Fig. 2.4). Pre-tests revealed that measurements in three different substratum depths do not increase the ecological information to an adequate extend in relation to the additional expenses. The most significant depth identified by pre-tests (5.0 cm substratum depth) was chosen for redox measurements and sampling of interstitial water in all experiments included in this thesis as described in Boeker & Geist (2015), Boeker & Geist (2016) and Boeker et al. (2016).

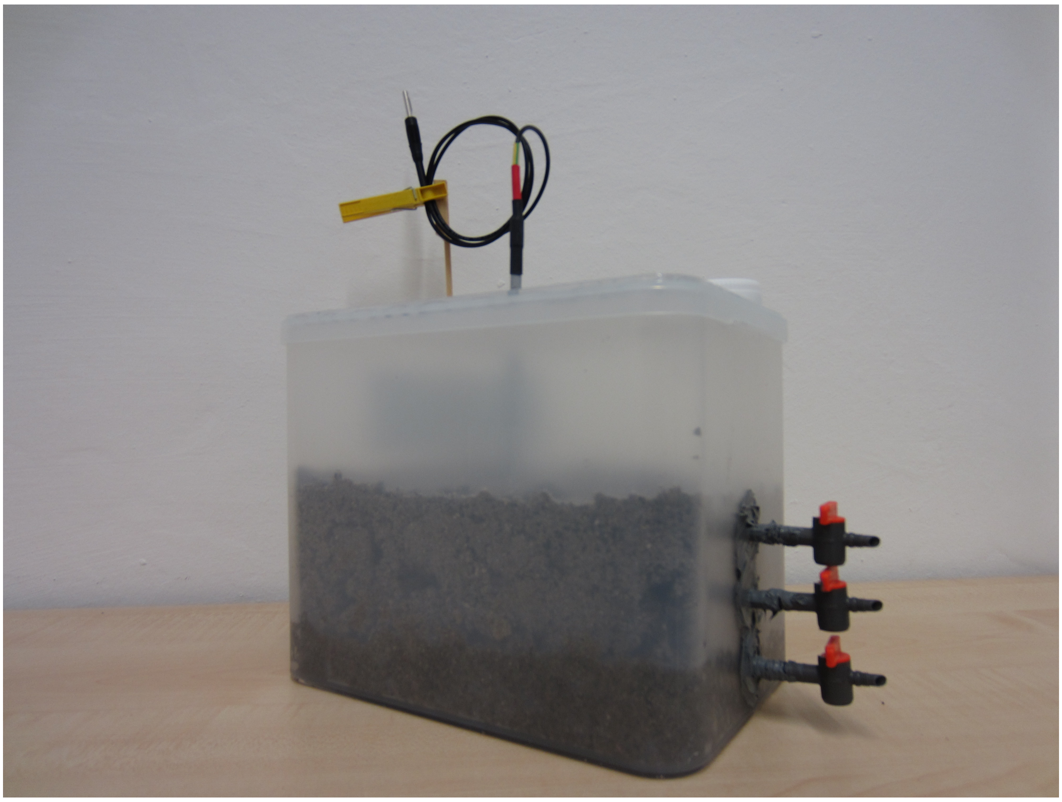

Fig. 2.4: Microcosm with installed redox platinum electrode and cable (black) on the top as well as fix installed pipes (dark grey) with control valves (red) for sampling of interstitial water.

2.4 Analyses of physico-chemical habitat properties

Physico-chemical parameters were measured in the open and interstitial water every five days. Measurements in open water were conducted 5 cm above the substratum surface. Samples of interstitial water were taken using the control valve of the installed pipe in 5 cm substratum depth after the volume of the pipe was purged. Physico-chemical measurements were conducted immediately in the water sample. Oxygen concentration and saturation as well as the redox potential as long-term oxygen indicator were measured because they are very important habitat properties for bacteria since some of the latter depend on certain oxygen concentrations (Kalvelage et al., 2011). The redox-potential in 5 cm substratum depth was measured according to Geist & Auerswald (2007). For further characterization of the interstitial zone and open water, pH and electric conductivity were measured. All measurements were conducted using a handheld WTW Multi 3430 Set G (Wissenschaftlich Technische Werkstätten, Weilheim, Germany) and a WTW pH 3110 Set-2 (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). In order to avoid cross contamination, all probes were rinsed with destilled water after measurements in each microcosm. Since different forms of N and other substrates for bacteria allow a more detailed and ecological relevant interpretation of physical and genetic results, water samples from days 0, 10 and 20 were immediately frozen at -20 °C for later ion detection because different bacterial community compositions are especially relevant in terms of nutrient cycling, for example N-cycling (Mao et al., 2013). For anion detection (i.g., Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}, F^-) with an ICS-1100 ion chromatograph, a AS-22 separation column after a AG-22 guard column was used in combination with a 1.8 mM dinatriumcarbonate and 1.7 mM natriumhydrogencarbonate eluent (all Thermo Fisher Scientific, Dreieich, Germany). A CG-12 guard column followed by a CS-12 separation column was used for cation detection (i.e., Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) with a 20 mM methanesulfonic acid as eluent in an ICS-1100 ion chromatograph (all Thermo Fisher Scientific, Dreieich, Germany) (Boeker & Geist, 2015; Boeker & Geist, 2016; Boeker et al., 2016).

2.5 Bacterial community analyses

The bacterial community composition was investigated using terminal restriction fragment length polymorphism (T-RFLP) fingerprinting as described in Boeker & Geist (2015), Boeker & Geist (2016), and Boeker et al. (2016) and cited references. This method allows a cost-effective and rapid overview on the composition of different operational taxonomic units (OTUs) with the potential to integrate taxonomic information (Witzig et al., 2015). For the purposes of this thesis the fingerprinting method is sufficient, because for the interpretation of the multivariate dataset (abiotic, biotic) the results of a more detailed investigation method such as pyro-sequencing would not be very meaningful in the holistic approach of the study.

For the detection of terminal restriction fragments (T-RFs) from samples taken from interstitial water, the latter were filtered through a 0.22 µm cellulose mixed ester (CME) membrane (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Since bacteria are normally bigger than 0.22 µm (Andersen et al., 2015) all bacteria in the samples of interstitial water were fixed to the CME membrane. The filters were frozen immediately in sterile Petri dishes at -20 °C. For bead-beating assisted DNA extraction the filters were cut into pieces of 0.5 cm^2 using a sterile scalpel as described in Mueller et al. (2013a) and put into a sterile 2.0 ml screw cap reaction tube filled with 0.1 zirconia/ silica beads (1:1). For the detection of bacterial T-RFs from substratum samples, 0.8 g of a representative substratum sample were put into a sterile 2.0 ml screw cap reaction tube filled with 0.1 zirconia/ silica beads (1:1) for bead-beating assisted DNA extraction. The adherent biofilm on the substratum could consequently be analysed. For the detection of bacterial T-RFs from mucus of experimental animals the sterile tissues with the adherent mucus sample were cut into pieces (0.5 cm^2) with a sterile scalpel according to Mueller et al. (2013a) and were put into a sterile 2.0 ml screw cap reaction tube filled with 0.1 zirconia/ silica beads (1:1) in order to conduct a bead-beating assisted DNA extraction.

The bead-beating procedure, which is an important cell lysis step, and subsequent phenol- chloroform DNA extraction were carried out as described in Pilloni et al. (2011) and Lueders et al. (2006). Polymerase chain reaction to generate FAM-labelled amplicons was conducted according to Pilloni et al. (2011). Additionally, a purification step after the PCR was performed using PCRExctract colums (PCRExctract Mini Kit, 5 PRIME GmbH, Hamburg, Germany) according to the manufacturer´s protocol to get clear results in the capillary electrophoresis (ABI 3730 Sequencer, Applied Biosystems, Carlsbad, California, USA). After amplification and purification, a restriction step using MspI followed before separating by capillary electrophoresis as described in detail by Lueders et al. (2006).

To evaluate the electropherograms generated by the sequencer, the software Gene Mapper 5.1 (Applied Biosystems, Carlsbad, California, USA) was used. To create a T-RF abundance data matrix from the Gene Mapper file, the free online tool T-REX (Culman et al., 2009) was used by aligning the peak heights. As described in Abdo et al. (2006) a default factor of 1.2 was set for background noise filtering. Using the default alignment method specified by Smith et al. (2005) the clustering threshold was set to 1.5 for peak alignments. Since the complexity of sequencing data was very high, T-RFs that occurred in less than ten percent of the total samples were eliminated according to Blackwood et al. (2007).

As described in Mueller et al. (2013a), different technical replicates of the T-RFLP fingerprinting exhibited the same OTU composition and only slight differences in relative abundances. Therefore, the studies included in this thesis contain nine biological replicates per treatment and no technical replicates which also agrees with a time a cost efficient method of operation.

One disadvantage of the high throughput T-RFLP-method is that no conclusions can be drawn concerning functional groups of the detected bacteria or any ecological meaning of the genetic data set. Combined with abiotic data such as values for oxygen concentration, the online tool MiCa (Microbial Community Analysis III) represents a potential instrument to get an indication what type of bacteria can be linked to the T-RFLP data in combination with the used primers and restriction enzymes. According to Shyu et al. (2007) the T-RFs generated in the studies of this thesis were analysed with MiCa database search with a sensitivity of maximum two mismatches within two bases from the 5´ end of the primer in the RDP (R10, U27) 16S bacterial rRNA database.

2.6 Experimental animals and permissions

All animals used in the experiments were caught and kept according to the national guidelines for the care and use of animals. Conformable to German law, for experiments with vertebrates permission has to be obtained (Az. 55.2-1-54-2532-180-13). Invertebrates could be used in experiments without permission.

2.6.1 Invertebrate ecosystem engineers

To study the effects of invertebrate ecosystem engineers three different bioturbators were chosen as test animals: the tubificid worm *Tubifex tubifex*, the nymph of the mayfly *Ephemera danica*, and the freshwater mussel *Anatina anatina*. On the one hand, they have in common that they live in the interstitial zone. One the other hand, each of the species belongs to different functional group of bioturbators (biodifusor, regenerator and upward-conveyor), characterising them as ideal targets for the study. To detect significant and ecologically effects but to generate comparable results at the same time, the densities of the animals were estimated by combining natural densities with the demand for a standardized laboratory experiment in terms of comparable dry weights of bioturbators between treatments. Howard and Cuffey (2006) tested densities of 20, 40 and 80 unionoid mussels per m^2 in their experiments, Krieger et al. (2007) reported on densities of mayfly nymphs of 1000 individuals per m^2 and Jabłońska (2014) examined oligochaetes with average densities between 6000 and 365,000 individuals per m^2. Based on that literature and in accordance to preferably similar dry weights, three *A. anatina* (mean dry weight per microcosm = 7.98 g), 40 *E. danica* nymphs (mean dry weight per box = 3.20 g) and approximately 6,000 *T. tubifex* (mean dry weight per box = 4.44 g) were separately introduced in each of nine microcosms per treatment (nine replicates per treatment). As a control, nine boxes remained unpopulated with macroinvertebrates.

All animals were kept under identical conditions for 14 days in basins at the Chair of Aquatic Systems Biology of the Technische Universtität München, Germany for acclimatization before they were introduced into the microcosms where they remained for 20 days. Once a week during acclimatization, the mussels were fed with 50 ml 0f 1:10 diluted Shellfish Diet 1800 (Reed Mariculture Inc., Campbell, USA) and the mayfly nymphs with 7.0 g of thawed chironomid larvae (Vivantis, Animal Quality GmbH, Isernhagen, Germay). During the experiment the animals were not fed to avoid additional nutrient load (e.g. NH_4^+) holding the risk of adulterating the water-chemical results.

2.6.2 Vertebrate ecosystem engineers

In addition to the tested invertebrates, the effects of a vertebrate ecosystem engineer exhibiting a different burrowing pattern compared to the invertebrates should be evaluated. According to German law, the experiment was permitted by the Bavarian State Ministry of the Environment and Consumer Protection (Az. 55.2-1-54-2532-180-13) and the sampling of lamprey larvae by the District Office of Deggendorf (Az. 32-7562/Ni). All national guidelines for the care and use of animals were followed. Every day, the health status was documented. No lamprey larvae died or had to be euthanized throughout the experiment. The lamprey larvae were caught at the Große Ohe River (Danube drainage, 48° 43´48.32” N, 13° 15´14.73” E) with permission of the regional authorities by excavating sediments from potential habitats using a shovel and searching them in the sediment by hand. Before the introduction into the microcosms all lamprey larvae (average size 9 ± 1.5 cm) were acclimatized under identical conditions for two days in basins with the same substrate and water as in the microcosms at the Aquatic Systems Biology Unit of Technische Universität München, Germany.

On experiment day 0, two lamprey larvae were put into each of nine microcosms. The same number of microcosms remained unpopulated with animals as a control. Test animals were not fed since natural open water containing particulate matter as food was supplied. After 20 days, the experiment was terminated. Acting in agreement with the permission, all lamprey larvae were returned to the site they were caught at the Große Ohe River after a positive health check.

2.6.3 Indigenous and invasive amphipods

Complementary to the effects of burrowing organisms, the indirect impacts of anthropogenic influences on the interstitial zone such as smoothing the way for invasive benthic species should be evaluated in this thesis. Appropriate test organisms in the context of the study were amphipods. They are well known organisms in ecotoxicological investigations, so there was enough information about how to keep and feed them in a laboratory system (Sroda & Cossu-Leguille, 2011). Additionally the invasive potential and invasion history of certain species is well documented (Rewicz et al., 2014). Moreover, there are still sites remaining where indigenous amphipods can be sampled. Therefore, indigenous amphipods (*Gammarus roeseli*) and invasive ones (*Dikerogammarus villosus*) were compared in regard to their effects on interstitial zone properties, leaf litter decomposition and the bacterial community composition.

Individuals of *D. villosus* were kick-sampled with permission of the fisheries association Kelheim, Germany in the Danube River at Kelheim (48° 54´ 35.5´´ N, 11° 51´ 07.4´´ E), those of *G. roeseli* were sampled in the Moosach River (48° 23´ 39.22´´ N; 11° 43´ 26.65´´ E, Danube drainage system, Freising, Germany). For standardization, only specimens which were small enough to pass through a sieve with a mesh width of 3.0 mm, and large enough to be retained on a sieve with a mesh width of 1.5 mm were used. Before the amphipods were introduced into the microcosms they were kept for one week in basins at the Chair of Aquatic Systems Biology of the Technische Universität München, Germany in the same water as that in the microcosms. During that time, they were fed with pre-incubated alder leaves(*Alnus glutinosa*). Alder leaves were collected in autumn and one standardized triangular piece (4.62 cm^2; 0.035 ± 0.007 g dry weight) was cut out of every leaf at the same position and dried at room temperature. Since the dry leaf pieces have to be settled by a biofilm before amphipods can feed on them, the leaves were pre-incubated for seven days (Dedourge-Gafford et al., 2009) in water from the Moosach River. Every second day, 80 % of the water for leaf pre-incubation was replaced by fresh water from the Moosach River. On day 0, 30 *D. villosus* or 30 *G. roeseli* were introduced into each of nine boxes for the respective treatments, whereas nine control boxes remained unpopulated with amphipods. The test animals remained in the microcosms for 20 days and were fed every day with one piece of a pre-incubated alder leaf per microcosm. The remainder of the triangular piece of leaf from the previous day was taken out of the microcosm and dried, to prevent from further decomposition by microorganisms, until analysis. All control microcosms were treated in the same way as those with test animals. To determine the feeding rate of the amphipods, the remaining leaves were photographed and the areas measured with Image J (version 1.48v). For statistical analyses, the areas of the leaf-pieces from the controls were taken as 0 % feeding rate. Dead specimens were removed and replaced daily.

2.7 Data analyses

Univariate statistics were calculated using R (version 3.0.2, www.r-project.com, 2013). Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene´s test). Pairwise comparisons were performed using the non-parametric pairwise Wilcox rank sum test with Bonferroni correction since no data were normally distributed. Significance in all tests was accepted at $p \leq 0.05$ (after Bonferroni correction). Boxplots were plotted using R.

For multivariate data analyses the Software PRIMER v.6 with the PERMANOVA+ add on (Plymouth Marine Laboratory, Plymouth, United Kingdom) was used. Based on the microbial community analysis, the DIVERSE function was used to compute different diversity indices (i.e., operational taxonomic unit (OTU) richness, Shannon diversity, Simpson and Evenness index including standard deviations (SD)). Similarity percentages (SIMPER) analyses using Euclidian distances were performed to reveal which TRFs contribute most to the average similarity per treatment. To test whether biotic or abiotic data differ between treatments or sampling time-points (day 0, 10 and 20), permutational multivariate analysis of variance (PERMANOVAs) were performed with 999 permutations under the assumption of a reduced model using the sum of squares type III (partial). In the PERMANOVA design, the depending factor was nested-fixed in the independent factor (e.g., factor treatment was nested fixed in the factor day). Distance based linear models (DISTLMs) were used to analyse which abiotic factors explain the variability in the bacterial community data set with a number of 999 permutations using Bray-Curtis similarities as distance measures. These relationships were illustrated by distance based redundancy analyses (dbRDAs). Biota and environment matchings (BESTs) were calculated using the Spearman rank correlation method to verify DISTLM data. Non-metric multidimensional scaling (NMDS) were plotted to display differences between bacterial communities between variable factors (e.g., treatments).

3. Alteration of physico-chemical and microbial properties in freshwater substrates by burrowing invertebrates

A similar version of this section is published:
Boeker C., Lueders T., Mueller M., Pander J., Geist J. (2016) Alteration of physico-chemical and microbial properties in freshwater substrates by burrowing invertebrates. *Limnologica – Ecology and Management of Inland Waters* 59:131-139.

3.1 Abstract

The hyporheic interstitial provides habitat for many different organisms - from bacteria to burrowing invertebrates. Due to their burrowing and sediment reworking behaviour, these ecosystem engineers have the potential to affect hyporheic processes such as respiration and nutrient cycling. However, there is a lack of studies that characterize the interactions between bioturbators, physico-chemical habitat properties and microbial communities in freshwater substrates. In a standardized laboratory experiment, we investigated the effects of three functionally different bioturbators, duck mussels (*Anodonta anatina*, Linnaeus 1758), mayfly nymphs (*Ephemera danica*, Müller 1764) and tubificid worms (*Tubifex tubifex*, Müller 1774), on the physico-chemical conditions and bacterial communities in hyporheic substrates. We hypothesized that different invertebrates distinctly alter habitat conditions and thus microbial community composition, depending on the depth and the manner of burrowing. *A. anatina* and *E. danica* caused an increase in interstitial oxygen concentration, whereas strong declines in oxygen concentration and redox potential were detected in the *T. tubifex* treatment. These effects on physico-chemical habitat properties were even detectable in open water. Mussels and tubificid worms also significantly influenced the composition of bacterial communities in the hyporheic zone. A loss or replacement of bioturbators in stream ecosystems due to anthropogenic habitat alterations is expected to result in shifts in microbial community compositions, with effects on nutrient fluxes, pollutant degradation and benthic food webs. An understanding of the effects of functionally different native and invasive bioturbators is crucial to predict changes in stream ecosystem functioning.

3.2 Introduction

The hyporheic zone is closely linked with ecosystem functions and services (Geist 2014). Due to its heterogeneity, it provides a variety of microhabitats inhabited by microscopic and macroscopic organisms (Braun et al., 2012; Lowell et al., 2009) such as the larvae of unionoid freshwater mussels (Geist 2010), Ephemeroptera (de Haas et al., 2005; Percival & Whitehead, 1926; Stief et al., 2004) and Oligochaeta (Mermillod-Blondin et al., 2001; Pelegri & Blackburn, 1995). Species inhabiting the hyporheic zone change their environment and as such alter substratum conditions. Bioturbating mussels or insect larvae burrow into the sediments and may therefore increase oxygen and nutrient exchange rates between open and interstitial water (de Haas et al., 2005; Howard & Cuffey, 2006; Vaughn & Hakenkamp, 2001). Benthic invertebrates can also influence the structure and physico-chemical conditions of the hyporheic zone (Mermillod-Blondin et al., 2001; Navel et al., 2012; Nogaro et al., 2006; Stief et al., 2004). Such alterations have the potential to influence microbial communities, which are likely the most important organisms of the hyporheic zone (Baerlocher et al., 2006; Cornut et al., 2010; Febria et al., 2011; Lowell et al., 2009), as they are the actual drivers of decomposition of organic material, nutrient turnover and pollutant degradation (reviewed in Marmonier et al., 2012).

Depending on their life style and burrowing behaviour, different ecosystem engineers can be expected to distinctly alter habitat properties. Gardner et al. (1987), François et al. (1997 and 2002) and Gerino et al. (2003) defined five functional groups of substrate reworking organisms: biodiffusors, upward conveyors, downward conveyors, regenerators and gallery diffusors. The species chosen in this study represent three of those functional groups. *A. anatina* acts as a regenerator causing sediment redistribution and creating open burrows by moving horizontally on the sediment as well as vertically in and out of it (Amyot & Downing, 1998; Zieritz et al., 2014). The burrows of indigenous European mussels can be up to 20 cm deep (Schwalb & Pusch, 2007). Inhabiting the top centimeters of stream substrata, larvae of the mayfly *E. danica* act as biodiffusors, causing omnidirectional substratum movements on the surface of streambeds. The hemimetabolic Ephemeroptera burrow U-shaped tubes of about three cm depth (Winkelmann & Koop, 2007), where they perform wavelike movements to filter food, such as detritus and algae (de Haas et al., 2005). *T. tubifex* belongs to the upward-conveyor group, feeding on sediment at depth and ejecting fecal pellets at the sediment water interface (Nogaro et al., 2009). In addition to sediment, tubificid worms also feed on detritus in the upper two to eight cm of the substratum (Pelegri & Blackburn, 1995). Due to the smaller diameter of *T. tubifex*, their burrows are smaller than those of Ephemeroptera, however, *T. tubifex* can reach higher densities up to 70,000 individuals per m^2 (Pelegri & Blackburn, 1995).

Much is known about hyporheic physical characteristics at the reach scale (Baker et al., 1999; Morrice et al., 1997), which is the basis for understanding hyporheic functioning. In contrast, knowledge about local interactions between abiotic and biotic drivers is scarce but necessary to explain the functioning of the whole hyporheic ecotone. Marmonier et al. (2012) identified many gaps in the current understanding of the dynamics of benthic invertebrate communities. To date, only few studies have specifically investigated the impact of invertebrates on microbial activity and processes (Mermillod-Blondin et al., 2001 and 2003). In particular, the interactions between burrowing organisms, streambed conditions, microbial diversity and biogeochemical processes are still unclear (Boulton, 2000a and 2000b; Marmonier et al., 2012). Microorganisms in the hyporheic zone influence the physico-chemical conditions and, in turn, their activity and diversity depend on those conditions (Navel et al., 2011, 2012; Stief et al., 2004).

The present study allows direct comparisons of the effects of functionally different bioturbators with a focus on the interaction between burrowing invertebrates, substratum conditions and bacterial diversity in the hyporheic zone. A standardized laboratory mesocosm experiment was set up with different macroinvertebrates and a control without organisms. Physico-chemical habitat properties and microbial diversity were analyzed in a multivariate statistical approach. The aims of the study were to evaluate the effects of three different (size, burrowing depth, burrowing style) macroinvertebrates (*E. danica*, *T. tubifex* and *A. anatina*) on physico-chemical parameters expected to be crucial for microbial diversity (pH, oxygen concentration and saturation, electric conductance, redox potential and N-cycle related ions) in the interstitial and open water. Moreover, differences in microbial community composition in the presence of burrowing organisms were assessed. We hypothesized that i) the analyzed organisms modulate physico-chemical conditions in the interstitial and open water distinctly which should be reflected in ii) different microbial community composition in the presence of *E. danica*, *T. tubifex* and *A. anatina* versus the control, respectively. From results of Vaughn and Hakenkamp (2001), we expected the mussels to alter habitat conditions in the direction to an oxygen-rich environment possibly leading to a microbial community dominated by obligate aerob or at least facultative aerob bacteria. The same was expected for the mayfly nymphs due to the findings of Wang et al. (2001) but to a lesser extent because of the smaller burrows of the insect larvae compared to those of the bivalves. The reported effects of tubificid worms reach from oxygen increase (Mermillod-Blondin & Lemoine, 2010) to oxygen decrease in substratums (Nogaro et al., 2007). We expected a slight oxygen decrease due to the deposition of faecal pellets probably resulting in high amounts of oxygen-consuming faeces-degrading bacteria.

In a standardized laboratory experiment, the effects of mayfly nymphs (*E. danica*), tubificid worms (*T. tubifex*) and duck mussels (*A. anatina*) on temperature, pH, oxygen concentration and saturation, redox potential, electric conductance and microbial community composition were investigated.

3.3 Material and methods

3.3.1 Study design

Plastic boxes (24.2 x 15.5 x 18.2 cm, Rotho Kunststoff AG, Würenlingen, Switzerland) were filled with a 10.0 cm substratum layer and randomly placed (Balanced latin square scheme, Evans, 1976) in three glass-fibre reinforced plastic-channels (3.55 x 0.45 x 0.17 m, AGK Kronawitter GmbH, Wallersdorf, Germany) for 35 days (Fig. 3.1a). Each box was filled with defined grain fractions (0.85, 0.63 and 0.063 mm in a proportion of 1.5: 0.5: 4.0) of washed and dried (100 °C) substratum from the Moosach River (a calcareous river, 48° 23´ 39.22´´ N; 11° 43´ 26.65´´ E) and constantly supplied with filtered water (mesh width 500 µm, Aquacultur Fischtechnik GmbH, Nienburg, Germany) from a drip-system from the same river with a flow rate of 3.5 – 4.0 l/h per box without simulating advective flow conditions. To prevent the macroinvertebrates from escaping, each box was equipped with a lid of gauze (mesh width 500 µm, Aquacultur Fischtechnik GmbH, Nienburg, Germany). For measuring the redox potential in the hyporheic zone without disturbing hyporheic processes, a redox electrode (ELANA, Boden und Wasser

Monitoring, Arendsee, Germany) was installed in each box at 5 cm below the substratum surface prior to the start of the experiment. To collect water samples from the hyporheic zone, a perforated pipe (Volume 5 ml, Gardena GmbH, Ulm, Germany) with a control valve on one end was placed into each box 5 cm below the substratum surface (Fig. 3.1b).

Fig. 3.1a. Photograph of two substratum-filled experimental boxes constantly supplied with natural water from the Moosach River using a drip-system and placed in a glass-fibre reinforced plastic-channel filled with overflowing water from the experimental boxes.

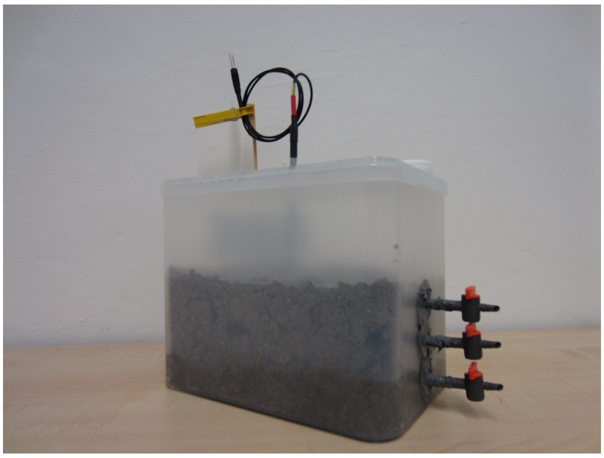

Fig. 3.1b. Photograph of a substratum-filled experimental box equipped with a lid of gauze (to prevent the test animals from escaping), an installed redox electrode for measuring the interstitial redox potential, and three control valves to take water samples from the substratum.

The experiment lasted for 35 days with a constant 12-hour light-dark cycle and a mean temperature in open water of 13.3 ± 1.1 °C. To generate similar initial conditions (i.e. physico-chemical parameters, microbial diversity), the microcosms were supplied with water but not seeded with test organisms for the first 15 days (day -15 to day 0). At day 0 the invertebrates were introduced into the boxes. To estimate appropriate densities of test animals we tried to mimic natural conditions on the one hand and to have an approximately equal biomass between the treatments on the other hand. Howard and Cuffey (2006) tested densities of 20, 40 and 80 unionoid mussels per m^2 in their experiments, Krieger et al. (2007) reported on densities of mayfly nymphs of 1,000 individuals per m^2 and Jabłońska (2014) examined oligochaetes with average densities between 6,000 and 365,000 individuals per m^2. Consequently, three mussels (mean dry weight per box = 7.98 g), 40 mayfly nymphs (mean dry weight per box = 3.20 g) and approximately 6,000 tubificid worms (mean dry weight per box = 4.44 g) were separately introduced in each of nine experimental boxes per treatment. Nine boxes remained unpopulated as control. Taken together, nine biological replicates were performed for each of the four different treatments. The organisms remained within the experimental boxes for 20 days. During the experiment the animals behaved normally and there was no increased mortality. Before the experiments, all test animals were held under identical conditions for two weeks in basins at the Aquatic Systems Biology Unit of the Technical University of Munich, Germany for acclimatization. During this period, the mussels were fed with 50 ml of 1:10 diluted Shellfish Diet 1800 (Reed Mariculture Inc., Campbell, USA) once a week. The mayfly nymphs were fed with 7.0 g of thawed chironomid larvae (Vivantis, Animal Quality GmbH, Isernhagen, Germany). During the experiment mesocosms were provided with natural water from the Moosach River. The animals were not fed to avoid additional nutrient loading (e.g., NH_4^+) and a possible falsification of results due to the feeding during the experimental phase.

3.3.2 Analysis of physico-chemical parameters

Physico-chemical water parameters (pH, oxygen concentration and saturation, electric conductance, temperature) were measured with a handheld WTW Multi 3430 SET G (Wissenschaftlich Technische Werkstätten, Weilheim, Germany) on days -15, -10, -5, 0, 5, 10, 15 and 20 of the experiment. Parameters in open water were measured 5 cm above the substratum surface. Hyporheic water samples (20 ml) were collected from each box using the pipe installed in 5 cm substratum depth. Before the 20 ml were sampled, the pipe was purged by opening the control valve completely and releasing the water in the pipe (volume 5 ml), which was discarded (Boeker & Geist, 2015). Redox potential in open water was measured 5 cm above substratum surface using a mobile redox electrode with a handheld WTW pH 3110 SET-2 (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). Redox potential in the hyporheic zone was measured 5 cm in the substratum using permanently installed redox electrodes (ELANA, Boden und Wasser Monitoring, Arendsee, Deutschland) with a handheld WTW pH 3110 SET-2 (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). Water samples from the hyporheic zone of experimental days 0, 10 and 20 were immediately frozen and stored at -20°C after measurements. These samples were used for ion detection (i. e. Li^+, Na^+, NH_4^+, K^+, Mg_2^+, Ca_2^+, Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}) by ion chromatography (ICS 1100, DIONEX, Waltham, USA) (Brielmann et al., 2009). A mixture of 1.8 mM dinatriumcarbonate and 1.7 mM natriumhydrogencarbonate was used to elute anions. For eluting cations, 20.0 mM methanesulfonic acid was used.

3.3.3 Analysis of bacterial diversity

Analysis of bacterial community structure in the hyporheic zone was conducted using DNA-based terminal restriction fragment length polymorphism (T-RFLP) fingerprinting. Sampling of substratum from the microcosms for analyses of bacterial diversity would have resulted in disturbance of the exposure units such as gradual reduction of substrate quantity, and surface water inflow, with possible effects on the development of microbial communities. In order to reduce such impact, standardized samples of interstital water (from 5 cm substratum depth) were taken on experiment days 0, 10 and 20 using the installed control valve. The sampled interstitial water was subsequently filtered through 0.22 µm CME membrane filters (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) following the methodology by Mueller et al. (2013a) who verified the validity of this method for charactization of microbial communities. Nevertheless, the sessile bacterial community (adherent on substratum particles) has not been completely disregarded since in every sample of interstitial water a small amount of substratum was present. Filters with adherent bacterial biomass and substratum were transferred to sterile Petri dishes and frozen immediately at -20°C. Since Mueller et al. (2013a) showed that technical replicates produce highly similar results, emphasis was put on including a high number of nine biological replicates per treatment. Membrane filters were cut into pieces (0.5 cm^2) using a sterile scalpel for DNA-isolation from each filter as described in Mueller et al. (2013a). After extraction, DNA-concentration was quantified by photometric measurement (ND-1000 Nanodrop Spectrophotometer, Peqlab, Erlangen, Germany) and samples were stored at -20°C until further processing. 16S rRNA gene-targeted T-RFLP fingerprinting was carried out as described in Pilloni et al. (2011) and Lueders et al. (2006). Additionally, amplicons were purified using PCRExtract columns (PCRExtract Mini Kit, 5 Prime GmbH, Hamburg, Germany) according to the manufacturer's protocol. After capillary electrophoresis, Gene Mapper 5.1 software (Applied Biosystems, Carlsbad, California, USA) was used to evaluate electropherograms. The online T-RF analysis software T-REX (Culman et al., 2009) was used to analyze T-RFLP data. A default factor of 1.2 was set for background noise filtering (Abdo et al., 2006). For peak alignments the clustering threshold was set to 1.5 using the default alignment method of Smith et al. (2005). Peak heights were used to create the T-RF abundance data matrix. T-RFs that occurred in less than 10 % of total samples were eliminated from the data set to reduce data complexity (Blackwood et al., 2007). The T-RF abundance table generated after aligning peaks to T-RFs was used for downstream statistical analyses, consequently including T-RF relative abundances. To correlate possible bacterial 16S rRNA gene sequences with the detected terminated restriction fragments, the Microbial Community Analysis III (MiCA) was applied (Shyu et al., 2007) using the PAT+ Method since only the forward primer was labeled. The settings were adjusted to the used primers Ba27f (5´FAM-aga gtt tga tcm tgg ctc ag-3´) and 907r (5´-ccg tca att cct ttg agt tt-3´) and the used restriction enzyme (MspI). A sensitivity of maximum three mismatches within two bases from the 5`end of the primer in the RDP (R10, U27) 16S bacterial rRNA database was selected for the database search, resulting in lists of microbial lineages which could be present in our samples.

3.3.4 Data analyses

Two different softwares were used for statistical data analyses. Multivariate data analyses were performed using the software PRIMER v.6 with PERMANOVA+ add on

(Plymouth Marine Laboratory, Plymouth, United Kingdom). For more specific comparisons (e.g., of single variables between treatments) standard univariate statistics were performed, which are implemented in the open software R (version 3.0.2, www.r-project.com, 2013).

Different statistical methods and models were used to evaluate the hypotheses. To test whether physico-chemical variables and composition of T-RFs differed between treatments and sampling time-points (day 0, 10 and 20) permutational multivariate analyses of variance (PERMANOVAs) were performed. Due to the permutation-based calculation of p-values the PERMANOVA method is independent of normal distribution and homoscedasticity. Prior to the PERMANOVA analyses data were normalized, due to differently scaled variables, by dividing the value minus its mean through its standard deviation. After normalization, a resemblance matrix was generated using Euclidian distances and 999 permutations under the assumption of a reduced model using the sum of squares type III (partial). The factor “treatment” was nested-fixed in the factor “day” (sampling time-point). To determine the proportion of explained variation in T-RF data by physico-chemical parameters of open water and interstitial water, as well as the ion composition in the interstitial water, nonparametric distance-based linear models (DistLMs) were performed with 999 permutations using Bray-Curtis similarities as distance measures. DistLM fits a linear model of matrix A (T-RF data) versus matrix B (e.g., abiotic habitat parameters). The total variation of T-RF data consists of the explained and unexplained part of variation. Using the DistLM procedure, only the explained amount of variation of a single or a set of variables (not completely independent from each other) was calculated. Thus, the sum of explained variation by different variables within a given data-set (e.g., explanation of variation in T-RF data by abiotic habitat parameters) is not inevitably 100 percent but refers to the asymptotically exact percentages of explained variation (Anderson et al., 2008). In this study the amounts of variation of variables within a given data set (e.g., abiotic habitat parameters) were calculated as for independent variables (so-called marginal tests). To visualize the similarity between and within T-RF data and the relationship between ion composition and T-RF data in respect to the factor “treatment”, a distance based redundancy analysis (dbRDA) was plotted. A dbRDA illustrates a fitted model, such as from the DistLM. Only environmental variables with a significant contribution to the relationship with T-RF data in the DistLMs were plotted in the dbRDA using Pearson correlation. For the purpose of verifying the DistLM data, biota and environment matchings (BESTs) were calculated using the Spearman rank correlation method with the same data. Like in the DistLM procedure, a BEST also fits a model of matrix A (T-RF data) versus matrix B (e.g., abiotic habitat parameters) to calculate the amounts of variation, but does not assume a linear type of relation. The contribution of individual bacterial 16S rRNA gene T-RFs to the average similarity within treatments based on the abundance of terminated restriction fragments was evaluated using a similarity percentage (SIMPER) analysis. The SIMPER analysis gives the percentage of similarity of factors (e.g., treatments or abiotic habitat parameters) and for specific levels of factors (for the factor treatment: *A. anatina*, *E. danica*, *T. tubifex*, control). The results give the percentage of contribution of variables that explain the similarity and how many variables explain for a certain amount of the similarity.

Differences between treatments and sampling time-points in abiotic variables and OTU diversity indices were analyzed using standard univariate statistics in R (version 3.0.2, www.r-project.com, 2013). Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene´s test). Since neither normal distribution nor homoscedasticity of data was fulfilled, pairwise comparisons between treatments and sampling time-points were performed using the non-parametric pairwise Wilcoxon rank sum test with Bonferroni correction since data were not normally distributed. Significance was accepted at $p \leq 0.05$. Significance levels were marked as follows: $p \leq 0.05$ labeled with *, 0.01 labeled with ** and 0.001 labeled with ***.

3.4 Results

3.4.1 Effects of burrowing stream invertebrates on physico-chemical habitat conditions

Before the introduction of burrowing macroinvertebrates (day 0), physico-chemical habitat conditions and ion composition in the interstitial and open water were not different between treatments (both PERMANOVA, $p > 0.05$).

After the introduction of macroinvertebrates, oxygen availability in open water measured 5 cm above the substratum surface changed as presented in detail in Table 3.1. Redox potential, pH value and electric conductance did not differ between treatments in open water and are therefore not presented.

Table 3.1: Results of Bonferroni corrected, pairwise Wilcoxon rank sum tests for differences in oxygen availability in open water between treatments over time (day 0, 10 and 20). Presented are only pairs of treatments (e. g., *A. anatina* versus *T. tubifex*) that were significantly different for the respective variable (e. g., oxygen saturation). SD = standard deviation.

Variable	**Treatment**	**Mean treatment ± SD**	**Treatment *T. tubifex***	**Mean treatment *T. tubifex* ± SD**	**p – value**
Oxygen saturation [%]	Control	86.4 ± 3.3	*T. tubifex*	72.2 ± 18.8	< 0.001
	E. danica	85.4 ± 3.2	*T. tubifex*	72.2 ± 18.8	< 0.01
Oxygen concentration [mg/l]	Control	8.7 ± 0.3	*T. tubifex*	7.3 ± 1.9	< 0.001
	E. danica	8.6 ± 0.3	*T. tubifex*	7.3 ± 1.9	< 0.01

More pronounced as in the open water, physico-chemical habitat properties in the hyporheic zone changed after the introduction of macroinvertebrates (Table 3.2). Tubificid worms most profoundly influenced physico-chemical parameters and ion composition compared to all other treatments. For instance, the average electric conductance in this treatment increased by 8 % from day 0 to day 20. In contrast, the average electric conductance decreased slightly over time in all other treatments (Fig. 3.2). Samples of interstitial water from boxes with tubificid worms had a 8 to 11 % higher electric conductance than those from control boxes or boxes containing mussels or mayfly nymphs. Oxygen saturation within the substrate of the treatment *A. anatina* was significantly higher (by 45 %) compared to the *T. tubifex* treatment. Oxygen saturation and concentration were higher in the treatments control and *E. danica* by up to 31 % compared to the *T. tubifex* treatment. The redox potential was higher in boxes with *A. anatina* and *E. danica* compared to those with *T. tubifex* (Fig. 3.3). The oxygen content in the water differed only slightly between control and tubifex treatment, but the redox potential changed drastically. This is most likely because the redox potential integrates over all solutes that react with the electrode. With *T. tubifex*, the pH value was slightly lowered compared to *A. anatina*, *E. danica* and the control.

Table 3.2: Results of Bonferroni corrected, pairwise Wilcoxon rank sum tests for differences in physico-chemical habitat properties of the interstitial zone between treatments over time (day 0, 10 and 20). Presented are only pairs of treatments (e.g., *A. anatina* versus *T. tubifex*) that were statistically significant different for the respective variable (e.g., oxygen saturation). SD = standard deviation.

Variable	**Treatment**	**Mean treatment ± SD**	**Treatment *T. tubifex***	**Mean treatment *T. tubifex* ± SD**	**p – value**
Oxygen saturation [%]	*A. anatina*	26.5 ± 12.3	*T. tubifex*	18.3 ± 3.2	< 0.05
	E. danica	24.0 ± 13.7	*T. tubifex*	18.3 ± 3.2	< 0.01
	Control	22.2 ± 11.8	*T. tubifex*	18.3 ± 3.2	< 0.001
Oxygen concentration [mg/l]	*E. danica*	2.3 ± 1.3	*T. tubifex*	1.8 ± 0.3	< 0.01
	Control	2.2 ± 1.1	*T. tubifex*	1.8 ± 0.3	< 0.001
Redox potential [mV]	*A. anatina*	122.4 ± 321.3	*T. tubifex*	-203.9 ± 166.9	< 0.001
	E. danica	28.6 ± 264.1	*T. tubifex*	-203.9 ± 166.9	< 0.01
	Control	-66.2 ± 225.4	*T. tubifex*	-203.9 ± 166.9	< 0.01
pH	*A. anatina*	7.9 ± 0.1	*T. tubifex*	7.8 ± 0.1	< 0.001
	E. danica	7.9 ± 0.1	*T. tubifex*	7.8 ± 0.1	< 0.01
	Control	7.9 ± 0.1	*T. tubifex*	7.8 ± 0.1	< 0.01
Electric conductance [µS/cm]	*A. anatina*	847.2 ± 43.4	*T. tubifex*	936.7 ± 51.1	< 0.001
	E. danica	856.5 ± 38.0	*T. tubifex*	936.7 ± 51.1	< 0.001
	Control	864.9 ± 34.2	*T. tubifex*	936.7 ± 51.1	< 0.001

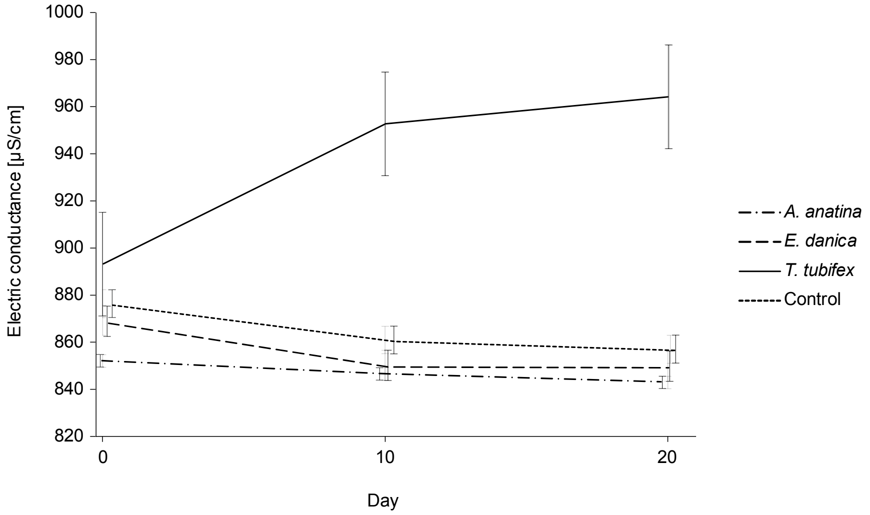

Fig. 3.2: Time course of electric conductance (including standard errors calculated from 9 biological replicates) in the interstitial zone (5 cm substratum depth) from day 0 to day 20 for all treatments. For an easier distinguishment of error bars between treatments, we shifted them artificially to the left and the right of the respective sampling time point.

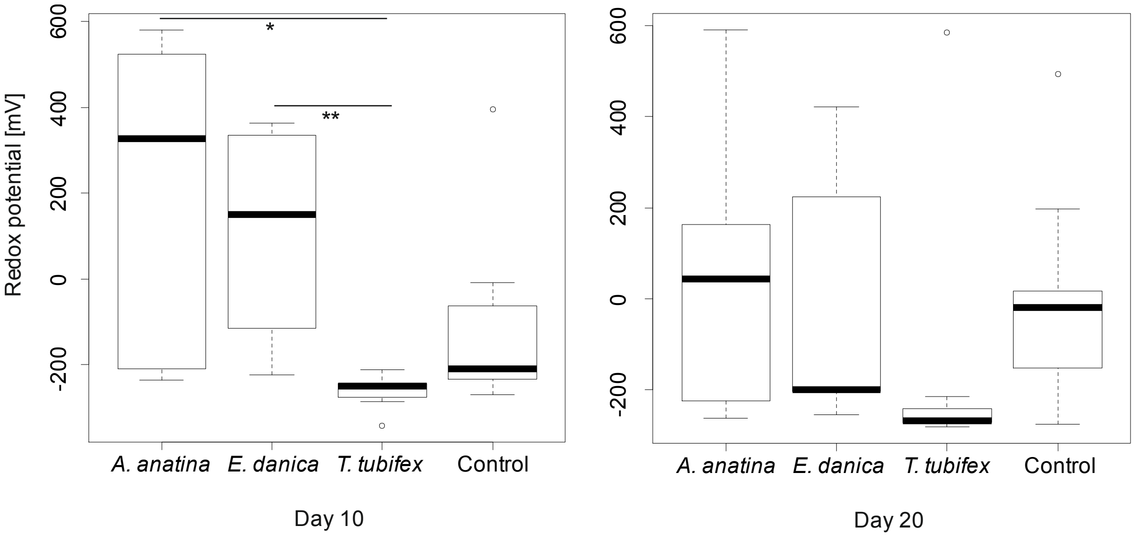

Fig. 3.3: Boxplot of the redox potentials in 5 cm substratum depth on experiment days 10 and 20 for all treatments including the control. Medians are displayed as thick horizontal bars. Boxes represent 0.25 and 0.75 quantiles and whiskers represent the range excluding outliers. Outliers (outside of the 1.5 x interquartile range interval from the whiskers) are indicated by circles. The horizontal bars with the stars indicate significant differences between groups at $p \leq 0.05$ (labeled with *), 0.01 (labeled with **) and 0.001 (labeled with ***) after Bonferroni correction.

The ion composition in the interstitial water changed after the introduction of organisms on day 0. Significantly higher nitrate concentrations (mean 7.63 mg/l) were detected in the treatment *A. anatina* compared to the control (mean 2.87 mg/l) and *T.*

tubifex (mean 1.38 mg/l) (pairwise Wilcoxon rank sum test, Bonferroni corrected; p < 0.001 between the treatment *A. anatina* and *T. tubifex*; p < 0.01 between treatment *A. anatina* and the control). Nitrate concentrations in the treatment *T. tubifex* (mean 1.38 mg/l) were lower than in treatment *E. danica* (mean 4.32 mg/l) (pairwise Wilcoxon rank sum test, Bonferroni corrected; p < 0.01). Figure 3.4 visualizes the treatment effects on day 10 and 20.

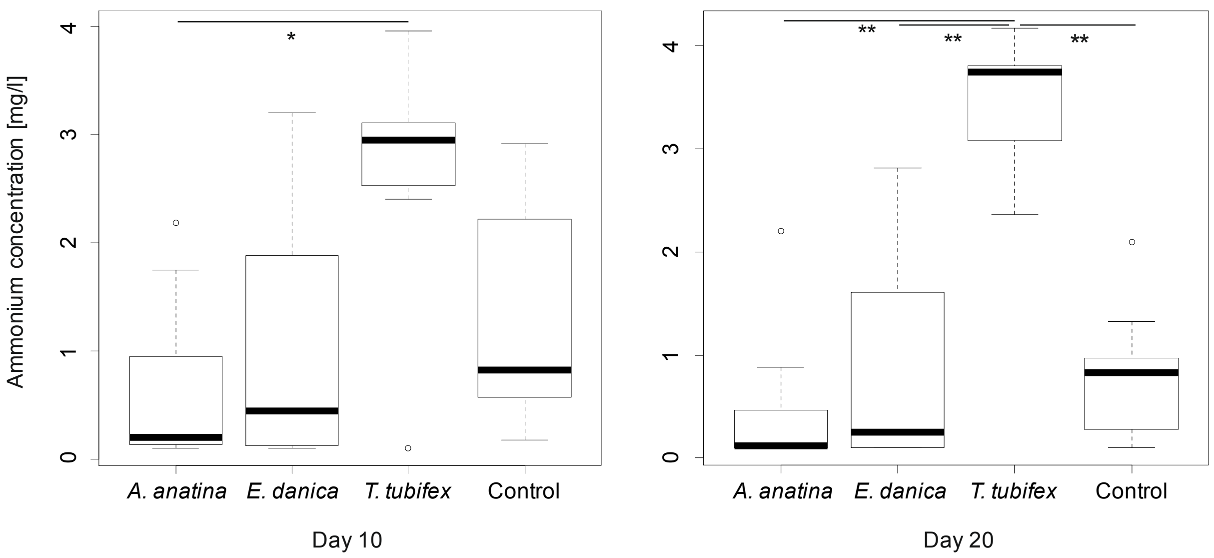

Fig. 3.4: Boxplot of ammonium concentrations in 5 cm substratum depth on day 10 and 20 for all treatments including control. Medians are displayed as thick horizontal bars. Boxes represent 0.25 and 0.75 quantiles and whiskers represent the range excluding outliers. Outliers (outside of the 1.5 x interquartile range interval from the whiskers) are labeled by circles. The horizontal bars with the stars indicate significant differences between groups at p ≤ 0.05 (labeled with *), 0.01 (labeled with **) and 0.001 (labeled with ***) after Bonferroni correction.

The effects on physico-chemical habitat conditions caused by the burrowing of the investigated stream invertebrates were detectable in interstitial water, and even extending into open water. Oxygen saturation and concentration in the *E. danica* treatment (means 85.4 % and 8.6 mg/l) and control (means 86.4 % and 8.7 mg/l) were significantly higher compared to *T. tubifex* (means 72.2 % and 7.3 mg/l) (Wilcoxon rank sum tests, Bonferroni corrected; p in all tests < 0.05).

3.4.2 Effects of burrowing stream invertebrates on microbial communities in the hyporheic zone

A total of nine T-RFLP fingerprints were generated in this study per treatment (3 + 1 control) and time point (3), as well as for the investigated water compartment (1). Before the invertebrates were introduced to the experimental boxes, bacterial community composition in the hyporheic zone as analyzed via 16S rRNA gene fingerprinting did not differ between the treatments (PERMANOVA, 2-factorial, nested fixed; $p > 0.05$). 105 different T-RFs were found over all treatments. After invertebrate amendment, slight differences in the numbers of T-RFs between the treatments were observed. Table 3.3 shows the variability of the bacterial community as detected by T-RFLP data using multivariate PERMANOVA analysis separated according to different sampling time points (day 0, 10 and 20). Especially the composition of T-RFLP fingerprints in the *T. tubifex* treatment differed from all other treatments at day 10 and 20 as indicated by p-values ≤ 0.01.

Table 3.3: Differences in bacterial community composition between treatments on different sampling time points (day 0, 10 and 20) evaluated by a PERMANOVA analysis based on T-RFLP fingerprints in a 2-factorial (sampling time point, treatment) fixed nested design. Only significant comparisons between treatments including the control are presented including the different sampling time points (day 0, 10 and 20).

Day	**Comparison of treatments**			**t - value**	**p - value**
0					no significant distinction
10	*A. anatina*	-	*T. tubifex*	3.33	0.001
	E. danica	-	*T. tubifex*	3.52	0.001
	Control	-	*T. tubifex*	3.72	0.001
20	*A. anatina*	-	*T. tubifex*	2.77	0.001
	E. danica	-	*T. tubifex*	2.04	0.007
	Control	-	*T. tubifex*	2.01	0.005

The SIMPER analysis, based on the abundance of terminated restriction fragments, revealed that six T-RFs (87, 127, 136, 139, 487 and 490 bp) were present in all treatments including the control (Fig. 3.5). However, the 136 bp T-RF had significantly less influence on average similarity in the *T. tubifex* treatment, while the 87 and 276 bp fragments clearly showed an elevated contribution under tubificid burrowing. Two T-RFs were uniquely present in the *A. anatina* treatment, of which especially the 154 bp fragment was distinctively abundant. Three T-RFs were exclusively detected in the treatment *E. danica*. In the control, one T-RF was solely present. Only one T-RF (84 bp) was unique for the *T. tubifex* treatment, albeit at low abundance which was unexpected considering the strong effects on the physico-chemical habitat properties.

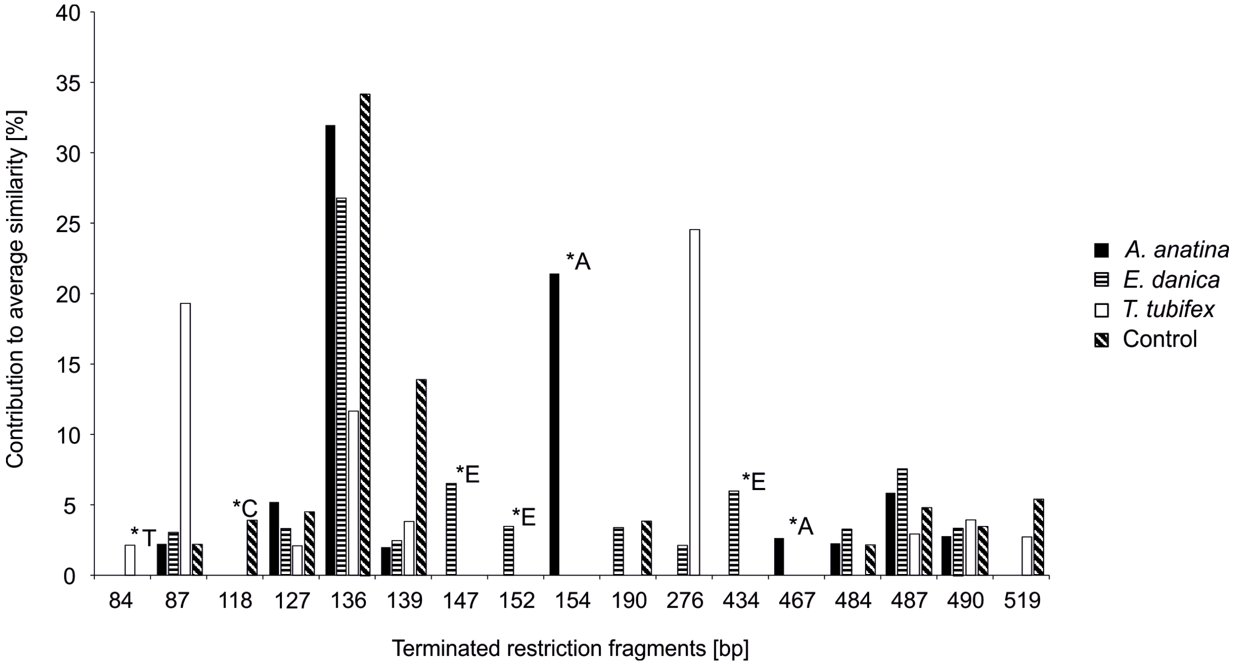

Fig. 3.5: Contribution of individual bacterial 16S rRNA gene T-RFs to the average similarity within treatments in a similarity percentage (SIMPER) analysis based on the abundance of terminated restriction fragments. Presented are T-RFs contributing more than 2 % to the average similarity. Different fillings of bars represent different treatments: T-RFs labeled with * A (*A. anatina*), * E (*E. danica*), * T (*T. tubifex*) or * C (Control) are exclusively present in the respective treatment.

The output of database search using MiCA was strongly different for the Tubifex treatment compared to all other treatments. For bacteria possibly represented by the T-RFs of the mussel treatment, the mayfly treatment and the control, more than 2,800 taxa were related. On the contrary, for the T-RFs detected in the *T. tubifex* treatment, the output of the search was less than 50 records of database entries. Many of the bacteria associated with the T-RFs of the non-tubificid treatments were facultative anaerobes, i.e. able to live in oxygen-rich and oxygen-poor environments. In contrast, many of the T-RFs associated in the *T. tubifex* treatment potentially represented denitrifying *Alphaproteobacteria* or several obligatly anaerobic members of the *Clostridiales*. The distinctive 154 bp T-RF observed in the mussel treatment was putatively linked to members of the genus *Pseudomonas*.

According to the DistLM analyses of T-RFLP data in interstitial water, electric conductance followed by redox potential and pH value explained 11.6 %, 7.8 % and 4.8 % respectively, of the variance in T-RFLP data (all $p < 0.01$). Further DistLM analyses revealed that the ion composition also explained a part of the variance in T-RFLP data. This was evident for NH_4^+ ($p < 0.01$, explained 10.8 % of T-RFLP data variance), Mg^{2+} ($p < 0.01$, explained 4.2 % of T-RFLP data variance), NO_3^- ($p < 0.01$, explained 5.6 % of T-RFLP data variance) and SO_4^{2-} ($p < 0.01$, explained 6.3 % of T-RFLP data variance) (Fig. 3.6). BEST analyses selected the same variables as DistLMs to partially explain the variance in T-RFLP data. The dbRDA (Fig. 6) revealed a highly similar multivariate pattern of OTU composition as the NMDS analyses (data not shown).

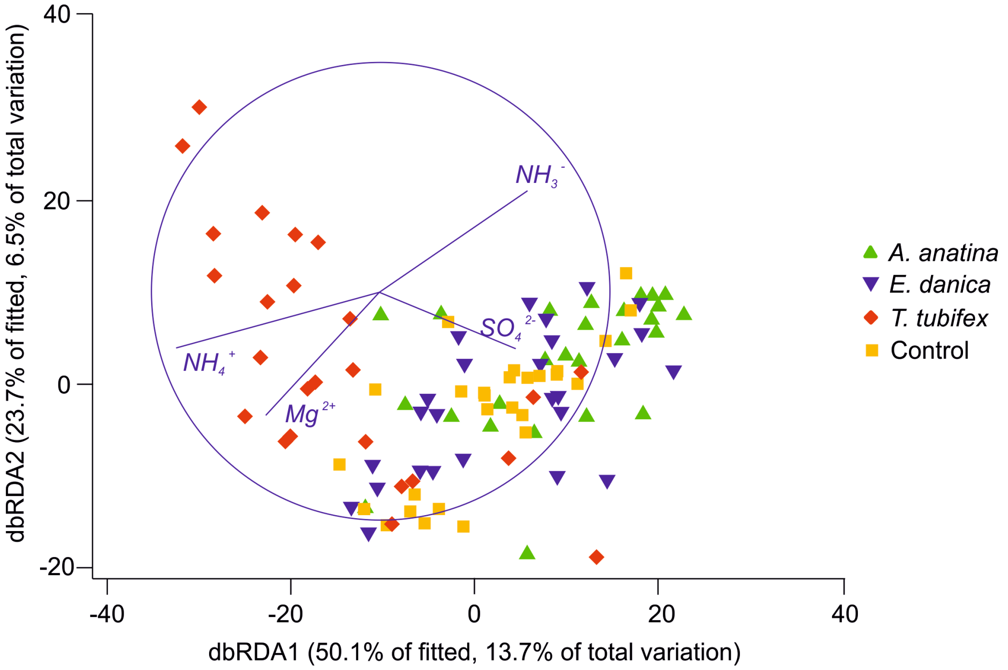

Fig. 3.6: Distance-based redundancy analysis (dbRDA) illustrating the overall microbial community structure of the hyporheic zone (T-RFLP data) in relation to environmental variables, separated according to treatment and pooled over time (day 0, 10 and 20). As evident from DistLM analyses using Bray-Curtis similarity, Mg^{2+}, NH_4^+, NO_3^-, SO_4^{2-} had a significant influence on the variability of T-RFLP data. For visualisation, these variables were correlated with the dbRDA plot using Pearson correlation. The strength of correlation is indicated by the length of the blue lines for the respective variable, with the radius of the blue circle representing 100 % correlation.

3.5 Discussion

This study provides first experimental evidence that physico-chemical and microbial properties can be differentially affected by burrowing macroinvertebrates of different functional groups. The observed alterations of the hyporheic zone between the investigated species can be explained by differences in their behaviour, as well as by the mode and location of feeding and excretion. As evident from the intensity of alteration, as well as from the finding that significant effects were even detected in open water caused by species such as *T. tubifex*, it appears likely that burrowing invertebrates have strong and currently underestimated effects on the functioning of aquatic ecosystems.

Consequently, also invertebrate species can fulfil the criteria of "ecosystem engineers". This concept has previously mostly been linked with vertebrates such as lampreys excerting pronounced effects on stream substrate conditions and microbial community structures (Boeker & Geist, 2016).

In line with our expectation, the results indicated high oxygen availability in the substratum of the mussel treatment, which can be explained by the voluminous burrows and high burrowing activity of the mussels (Zieritz et al., 2014), resulting in high influx rates of oxygen-rich surface water. As opposed to the tubificid worms, mussels may not have consumed oxygen from the hyporheic zone because they use their siphon for the respiration of oxygen from open water. In such an oxic environment, nitrate is probably not subject to denitrification, which explains the measured constant nitrate concentrations. Vaughn & Hakenkamp (2001) reported on mussels´ excretion of pseudofeces into open water, which probably caused a low biodeposition of feces and thus a low ammonium concentration in the hyporheic zone. As indicated by our findings, the high impact of the mussels´ sediment-reworking behaviour changes bacterial community composition. Here, the higher oxygen availability in the *A. anatina* microcosms resulted in a high abundance of T-RFs possibly representing obligate aerobes.

The indicators of oxygen supply (oxygen saturation and concentration, redox potential) in the *E. danica* treatment were approximately 1.5-fold decreased in comparison to *A. anatina*. In contrast to the mussels, mayfly nymphs respire oxygen available in substratum burrows. Taking *Ephemera simulans* as an example, 0.25 – 0.35 ml oxygen per gram dry weight per hour are consumed (Eriksen, 1963). Furthermore, the burrows of the mayfly nymphs are shallower and less voluminous than the mussels´ burrows suggesting that *E. danica* caused a lower influx of oxygen-rich open water. Due to an increased oxygen availability, compared to the control and the tubificid worms, the measured nitrate concentrations in *E. danica*-microcosms suggest a lower denitrification rate. The effects of *E. danica* nymphs burrowing in around 3 cm substratum-depth (Winkelmann & Koop, 2007) were probably diluted towards a substratum depth greater than 3-4 cm. As our measurements were performed in 5 cm depth, the observed alterations may even be greater than evident from our results. The impact of mayfly nymphs on microbial community composition in a certain direction was not as pronounced as in the other treatments. Thus, our results reveal an increased impact of burrowing invertebrates on hyporheic microbial community composition with bioturbation intensity. Nogaro et al. (2009) stated that the burrowing depth is the most important factor defining the role of bioturbators in fine sediments, yet their results did not consider sediment microbes. Additionally, our results indicate that the way how organisms burrow (e. g. frequency of position change and density of burrows) is also important. This further substantiates Hancock's statement (2002) that the "exchange between surface and subsurface water may be the most important regulator of biological activity in the hyporheic zone" as well as the work of Battin (2000).

T. tubifex caused the strongest impact on physico-chemical parameters in the microcosm-substratum. Oxygen-related parameters clearly indicated low oxygen availability in these boxes. This suggests that either the oxygen consumption by tubificids or by aerobic microbes in the habitat was higher than the oxygen-influx from surface water and via burrowing activity. Assuming the simplified consideration that under oxic conditions bacteria in the control boxes and in boxes inhabited by tubificid worms have the same respiration rate, the oxygen consumption due to bacteria and tubificid worms can be estimated. From our data a tubificid respiration rate of approximately 16 % of the total oxygen consumption in the hyporheic zone can be calculated. Thus, it is unlikely that the low oxygen consumption by tubificids outweights that of the microbes. This is further confirmed by our findigs that nitrate in *T. tubifex* microcosms was lower compared to the concentrations found in the controls, assuming that local oxygen depletion coincided with denitrification in this treatment. The flux of nitrate as electron acceptor for denitrifiers towards deeper sediments may have been increased by the burrowing networks of tubificid worms. The high ammonium concentrations and a temporary increase of nitrite in the substratum might be explained by a continuous transport of feces to the sediment surface by *T. tubifex*. Gardner et al. (1983) reported on the direct excretion of ammonium by oligochaetes. High ammonium loads increase oxygen consumption via nitrification, an effect which was also detected in our study. The mobilization of ions to the interstitial water is probably enhanced by higher numbers and activities of bacteria at the burrow walls (Henriksen et al., 1983), explaining the higher electric conductance in the interstitial. Also in bacterial community composition, the *T. tubifex* treatment displayed a strong difference compared to all other treatments. It is likely that *T. tubifex* caused a shift in microbial communities towards an increased abundance of anaerobic bacteria as suggested by our MiCa database search. Nevertheless, we are fully aware that taxonomic or functional interpretation of microbial communities is not possible based on T-RFLP data alone (Blackwood et al., 2007). For this, more elaborate community analyses based on marker gene sequencing or metagenomics would be the next step. Nevertheless, our approach of associating T-RFLP patterns with physico-chemical habitat properties provides a link between abiotic and biotic habitat properties (strong decline in oxygen concentration, low redox potential).

Predicting anthropogenic impacts on streams depends on the understanding of the intertwined effects on geological, hydrological, biological, physico-chemical, and microbial processes. The results of this study confirmed our hypotheses and point towards distinct shifts in bacterial community composition depending on the functional group of bioturbators. The present findings emphasize the importance of studying those interactions in a standardized comparative approach. It is well established that bacteria play a key role in aquatic nutrient turnover and pollutant degradation, as well as in aquatic food webs (Findlay, 2010). As revealed for N-cycling in the present study, a shift in bacterial community composition is likely to relate to altered nutrient and pollutant turnover potentials. In natural systems, the growth of macrophytes, periphyton, and algae and thereby the balance of the riverine food web can be expected to change. Analogously, Fleming et al. (2014) studied such feedbacks in a terrestrial context and concluded that a loss of Australian digging mammals affected the local loss of ecosystem function. Since we mainly investigated free bacteria, not bound to biofilms, which are known to represent the minor fraction of hyporheic microbes, the effects on the entire hyporheic microbial community due to bioturbation is likely to even be greater than revealed here.

3.6 Conclusions

The results of our study suggest that invertebrate bioturbators play a key role in freshwater ecosystem functioning affecting physico-chemical properties of the substrate, as well as microbial community structure. Additionally, the presented findings underline that the functional group approach (Poff et al., 2006) is appropriate to determine influences of different taxa on biogeochemical functioning and microbial diversity in freshwater substrata. Increased loads of fine sediment are known to be one of the major problems of stream headwater reaches and are thus a main target of habitat restoration (Geist, 2015; Mueller et al., 2014; Pander & Geist, 2013; Pander et al., 2015). The pronounced impacts of alteration of stream bed structure (e.g., fine sediment input, construction of weirs, alterations of flow regimes and sediment transport rates) on multiple substrate-dependent macroscopic taxa such as endangered salmonids (Sternecker et al., 2013a and 2013b) and freshwater mussels (Geist & Auerswald, 2007; Denic & Geist 2014; Denic et al., 2014a; Scheder et al., 2014) are well understood. Our study suggests that the currently less considered effects on microbiota, as well as the interaction between micro- and macrobiota are equally important. Especially the presence of mussels and tubificids in dense populations clearly has the potential to modulate (mussels) or enhance (tubificids) anthropogenic pressures such as increased sedimentation, as previously also reported for lampreys (Boeker & Geist, 2016). Consequently, the loss or replacement of specific bioturbators can lead to changes in stream ecosystem function, extending to the ecosystem services provided by streams. Therefore, ecological interactions at the interface between open water and the stream bed, as well as the important roles of habitat engineers should be taken into account in hydraulic engineering plans and conservation management.

4. Lampreys as ecosystem engineers: burrows of *Eudontomyzon* sp. and their impact on physical, chemical and microbial properties in freshwater substrates

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4.1 Abstract

The stream bed is considered an important key habitat, yet there is limited knowledge on the effects of burrowing organisms and the resulting bioturbation on this habitat. In this study, a standardized laboratory experiment was used to compare the impact of burrowing lamprey larvae (*Eudontomyzon* sp.) on physical and chemical habitat properties and microbial community composition in freshwater substrates to a control without animals. We hypothesized that the burrowing activity of the lamprey larvae increases the influx of open water into interstitial areas with consequences for oxygen availability and thus for microbial community composition. The study revealed a strong increase in oxygen availability (oxygen concentration and redox potential) and nitrate concentrations in interstitial water in the lamprey treatment, indicative of an increased exchange with open water. Over time, a shift in microbial community composition was observed in the lamprey treatment, which was dominated by aerobe bacteria. Because the functional relevance of lamprey larvae for freshwater ecosystems is evidentially high, they can clearly be considered ecosystem engineers. Their important ecosystem functions should be recognized in conservation and management plans.

4.2 Introduction

Freshwater substrates provide a key habitat for many different species and life stages, and the physical and chemical properties of the stream bed govern the patterns of individual species and communities in this ecotone (e.g., Geist, 2010; Sternecker et al., 2013a, 2013b; Denic et al., 2014a). Alternatively, many aquatic organisms themselves have the potential to alter stream bed properties through processes commonly referred to as ´bioturbation´ or ´ecosystem engineering´. For instance, nest-building activities of gravel-spawning fishes can greatly disturb natural freshwater substrates, washing out fine sediments, and creating more oxygen-rich conditions that increase hatching success (Hogg et al., 2014; Montgomery et al., 1996). Thus, effective conservation management of freshwater ecosystems requires an understanding of such processes in freshwater substrates (Geist, 2015). Species that are bound to the streambed in any form are particularly susceptible to habitat degradation (Mateus et al., 2012). Brook lampreys (*Eudontomyzon* sp.) are classical examples because they highly depend on freshwater substrates in all life stages: the eggs develop in the gravel bed, larvae use substrates as permanent habitat and feed on organic material at the substrate surface, and adults require an intact gravel bed for spawning (Hardisty, 1979). Consequently, the loss of suitable habitats through channel and bank regulation is the biggest threat to lampreys (Renaud, 1997; Mateus et al., 2012). As the input of fine sediments and the resulting colmation of substrates negatively affect benthic organisms (Murphy et al., 2015), it is likely that fine sediment input is also a threat to lampreys.

Lampreys are considered an important and fascinating group of animals worldwide because of their cultural, ecological and scientific significance (Docker et al. 2015). Due to their broad distributions and high densities, especially in regions of the Northern Hemisphere, lampreys are thought to have a significant impact on freshwater substrates and ecosystems among the benthic fauna (Shirakawa et al., 2013). In particular, their burrowing behavior during the larval phase (Hardisty, 1979) qualifies them as potential ´ecosystem engineers´ (Jones et al., 1994). They dig themselves with whiplash contractions of the tail into the sediment and subsequently come back to the substrate surface to filter feed on microbiota such as Diatoms, Chlorophyta, Chrysophyta and Euglenophyta (Hardisty, 1979). Larvae change their positions several times during the night, and consequently the resultant burrows allow an influx of open water, thereby potentially affecting interstitial abiotic habitat properties (Shirakawa et al. 2013), as well as the cycling of nutrients and energy between open and interstitial water. While the impacts of benthic macroinvertebrates on physical, chemical and microbial habitat properties are well studied (Marmonier et al., 2012), the impacts of vertebrates such as lamprey larvae are still poorly understood warranting further research “in and around larval habitats” (Shirakawa et al., 2013). In particular, the impact of larval lampreys on the composition of microbial communities, to our knowledge, has not been studied. The composition of microbial communities is crucial for key processes such as nutrient cycling (e.g., nitrogen cycle) and pollutant degradation (Findlay, 2010), but the lack of information on these topics currently prevents linking ecological processes such as ecosystem engineering with microbial diversity.

Research topics addressing the interstitial zone can greatly benefit from integrating effects on biodiversity (Mermillod-Blondin et al., 2003). Feris et al. (2004) and Williams et al. (2009) stated that parameters that control contaminant fluxes (xenobiotics, pesticides etc.) between free flowing and interstitial water are equally important. Connecting both aspects is important for the assessment of biological functionality, and an approach considering both microscopic and macroscopic levels of biological organization is advantageous (Geist, 2011; Pander & Geist, 2013). Before exploring and understanding complex circumstances like contaminant fluxes in the field, mechanistically elucidating the effects of different biota and their multi-level interactions in controlled environments can be a useful first step. Thus, the present study focused on the impact of lamprey larvae (macroscopic biological level) on physical and chemical habitat properties and microbial diversity (microscopic biological level) in the interstitial zone under standardized laboratory conditions. We compared the effects of lamprey larvae on abiotic and biotic habitat properties in the interstitial zone to a control without animals using standardized microcosms supplied by stream water. The interactions of lamprey-induced abiotic and biotic effects were tested with a multivariate statistical approach.

Changes in interstitial physical and chemical habitat conditions in presence of lamprey larvae were expected from a field study (Shirakawa et al., 2013). As additionally abiotic habitat properties are crucial for the microbial community composition (Stief et al., 2004; Navel et al., 2012), we hypothesized that i) physical and chemical habitat properties and ii) microbial community composition between the controls and the lamprey treatment differ with regard to a more aerobic micro-environment in the lamprey treatment. We further expected alterations in N-cycling as a consequence of a shift in microbial community composition after the introduction of lamprey larvae into the microcosms.

4.3 Methods

4.3.1 Study design

The impacts of the burrowing behavior of lamprey larvae (*Eudontomyzon* sp.) on physical and chemical habitat properties and microbial community composition in freshwater substrates were evaluated in a 35-day laboratory experiment under standardized conditions. Nine biological replicates of microcosms per treatment were used to compare the effects of the lamprey larvae to a control without animals. The experiment was performed during April and March 2014 with a constant 12-hour light-dark cycle and a mean temperature in open water of 12.4 ± 0.9 °C.

Eighteen microcosm boxes, solid on five sides (24.2 x 15.5 x 18.2 cm, Rotho Kunststoff AG, Würenlingen, Switzerland) were placed randomly in two fiberglass reinforced plastic-channels (3.55 m x 0.45 m x 0.17 m, AGK Kronawitter GmbH, Wallersdorf, Germany) according to the balanced latin square scheme. The microcosms were filled with a 10 cm substrate layer of sieved and dried (90°C, 24 h) substrate particles (0.85, 0.63 and 0.063 mm in a proportion of 1.5: 0.5: 4.0) collected from the Moosach River (Danube drainage, 48° 23´ 39.22´´ N; 11° 43´ 26.65´´ E). The substrates did not contain any animals when they were filled into the microcosms. A lid of gauze (mesh width 500 µm, Aquacultur Fischtechnik GmbH, Nienburg, Germany) covered each microcosm box and allowed a constant top-down water flow of 3.5 – 4.0 l per hour drawn from the Moosach River using a dripping system. A redox electrode (ELANA, Boden und Wasser Monitoring, Arendsee,

Germany) was installed in each microcosm at a substrate depth of 5 cm to collect redox measurements from the interstitial zone. A perforated pipe (Volume 5 ml, Gardena GmbH, Ulm, Germany) with a control valve on one end was also placed at a substrate depth of 5 cm in each box for collecting water samples from the interstitial zone.

As similar initial conditions (i.e. physical and chemical parameters) as well as a certain degree of succession of microbial communities are crucial for the assessment of treatment-effects, all microcosms were supplied with water for the first 15 days (day -15 to day 0) but not stocked with lamprey larvae. The lamprey larvae were captured from the Große Ohe River (Danube drainage, 48° 43´48.32” N, 13° 15´14.73” E) by excavating sediments from potential habitats using a shovel and searching for them in the sediment by hand. All national guidelines for the care and use of animals were followed. The experiment was permitted by the Bavarian State Ministry of the Environment and Consumer Protection (Az. 55.2-1-54-2532-180-13) and the sampling of lamprey larvae by the District Office of Deggendorf (Az. 32-7562/Ni). Five tissue samples of lampreys were genetically analyzed at the Bavarian State Collection of Zoology (Munich, Germany). The calculated phylogenetic tree illustrated a grouping of the tested samples among samples of different species of the genus *Eudontomyzon* (*E. vladykovi*, Olivia & Zanandrea 1959; *E. mariae,* Berg 1931*; E. danfordi,* Regan 1911) but a distinct determination to species level could not be drawn from the analyses. Consequently, the lampreys are described on genus level in this work. For the experiment all lamprey larvae (average size 9 ± 1.5 cm) were held under identical laboratory conditions for two days in substrate filled basins at the Aquatic Systems Biology Unit of Technical University of Munich, Germany for acclimatization. Substrate and water conditions in the acclimation basins were consistent with microcosm conditions. On experiment day 0, two lamprey larvae were put into each of nine microcosms. The same number of microcosms remained unpopulated with animals as a control. Test animals were not fed since natural open water containing particulate matter as food was supplied. No lamprey larvae died or had to be euthanized throughout the experiment duration of 20 days.

4.3.2 Analysis of physical and chemical parameters

All physical and chemical parameters were measured on days -15, -10, -5, 0, 5, 10, 15 and 20 of the experiment between 9:00 and 12:00 am. Open water measurements were made 5 cm above the substrate surface. Samples from interstitial water were taken using the control valve of the perforated pipe installed into the microcosm boxes. Twenty ml of water were collected after 5 ml (volume of the pipe) were purged. Oxygen concentration, saturation and redox potential were measured because these indicators represent important habitat properties for biological processes, including microbial metabolism. Redox potential at a substrate depth of 5 cm was measured as described in Geist & Auerswald (2007) with a platinum electrode which was fixed in a plastic tube. Moreover, pH and electric conductance were measured for further characterization of the microhabitats. All physical and chemical measurements were conducted using a WTW Multi 3430 SET G and a WTW pH 3110 SET-2 (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). The probes were rinsed with distilled water after collecting measurements from each microcosm. On experimental days 0, 10 and 20, samples from interstitial water were immediately frozen at -20°C for ion analysis, which is important for evaluating treatment-specific effects on water chemistry such as presence or absence of different forms of N (indicative of N-cycling). A mixture of 1.8 mM dinatriumcarbonate and 1.7 mM natriumhydrogencarbonate was used for eluting anions (i.e., Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}, F^-; AG-22 as guard column and AS-22 separation column) and 20 mM methanesulfonic acid for eluting cations (i.e., Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}; CG-12 as guard column in line with a CS-12 separation column). Ion chromatographs and columns were manufactured by Thermo Scientific, Dreieich, Germany.

4.3.3 Analysis of microbial diversity

The microbial community composition was analyzed using DNA-based terminal restriction fragment length polymorphism (T-RFLP) fingerprinting. For investigating microbial communities at 5 cm substrate depth, samples of interstitial water (taken on experiment days 0, 10 and 20) were filtered through 0.22 µm cellulose mixed ester membrane filters (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), which were subsequently stored in sterile Petri dishes at -20 °C. For each treatment, nine biological replicates (one from each box) were analyzed (18 in total). Membrane filters were cut into pieces (0.5 cm^2) using a sterile scalpel for DNA-isolation, as well as a sample of epidermal mucus from two different lamprey larvae, according to the protocol in Mueller et al. (2013a). DNA-concentration was quantified using a ND-1000 Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany) and samples were stored at -20°C until further processing. As described in Pilloni et al. (2011) and Lueders et al. (2006), 16S rRNA gene-targeted T-RFLP fingerprinting was carried out; however we conducted an additional purification step using PCRExtract columns (PCRExtract Mini Kit, 5 Prime GmbH, Hamburg, Germany) according to the manufacturer's protocol. In total, a sum of 54 T-RFLP fingerprints were generated: nine biological replicates per treatment (n=2) and sampling time point (n=3). To evaluate electropherograms after capillary electrophoresis, the Gene Mapper 5.1 software (Applied Biosystems, Carlsbad, California, USA) was used and T-RFLP data were analyzed during the subsequent step with the free online-tool T-REX (Culman et al., 2009) using the peak heights to create the T-RF abundance data matrix. A default factor of 1.2 was set for background noise filtering consistent with methods described by Abdo et al. (2006). The clustering threshold was set to 1.5 using the default alignment method of Smith et al. (2005) for peak alignments. We eliminated T-RFs that occurred in less than 10% of the total samples in order to reduce data complexity (Blackwood et al. 2007). The terminated restriction fragments were analyzed with the Microbial Community Analysis III (MiCA) database search in order to assign bacterial lineages to the detected terminated restriction fragments. A sensitivity threshold of two mismatches within two bases from the 5´end of the primer in the Ribosomal Database Project (RDP; R10, U27; 16S bacterial rRNA database) was applied following adjustment according to Shyu et al. (2007). Consequently, the assigned bacterial lineages to the control and lamprey treatment could be checked for functional differences.

4.3.4 Data analysis

Multivariate data analyses were performed using the software PRIMER v.6 with the PERMANOVA+ add on (Plymouth Marine Laboratory, Plymouth, United Kingdom). Similarity percentage (SIMPER) analyses using Euclidian distances were performed to reveal which T-RFs contribute most to the average similarity per treatment. Differences of physical and chemical variables and T-RF-compositions between treatments and sampling time-points (day 0, 10, and 20), were tested by conducting permutational multivariate analysis of variance (PERMANOVAs) with 999 permutations under the assumption of a reduced model using the sum of squares type III (partial). In the PERMANOVA design, the factor ´treatment´ was nested-fixed in the factor day (sampling time-point). The relationships between T-RF data and the physical and chemical parameters of open and interstitial water, as well as the ion composition in the interstitial water, were evaluated by computing separate distance-based linear models (DISTLMs). We calculated DISTLMs using 999 permutations of a Bray-Curtis similarity matrix. Non-metric multidimensional scalings (NMDS) were plotted to display the differences between microbial communities at different sampling time points, and between treatments and T-RFs in mucosal samples from lamprey larvae. Differences between treatments and sampling time-points in abiotic variables were analyzed using standard univariate statistical methods in R (version 3.0.2, www.r-project.org, 2013). Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene´s test). Pairwise comparisons between treatments and sampling time-points were performed using the non-parametric pairwise Wilcoxon rank sum test with Bonferroni correction because data were not normally distributed. Significance was accepted at a level of $\alpha \leq 0.05$ after Bonferroni correction.

4.4 Results

Our study revealed pronounced and distinct effects of lamprey larvae on physical and chemical habitat properties as well as on microbial community composition in the substrate. In microcosms with lamprey larvae, oxygen-levels were increased compared to those of the controls. This was also reflected in an aerobe microbial community in these treatments.

4.4.1 Impacts of lamprey larvae on abiotic habitat properties

Before the animals were introduced into the microcosms, no significant differences in physical and chemical properties and ion composition between the lamprey treatments and the controls were detectable neither in open nor in interstitial water (PERMANOVA, 2-factorial, nested fixed, all $p > 0.05$), indicating highly similar initial conditions. Between subsequent sampling time points, significant differences in abiotic habitat properties were detected between the lamprey treatments and the controls (PERMANOVA, 2-factorial, nested fixed, all $p \leq 0.001$). From day 0 to day 20, temperature in open and interstitial water increased for seasonal reasons.

In contrast to the similar abiotic habitat properties between lamprey treatments and controls in open water, those in the interstitial water differed significantly at day 10 and 20 (PERMANOVA, 2-factorial, nested fixed, all $p \leq 0.01$). The most pronounced effects were observed in the interstitial water oxygen availability. Before the introduction of lamprey larvae (day 0), the oxygen concentration and redox potential in the interstitial water were not significantly different between control and lamprey microcosms. Pairwise Wilcoxon rank sum tests revealed significantly higher values in the lamprey treatments on day 10 for oxygen concentration ($p < 0.05$), oxygen saturation ($p < 0.05$), redox potential ($p < 0.01$), and pH ($p < 0.01$). In the case of biologically important oxygen concentration and redox potential, values in the lamprey treatments were more than 1.5 fold higher than those in the controls (redox: 603 vs. 397 mV; oxygen concentration: 7.47 vs. 4.56 mg/l). Even stronger differences between treatments were detected on day 20 for oxygen concentration ($p < 0.001$), oxygen saturation ($p < 0.001$), redox potential ($p < 0.001$), and pH ($p < 0.001$) which were significantly higher in the lamprey treatments compared to the controls (Fig. 3.2.1 and Table 3.2). At the end of the experiment (day 20), the oxygen concentration in the interstitial waters of those microcosms stocked with lamprey larvae increased by a factor of 2.2 to 6.68 mg/l (± 0.22), strongly exceeding the values of the controls (2.66 ± 0.33 mg/l). Similarly, redox potential in the lamprey treatments increased by a factor of 2.6 in the interstices, whereas a slight decrease was observed in the controls (Fig. 4.1). In addition to oxygen-related abiotic habitat parameters, electric conductivity differed between treatments. On day 10, the electric conductivity was significantly lower in the lamprey treatments compared to the controls ($p < 0.05$) as well as on day 20 ($p < 0.001$; Table 4.1).

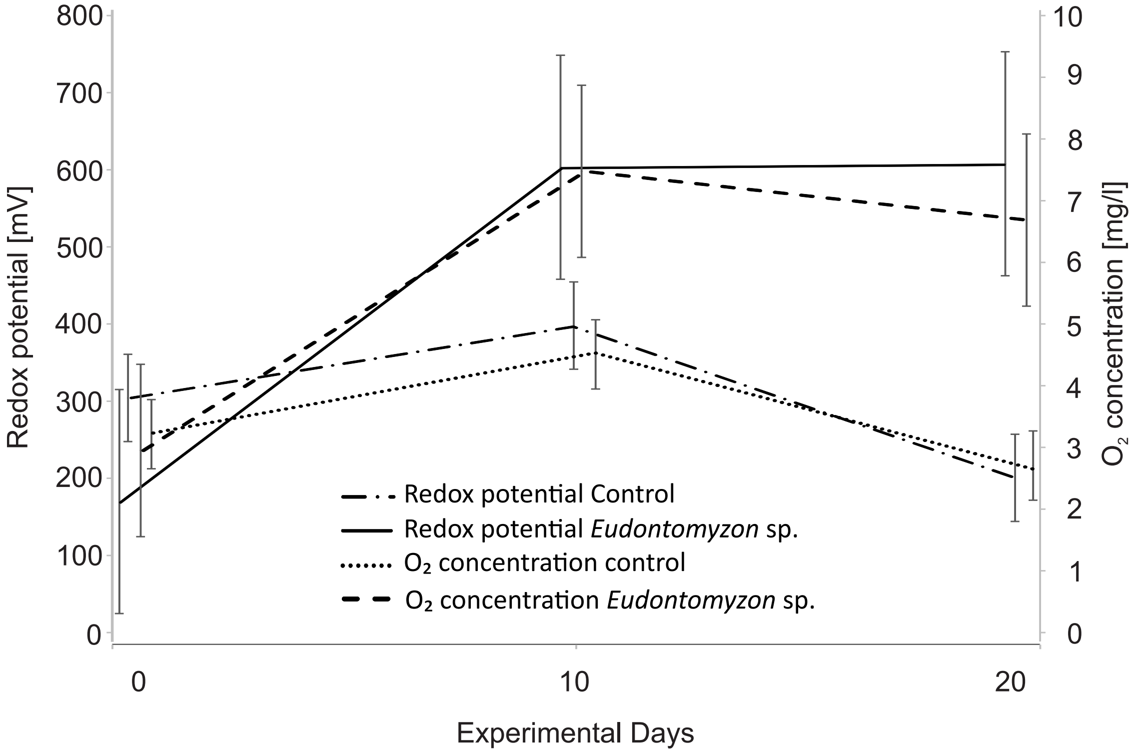

Fig. 4.1: Timeline of redox potential (continuous line for *Eudontomyzon* sp., broken line with dots for control) and oxygen concentration (broken line for *Eudontomyzon* sp., dotted line for control) in the interstitial zone over time (means with standard error). A strong increase in oxygen availability in the lamprey treatment is evident from the beginning to the end of the experiment

On day 10 and 20, the sulfate concentrations in the controls were significantly increased by more than 10% compared to those in the lamprey treatments (pairwise Wilcoxon rank sum test, all $p < 0.05$). The opposite pattern was found for nitrate concentration on day 20. Nitrate in the lamprey treatments was more than 40 % higher compared to the controls (pairwise Wilcoxon rank sum test, all $p < 0.01$).

Table 4.2: Treatment specific abiotic variables (means ± SDs) in the interstitial zone on day 0, day 10 and day 20.

	Day 0		Day 10		Day 20	
Variable	Control	*Eudontomyzon* sp.	Control	*Eudontomyzon* sp.	Control	*Eudontomyzon* sp.
PH	7.68 ± 0.07	7.66 ± 0.10	7.70 ± 0.11	7.89 ± 0.03	7.70 ± 0.08	7.89 ± 0.04
Oxygen saturation (%)	32.9 ± 18.26	30.0 ± 18.10	46.40 ± 25.47	75.60 ± 4.95	24.8 ± 7.10	70.2 ± 7.31
Electric conductivity	815.22 ± 27.33	807.0 ± 24.24	801.67 ± 27.58	781.78 ± 2.22	820.25 ± 27.46	780.5 ± 3.51
Sulfate concentration (mg/l)	39.74 ± 18.97	44.79 ± 11.53	36.8 ± 8.48	30.41 ± 3.43	35.04 ± 8.25	31.13 ± 0.42
Nitrate concentration (mg/l)	12.77 ± 8.76	8.12 ± 6.41	16.18 ± 7.18	21.3 ± 0.37	12.27 ± 6.11	20.84 ± 0.31

4.4.2 Impacts of lamprey larvae on microbial communities

According to our hypothesis and in line with the observed alterations in physical and chemical habitat properties, the microbial community composition differed between the lamprey treatments and the controls. Before the introduction of lamprey larvae, no differences in microbial communities between microcosms were detected (PERMANOVA, $p > 0.05$). A significant differentiation between microbial communities in treated and untreated microcosms was detected on day 20 (PERMANOVA, $p < 0.01$). Along a timeline, an increasing OTU composition differentiation between the lamprey treatments and the controls was evident, especially between day 10 and 20 (Fig. 4.2).

Certain T-RFs, which were solely present in the lamprey treatments (145 base pairs (bp), 437 bp, and 483 bp), as well as one T-RF (488 bp), which was exclusively present in the controls, mostly contributed to the observed differences in OTU composition (Fig. 4.3). According to the MiCA database search and in line with the physical and chemical differences, different proportions of aerobe and anaerobe bacteria were found between lamprey treatments and controls. In the lamprey treatments, the percentage of aerobe bacteria was higher compared to that of anaerobes. In the controls, the proportion of aerobe and anaerobe bacteria was nearly the same.

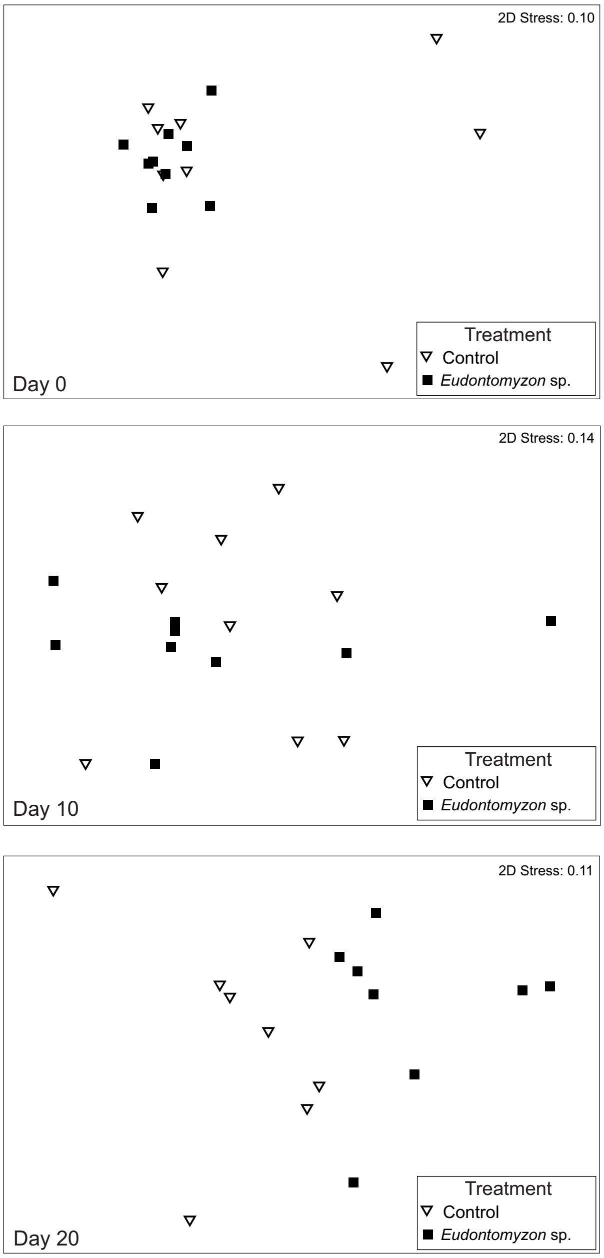

Fig. 4.2: Increasing differentiation of the T-RF composition in the interstitial zone between the control (empty triangles) and the lamprey treatment (filled quadrats) over a timeline. On day 0 the microbial community composition is very similar among treatments. The 2-dimensional NMDS based on Bray-Curtis similarity reveals a clear differentiation of the microbial community composition over time: On day 10 a slight differentiation of microbial communities between treatments is displayed, followed by a statistically significant differentiation on day 20. The variation explained by the horizontal and vertical axis is 16.8 % and 15.2 % for day 0, 8.0 % and 10.8 % for day 10, and 7.1 % and 12.5 % for day 20.

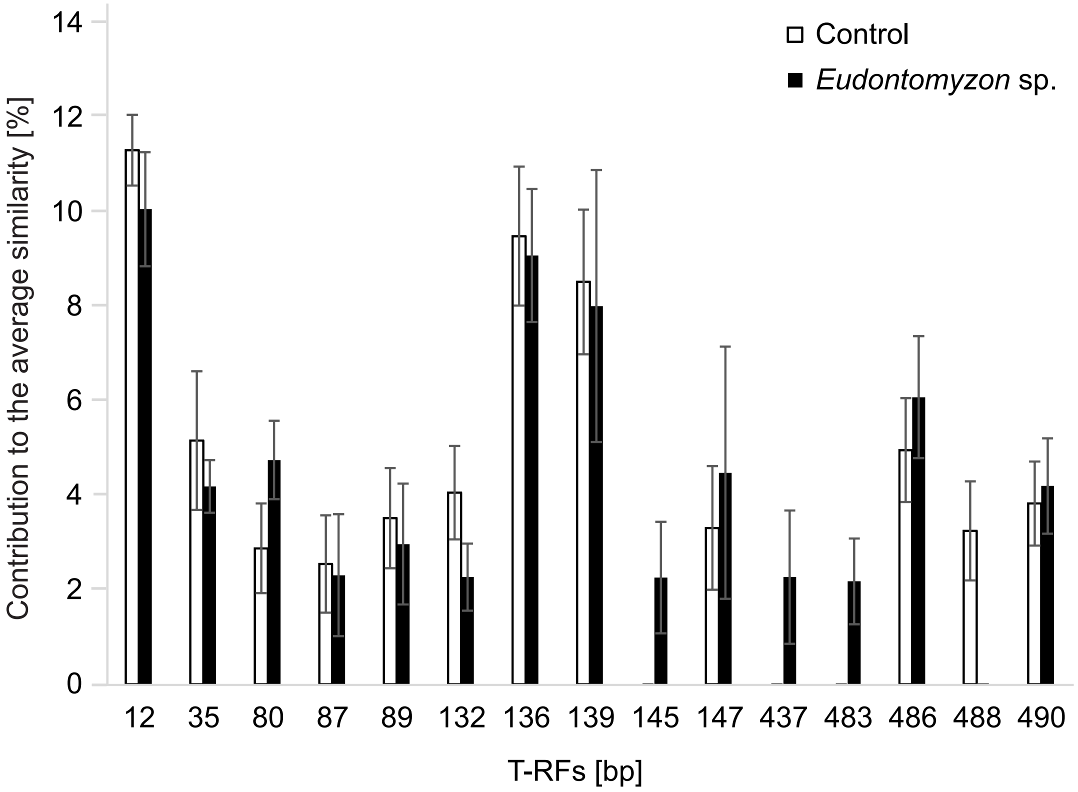

Fig. 4.3: Contribution of single T-RFs to the average similarity. The 145, 437 and 483 bp T-RFs exclusively contribute to the average similarity in the lamprey treatment (filled bars). Error bars indicate the standard deviation within 9 biological replicates in the lamprey treatments and the controls.

Physical and chemical variables that significantly influenced the observed microbial community compositions were revealed through DISTLMs: oxygen concentration (explained 7.5 % of the variance in T-RF data, $p \leq 0.001$), pH (7.3 %, $p \leq 0.001$), redox potential (6.4 %, $p < 0.01$), and electric conductivity (3.8 %, $p < 0.01$). Nitrate concentrations were also related to the microbial community composition in the interstitial zone (7.1 %, $p \leq 0.001$), as well as magnesium (5.5 %, $p < 0.01$), potassium (3.5 %, $p < 0.05$), chloride (3.5 %, $p < 0.05$) and sulfate concentrations (3.4 %, $p < 0.05$). A NMDS between T-RFs of the lamprey treatments, the controls, and samples taken from the epidermal mucus of lampreys (all pooled over time) revealed a clear discrimination of the mucosal T-RFs to those of the filter samples from the controls and the lamprey treatments (Fig. 4.4). NMDS scores of the lamprey treatments occupied intermediate positions in the ordination space relative to the scores of controls and lamprey mucus samples.

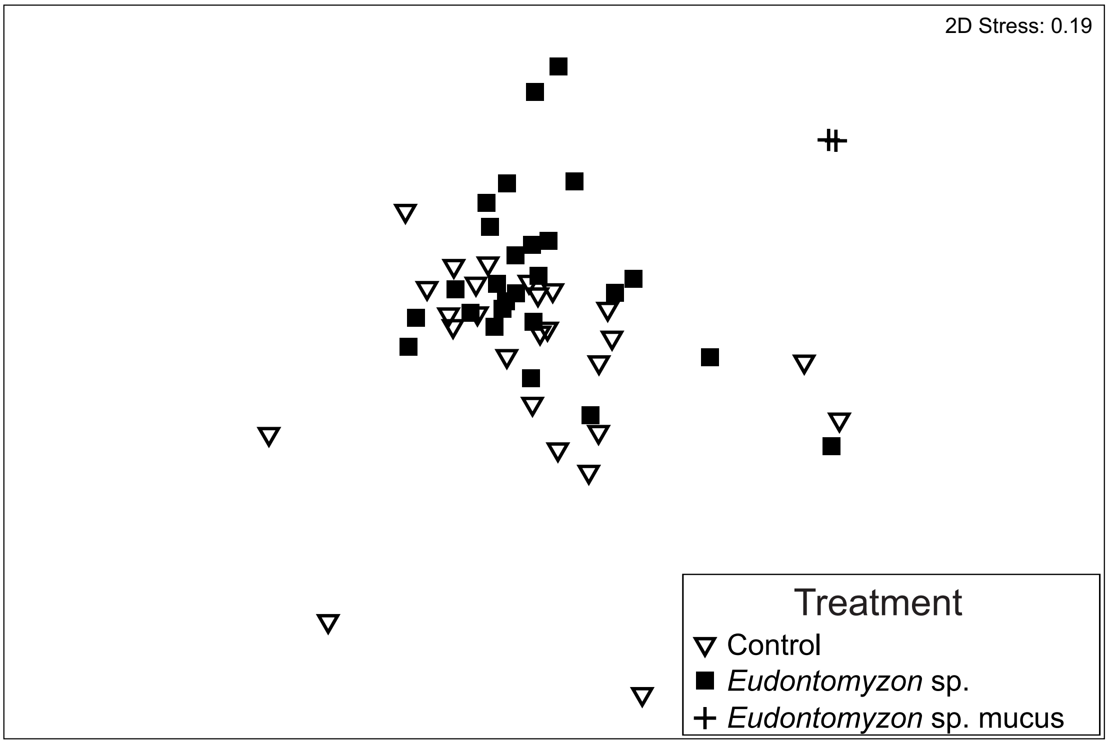

Fig. 4.4: 2-dimensional NMDS (based on Bray-Curtis similarity) of T-RF composition in the control (empty triangles) and the lamprey treatment (filled quadrats) pooled over time. In addition, the T-RF composition of adherent mucus of lamprey larvae (crosses) is visualized. The variation explained by the horizontal and vertical axis is 6.4 % and 5.0 %.

4.5 Discussion

The presented results support our hypotheses and clearly substantiate that lamprey larvae have an important impact on physical and chemical habitat properties and the microbial community composition in the interstitial zone, justifying the classification of larval lampreys as ´ecosystem engineers´. Lamprey-induced bioturbations increased oxygen availability and increased redox potentials within the stream bed, which can be considered an important ecosystem service, at least for specific life-history stages of various oxygen-dependent species such as freshwater mussels and gravel-spawning fishes (Geist & Auerswald, 2007; Sternecker et al., 2013a; 2013b; Denic et al., 2014a and 2014b).

The observed strong differences in interstitial oxygen availability and redox potentials between microcosms with and without lamprey larvae in the presented standardized laboratory experiment are in line with the field study findings of Shirakawa et al. (2013)

who reported high redox potentials in substrates with lamprey larvae. The increased oxygen availability within microhabitats adjacent to lamprey burrows is likely a result of the increased exchange between open and interstitial water. In addition to these physical interchanges, we suggest that the nocturnal changes of position of the larvae (Hardisty, 1979) further contribute to the distribution of oxygen-rich open water throughout the interstitial zone. Detailed physical reasons for a development of oxygen-rich microhabitats surrounding the burrows of lamprey larvae can be found in the publication of Li & Liu (2015) who studied shear flow interactions between tubes and surrounding matrices in the context of aquifers. They concluded that different water bodies mix up in the matrix around tubes because there are pressure gradients along and perpendicular to the tubes. Thus, downwelling water flowing into the tubes in addition to water flowing perpendicular to the tubes, can explain the mass transport of oxygen-rich open water throughout the interstitial zone. Newly established microhabitats around burrows of benthic organisms can be used by other benthic meiofauna (Reise & Ax, 1979) which can in turn influence physical and chemical habitat properties, as reported for alderfly nymphs (Wang et al., 2001). Our results may compare to studies examining bioturbations from benthic macroinvertebrates, where the character and extent of such modifications does not solely depend on the mode of bioturbation (Nogaro et al., 2009), but also on body size of bioturbators. The establishment of meiofauna in close proximity to burrows of ecosystem engineers may be a crucial part of the food web and therefore can be equally important for ecosystem functioning.

Many studies have highlighted the key-role of stream-bed habitats for the ecological functioning of lotic ecosystems (Boulton et al., 1998; Geist, 2011; Mueller et al., 2013b; Sternecker et al., 2013a). Our results emphasize that the burrowing behavior of lamprey larvae is crucial for the maintenance of aerobic physical and chemical habitat properties in the interstitial zone. These modifications on the microhabitat scale are essential for many different species and life stages, and may be particularly important in the absence of substrate-resorting floods in anthropogenically modified flow regimes. Oxygen availability in the interstices of the stream-bed is important for every life stage of brook lampreys (Hardisty, 1979) as well as gravel spawning salmonids. Interstitial oxygen availability is linked to high hatching success for salmonids (Sternecker et al., 2013a, 2013b) and macroinvertebrate colonization at sites with increased oxygen availability may provide an important prey source for salmonids throughout the duration of their life-cycles. Additionally, many freshwater mussels need stable substrates with high oxygen availability during the critical post-parasitical phase (Geist & Auerswald, 2007; Geist, 2010) as well as in adult life stages (Denic et al., 2014a). Lampreys, salmonids, freshwater pearl mussels (*Margaritifera margaritifera,* Linnaeus 1758) and thick shelled river mussels (*Unio crassus*, Philipsson 1788) co-exist at the site from which lamprey larvae were collected for this experiment (personal supplemental comment to Mueller et al., 2014). This naturally occurring co-existence of species depending on a well-oxygenated stream-bed coupled with the presence of a species that increases and maintains oxygen supplies exemplifies the synecological network and points out the relevance of the observed results on a greater ecological scale.

In addition to the observed changes in abiotic habitat properties, a currently undescribed differentiation in microbial community composition was detectable between controls and the lamprey treatments. We suggest that the strong differentiation in the microbial OTU composition over time is partly induced by the treatment-specific modification of physical and chemical habitat properties. First, our argument is supported by the steady divergence of physical and chemical variables over time, which was similarly reflected by the T-RF development between treatments. Second, a shift in microbial community composition exclusively due to the introduction of mucosal bacteria of lamprey larvae can be excluded because the mucosal T-RF fingerprint strongly differed from that of the filter samples from the lamprey microcosms. Had the mucosal bacteria inoculated a developing microbial community in the lamprey treatment, highly similar T-RF fingerprints in filter samples and lamprey mucus would have been observed. Therefore, it is most likely that both effects (changing physical and chemical parameters over time and the introduction of new OTUs with the epidermal mucus) influence the microbial community composition (Benhamed et al., 2014). Possibly, the role of the alterations in physical and chemical habitat properties is somewhat larger because the epidermal mucus of fishes hosts bactericidal proteins and other innate immune defense molecules (Subramanian et al., 2007 and 2008). The specific microbial community that evolves in the nearer environment of burrows created by ecosystem engineers is likely to contribute to the overall patchiness (spatial habitat variability) in the interstitial zone, which is driven by different abiotic and biotic factors (Braun et al., 2012). Habitat variability is of great importance for the functioning of freshwater ecosystems. Habitat variability provides for diverse ecological niches thereby driving the establishment of a high overall biodiversity (Baltensperger, 2015) and the preservation of biodiversity is one of the main goals in present management plans for freshwater ecosystems (Ward et al., 1999; Geist, 2015).

Our results illustrate that the ecosystem services provided by larval lamprey bioturbations not only contribute to physical habitat variability, but also facilitate an interstitial aerobic bacterial metabolism which is part of the complex microbial loop and a key component in nutrient recycling (Azam et al, 1983), indispensable for river functioning. Oxygen and nitrate concentrations measured from our experimental microcosms revealed that the bacterial metabolic activities likely affect riverine N-cycling. We observed higher nitrate concentrations in microcosms that contained larval lampreys and these higher concentrations likely explained part of the variability in the microbial OTU composition.

The ecological relevance of our results might be underestimated because the density of lamprey larvae in the microcosm boxes (25 per m^2) was less than natural densities observed in the Große Ohe (33 per m^2) (personal comment Müller & Pander 2010, 2011). With higher densities in a natural riverine ecosystem the observed experimental effects might be more pronounced. Our results substantiate that the functional relevance and the ecosystem services lamprey larvae provide to riverine ecosystems is evidentially high. Because lampreys generate suitable habitats for other species including meiofauna in this study and macrofauna in other studies (e.g. salmonids and freshwater mussels), lamprey larvae can clearly be considered ecosystem engineers, following the definition of Braeckman et al. (2014). The conservation management of far-reaching ecosystem services provided by lampreys as keystone fauna should be of great priority.

5. Effects of invasive and indigenous amphipods on physico-chemical and microbial properties in freshwater substrates

A similar version of this section is published:
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5.1 Abstract

Native benthic amphipods play a key role in freshwater ecosystem services such as leaf litter decomposition. The replacement of native by invasive species with different functional traits has the potential to alter hyporheic processes, communities, and food webs. Despite the increasing number of publications on invasive species, there is a lack of studies that compare the effects of native versus invasive amphipods on physico-chemical habitat properties and microbial communities in the substrate. We compared the effects of an indigenous (*Gammarus roeseli*, Gervais 1835) and an invasive (*Dikerogammarus villosus*, Sowinsky 1894) amphipod on leaf litter decomposition, as well as on bacterial communities and physico-chemical conditions in freshwater substrata. We hypothesized that the different amphipod species distinctly alter habitat conditions and microbial community composition, depending on functional differences in leaf litter decomposition. We detected strong differences between *G. roeseli* and *D. villosus* in the feeding rates on alder leaves, with eleven-fold greater decomposition of alder leaves by the native species. Additionally, our study revealed differences in microbial community composition between treatments at the substrate surface, but almost no differences in physico-chemical parameters in the interstitial and open water. These results support the hypothesis that the replacement of indigenous amphipod species by functionally different invasive amphipods can lead to a decrease in leaf litter decomposition and an altered microbial community composition with possible effects on benthic food webs. These effects on ecosystem services should be taken into account when assessing the impacts of invasive species on freshwater habitats.

5.2 Introduction

Aquatic ecosystems are important to mankind due to the many ecosystem functions and services they provide (e.g., Geist 2011). In particular, biological communities in stream ecosystems are important for the decomposition of leaf litter and the recycling of nutrients via the microbial loop (Azam et al. 1983). Multiple anthropogenic impacts are hypothesized to alter the biological communities in streams, and consequently their ecosystem services. Globally, invasions by non-native species are among the five major threats to aquatic ecosystems (Dudgeon et al. 2006). Several non-native species have been identified that can directly impact indigenous species and communities, in turn affecting the structure (e.g., by invasive ecosystem engineers) and functioning (e.g., by disrupting food webs) of ecosystems (Allen et al. 2013 and references therein). Whilst many of the direct effects of invasive species on indigenous species such as competition and predation are well understood (reviewed in Simberloff et al. 2013), there is much less information on how functional differences between invasive and indigenous species alter ecosystem services (Levine et al. 2003; Dukes & Mooney 2004; Vellend et al. 2007). An assessment of threats to ecosystem services by alien species requires the identification of their impacts on keystone elements for the functioning of these systems, such as food web structure and the network of important services like leaf litter decomposition, nutrient cycling and microbial communities as they affect the global metabolism of rivers (Marmonier et al. 2012).

In forested headwater streams with low primary production, the input and decomposition of allochthonous organic matter is crucial for the energy supply and the in-stream nutrient cycling (Vannote et al. 1980). Shredder organisms are particularly important in the recycling and bioavailability of nutrients because they break the organic matter up into finer particles. This increases the surface area for biofilms (Graca et al. 2001) which are essential in the further degradation and nutrient recycling in the microbial loop (Azam et al. 1983). In freshwater ecosystems, amphipods are among the most important shredder organisms and thus play a key role in leaf litter decomposition (Navel et al. 2010). These processes might get increasingly disturbed through the replacement of native species by their invasive relatives. For instance, the invasive ponto-caspian amphipod *D. villosus* has mostly replaced the indigenous *G. roeseli* in the upper Danube river within a few years (Kley & Maier 2006, Brandner et al. 2013a), with unknown consequences on ecosystem services. Many studies analyzed the invasion success of *D. villosus* in relation to morphology, physiological tolerance, life-history strategies (all reviewed in Rewicz et al. 2014), anatomy (Mayer et al. 2009) and nutrition (Truhlar et al. 2014). All of those studies highlighted important aspects that explain the success of the species without taking the entire ecosystem functioning into account. In contrast, Piscart et al. (2011) reported on the impact of invasive amphipods on leaf litter recycling in aquatic ecosystems, but their study did not include the effects on physico-chemical habitat properties, nor those on microbiota.

A holistic assessment of the replacement of native amphipods by non-native ones should also take the versatile effects on the supporting function of ecosystems and their services into account. Many theories in invasion biology try to explain the success of alien species (Catford et al. 2009) but some of them are restricted to certain aspects of ecology like the novel weapons or enemy release hypothesis (reviewed in Catford et al. 2009). Nevertheless, some are appropriate to evaluate the effects of invasive species on newly invaded areas in a holistic way (on different levels of taxonomic organization including abiotic factors) such as the limiting similarity hypothesis (MacArthur & Levins 1967). Similarity to native species can include morphologic, trophic, genetic, as well as functional similarity. In plant invasions, like described for the Canada thistle (*Cirsium arvense*), potential invaders that are functionally similar to resident plant species are less likely to establish than those being functionally distinct (Larson et al. 2013). The aim of the present study was to evaluate the functional differences of an invasive (*D. villosus*) and a native (*G. roeseli*) amphipod species to draw conclusions on the effects on ecosystem services due to a replacement of native by invasive amphipods. To our knowledge, no study has yet analyzed the effects of the invasive *D. villosus* on ecosystem services in direct comparison to the indigenous *G. roeseli* in a standardized and multivariate approach. The present study provides insight into the complex relationship of the presence and absence of invasive and indigenous amphipods on leaf litter decomposition, abiotic habitat properties and microbiota. A standardized microcosm experiment was set up with indigenous (*G. roeseli*) and invasive (*D. villosus*) amphipods and a control without organisms. Feeding rates, physico-chemical habitat properties (including standard ion concentrations) and microbial community composition were analyzed with a multivariate statistical approach to determine the effects of an alteration in species composition on supporting ecosystem services such as leaf litter decomposition and nutrient turnover.

According to the limiting similarity hypothesis, suggesting that successful invaders are functionally distinct from species in the recipient community (reviewed in Catford et al. 2009), we assumed that the invasive *D. villosus* is functionally different compared to the indigenous *G. roeseli*. These differences could be in burrowing behavior and in leaf litter processing due to a different nutrition which in turn has the potential to affect physico-chemical habitat parameters, and indirectly microbial communities. Therefore, we compared the two species for i) differences in the feeding rate on alder leaves, ii) the alteration of physico-chemical habitat properties in open and interstitial water, and consequently, iii) differences in microbial community composition on the substrate surface and at five centimeters substrate depth.

5.3 Methods

5.3.1 Study design

The effects of the indigenous (*G. roeseli*) and invasive *(D. villosus*) amphipods on abiotic habitat properties and microbial community composition were investigated in a 35-day laboratory experiment under standardized conditions. The experiment included nine biological replicates for each of the treatments and the control without animals.
Plastic boxes, solid on five sides (24.2 x 15.5 x 18.2 cm, Rotho Kunststoff AG, Würenlingen, Switzerland) and equipped with a lid of gauze (mesh width 500 µm, Aquacultur Fischtechnik GmbH, Nienburg, Germany), were filled with a 10.0 cm substrate layer. Experimental boxes were randomly placed (balanced latin square scheme) in three fiberglass reinforced plastic-channels (3.55 m x 0.45 m x 0.17 m, AGK Kronawitter GmbH, Wallersdorf, Germany) with constant water flow to ensure identical exposure temperatures. The substrate layer consisted of defined grain fractions (0.85, 0.63 and 0.063 mm in a proportion of 1.5: 0.5: 4.0) of washed substrate from the Moosach River (a calcareous river, 48° 23´ 39.22´´ N; 11° 43´ 26.65´´ E). Every microcosm was constantly supplied with water from the same river by a dripping-

system with a flow rate of 3.5 – 4.0 l/h per box. One redox electrode (ELANA, Boden und Wasser Monitoring, Arendsee, Germany) per box was installed five cm below the substrate surface to ensure non-invasive measurements. In each box, a perforated pipe (Volume 5 ml, Gardena GmbH, Ulm, Germany) with a control valve on one end was placed in each box at the same substrate depth as the redox electrode to collect water samples from the substrate.

To assess the leaf litter decomposition abilities by both amphipod species, alder leaves (*Alnus glutinosa*) were collected in autumn to feed the animals. For standardization, one triangular piece (4.62 cm^2; 0.035 ± 0.007 g dry weight) was cut out of every leaf at the same position and dried at room temperature. Before the test animals were fed with the triangular leaf pieces, the leaf pieces were pre-incubated for seven days (Dedourge-Gafford et al. 2009) in water from the Moosach River. Every second day, 80 % of the water for leaf pre-incubation was replaced by fresh water from the Moosach River.

The experiment was performed during February and March 2014 with a constant 12-hour light-dark cycle and a mean temperature in open water of 10.5 ± 1.2 °C. Due to the importance of similar initial conditions (i.e. physicochemical parameters, microbial diversity), the microcosms were supplied with water for the first 15 days (day -15 to day 0) but not stocked with the amphipods. Each treatment consisted of nine biological replicates. On day 0, 30 *D. villosus* or 30 *G. roeseli* were introduced into each of nine boxes for the respective treatments. Nine control boxes remained unpopulated with amphipods. The invasive *D. villosus* were kick-sampled at the Upper Danube River (48° 54′ 35.5′′ N, 11° 51′ 07.4′′ E) one week before the introduction into the microcosms. The native *G. roeseli* were kick-sampled at the Moosach River (48° 23′ 39.22′′ N; 11° 43′ 26.65′′ E) which also belongs to the Danube drainage. For standardization, we used 30 test animals of the same size per box. Only specimens which were small enough to pass through a sieve with a mesh width of 3.0 mm, and large enough to be retained on a sieve with a mesh width of 1.5 mm were used. Before the experiments, all test animals were held under identical conditions for one week in basins at the Chair of Aquatic Systems Biology of the Technische Universität München, Germany for acclimatization. The test animals remained in the experimental boxes for 20 days and were fed every day with one piece of a pre-incubated alder leaf per box. The remainder of the triangular piece of leaf from the previous day was taken out of the box and dried, to prevent from further decomposition by microorganisms, until analysis. All control boxes were treated in the same way as the boxes with test animals. To determine the feeding rate of the amphipods, the remaining leaves were photographed and the areas measured with Image J (version 1.48v). For statistical analyses, the area of the leaf-pieces from the control boxes were taken as 0 % feeding rate. Dead specimens were removed and replaced daily.

5.3.2 Analysis of physico-chemical parameters

Oxygen concentration and saturation, as well as redox potential as long term oxygen indicator, were measured representing important habitat properties for bacterial metabolism. Values in redox potential > 300 mV indicate oxic conditions, crucial for obligate aerobic bacteria. The redox potential in 5 cm substratum depth was measured using a Platinum electrode fixed in a plastic tube as described in Geist & Auerswald (2007). Physicochemical water parameters (pH, oxygen concentration and saturation, electric conductance, and redox potential) were measured with a handheld WTW Multi 3430 SET G and a WTW pH 3110 SET-2 (Wissenschaftlich Technische Werkstätten, Weilheim, Germany) on days -15, -10, -5, 0, 5, 10, 15 and 20 of the experiment at nearly the same time between 9:00 am and 1:30 pm in random order. Parameters in open water were measured five cm above the substrate surface. Water samples (20 ml) of the interstitial zone were collected from each box using the valve of the installed and perforated pipe. To minimize sampling bias, 5 ml (the volume of the pipe) were purged before the interstitial water was sampled. Samples of interstitial water collected on experimental days 0, 10 and 20 were immediately frozen and stored at -20°C. To evaluate treatment-related differences (including different forms of N indicative of N-cycling), these samples were used for ion detection (i.e., Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}, and F^-) using two ICS 1100 ion chromatographs, one with a AG-22 as guard column and AS-22 column for anion separation, and the other one with a CG-12 as guard column in line with a CS-12 column for cation separation (Ion chromatographs and colums from Thermo Scientific, Dreieich, Germany). As eluent for anions, a mixture of 1.8 mM dinatriumcarbonate and 1.7 mM natriumhydrogencarbonate was used. 20 mM methansulfonacid was used for eluting cations.

5.3.3 Analysis of bacterial diversity

The bacterial community composition was analyzed based on DNA-based terminal restriction fragment length polymorphism (T-RFLP) fingerprinting. For investigating bacterial communities at 5 cm substrate depth, samples of interstitial water (taken on experiment days 0, 10 and 20) were filtered through 0.22 µm CME membrane filters (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), which were subsequently stored in sterile Petri dishes at -20 °C. For each treatment, nine biological replicates (one from each box) were analyzed. Membrane filters were cut into pieces (0.5 cm^2) using a sterile scalpel for DNA-isolation as described in Mueller et al. (2013a). For investigating bacterial communities in the upper 1 to 3 mm of the substrate, a representative substrate sample was taken with a spoon by "writing" a Z on the substrate surface. The samples were put in sterile screw cap plastic vessels and stored at -20°C. For DNA extraction, 0.8 g of each substrate sample with the adherent bacterial biofilm were put into a sterile 2.0 ml screw cap reaction tube filled with 0.l mm zirconia/silica beads (1:1) and DNA was extracted as described in Lueders et al. (2004). DNA-concentration was quantified using a ND-1000 Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany) and samples were stored at -20°C until further processing. As described in Lueders et al. (2006) and Pilloni et al. (2011), 16S rRNA gene-targeted T-RFLP fingerprinting was carried out but with an additional purification step using PCRExtract columns (PCRExtract Mini Kit, 5 Prime GmbH, Hamburg, Germany) according to the manufacturer's protocol. In total, a sum of 162 T-RFLP fingerprints were generated: nine biological replicates per treatment (n=3), sampling time point (n=3) and substrate depth (n=2). To evaluate electropherograms after capillary electrophoresis, the Gene Mapper 5.1 software (Applied Biosystems, Carlsbad, California, USA) was used and in the subsequential step T-RFLP data were analyzed with the free online-tool T-REX (Culman et al. 2009) using the peak heights to create the T-RF abundance data matrix. According to Abdo et al. (2006) a default factor of 1.2 was set for background noise filtering. The clustering threshold was set to 1.5 using the default alignment method of Smith et al. (2005) for peak alignments. To reduce data complexity, T-RFs that occurred in less than 10% of total samples were eliminated from the data set (Blackwood et al. 2007). To check which bacteria differ between treatments, and consequently, which functional differences result from our findings of distinct OTU compositions between the treatments, the terminated restriction fragments were further analyzed with the MiCa database search. Bacterial lineages were assigned to the detected terminated restriction fragments using the Microbial Community Analysis III (MiCA) with a sensitivity of maximum two mismatches within two bases from the 5´end of the primer in the RDP (R10, U27) 16S bacterial rRNA database (Shyu et al. 2007).

5.3.4 Data analyses

Multivariate data analyses were performed using the software PRIMER v.6 with PERMANOVA+ add on (Plymouth Marine Laboratory, Plymouth, United Kingdom). Based on the microbial community analysis, the DIVERSE function was used to compute different diversity indices (i.e., operational taxonomic unit (OTU) richness, Shannon diversity, Simpson and Evenness index including standard deviations (SD)). SIMPER (similarity percentages) analyses using Euclidian distances were performed to reveal which TRFs contribute most to the average similarity per treatment. To test whether physico-chemical variables and T-RF-compositions differ between treatments and sampling time-points (day 0, 10 and 20), PERMANOVAs (permutational multivariate analysis of variance) were performed using 999 permutations under the assumption of a reduced model using the sum of squares type III (partial). In the PERMANOVA design, the factor ´treatment´ was nested-fixed in the factor day (sampling time-point). To analyze the relationship between T-RF data and the physicochemical parameters of open and interstitial water, as well as the ion composition in the interstitial water, separate distance-based linear models (DISTLMs) were computed. For calculations of the DISTLMs, a number of 999 permutations using Bray-Curtis similarities as distance measures was defined. A non-metric multidimensional scaling (NMDS) was plotted to display the differences between bacterial communities at the substrate surface and at 5 cm substrate depth. Differences between treatments and sampling time-points in abiotic variables and OTU diversity indices, as well as differences in feedings rates between treatments, were analyzed using standard univariate statistics in R (version 3.0.2, www.r-project.com, 2013). Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene´s test). Pairwise comparisons between treatments and sampling time-points as well as those of feeding rates between treatments were performed using the non-parametric pairwise Wilcoxon rank sum test with Bonferroni correction since data were not normally distributed. Significance was accepted at $p \leq 0.05$ after Bonferroni correction.

5.4 Results

5.4.1 Differences in the feeding rate of *G. roeseli* and *D. villosus*

In line with our hypothesis, pronounced and significant differences in the feeding rates of invasive and indigenous amphipods on alder leaves were observed. The feeding rate of *G. roeseli* on the pre-incubated pieces of alder leaves was more than eleven times higher compared to that of *D. villosus*. The average area of the leaf pieces consumed by *G. roeseli* was 8.0 ± 2.5 $mm^2 d^{-1}$ per individual whereas that of *D. villosus* was only 0.7 ± 0.6 $mm^2 d^{-1}$ per individual. Figure 5.1 displays the feeding rates of alder leaves observed in all treatments in $mm^2 d^{-1}$ per individual.

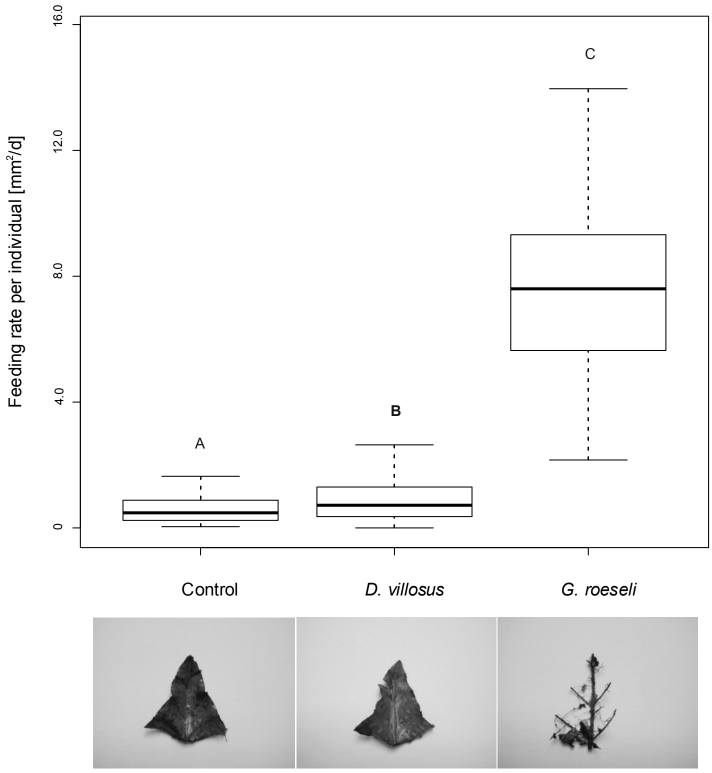


Fig. 5.1: Feeding rates of indigenous *G. roeseli* and invasive *D. villosus* compared to a control without animals on alder leaves after 24 h exposure. Different letters represent statistically

significant differences between treatments (pairwise Wilcoxon rank sum tests, Bonferroni corrected, both $p < 0.001$). Additionally, representative photographs illustrate an alder leaf out of a control box (left, no feeding rate), the rest of leaf consumed by *D. villosus* (middle, low feeding rate) and *G. roeseli* (right, high feeding rate) after 24 h exposure.

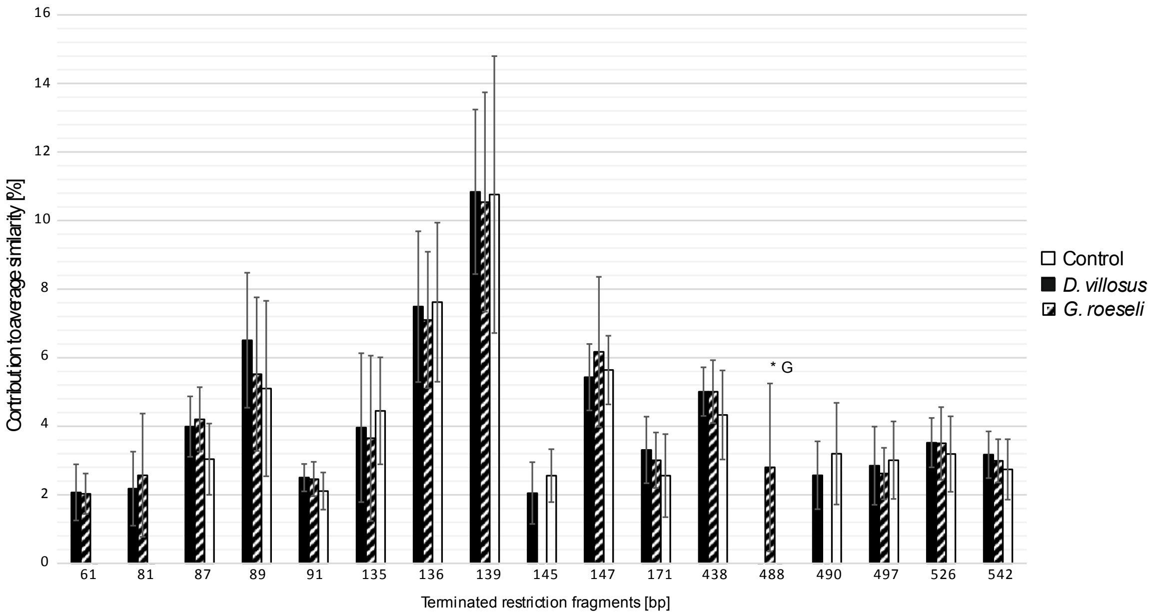
5.4.2 Effects of invasive and non-invasive amphipods on microbial communities in the interstitial zone

In line with the hypotheses, the microbial community composition differed significantly between treatments at the substrate surface. These effects were less pronounced and not statistically significant at 5 cm substrate depth.

Before the introduction of amphipods, OTU composition between treatments was highly similar, both at the substrate surface (PERMANOVA, 2-factorial, nested fixed, $p > 0.05$), as well as at 5 cm substrate depth (PERMANOVA, 2-factorial, nested fixed, $p > 0.05$).
The OTU composition at the substrate surface differed most strongly between all treatments at day 20 (PERMANOVA, 2-factorial, nested fixed, p (control – *D. villosus*) < 0.05; p (control – *G. roeseli*) < 0.01; p (*D. villosus* – *G. roeseli*) ≤ 0.001). This significant difference between treatments was not evident from comparisons of the common diversity indices (OTU richness, Evenness, Shannon, Simpson) (pairwise Wilcoxon rank sum tests, Bonferroni corrected; all p-values > 0.05). Additional differences in microbial community composition were detectable between the substrate surface and 5 cm substrate depth as evident from the number of different TRFs: 87 were found in samples collected from the substrate surface and 110 in those from 5 cm substrate depth. A PERMANOVA further substantiated the differences in OTU diversity between different substrate depths within all treatments (2-factorial, nested fixed, all $p \leq 0.001$) (Fig. 5.2). At 5 cm substrate depth, neither the OTU composition between treatments, nor that between sampling time points differed (PERMANOVA, 2-factorial, nested fixed, all $p > 0.05$). These findings were also confirmed by the comparison of calculated diversity indices which revealed no differences between treatments (pairwise Wilcoxon rank sum tests, Bonferroni corrected; all p-values > 0.05).

Fig. 5.2: Non-metric multidimensional scaling plot of operational taxonomic unit (OTU) composition of samples from the substrate surface (filled circles) and from 5 cm substrate depth (empty quadrats) overall sampling time points and treatments. The OTU composition in different substrate depths differs strongly.

Distinct T-RFs contributed significantly to the observed microbial differences. At the substrate surface, the 145 bp and 490 bp fragments contributed to the average similarity (SIMPER analysis) in the control and the *D. villosus* treatment, but they were not abundant in the *G. roeseli* treatment (Fig. 5.3a). In contrast, the 488 bp fragment contributed solely to the average similarity in the *G. roeseli* treatment. The 61 bp and 81 bp T-RF were only abundant in treatments containing amphipods. Generally, the contribution of T-RFs to the average similarity was dominated by two fragments, which were detectable in all treatments: the 139 bp (SIMPER; 10.71 ± 0.16 %) and the 136 bp fragment (SIMPER; 7.40 ± 0.27), almost without differences in abundance between treatments. At 5 cm substrate depth, the 205 bp and 287 bp T-RF was exclusively detected in presence of *D. villosus* (Fig. 5.3b). Additionally, the 118 bp and the 160 bp fragment was solely present in samples of boxes populated with amphipods. The T-RF with 216 bp was most abundant in all treatments with slight differences between *D. villosus*, *G. roeseli* and the control (SIMPER; 9.96 ± 1.69 %), followed by the 490 bp fragment which exhibited no differences in abundance between treatments (SIMPER; 7.66 ± 0.25 %).



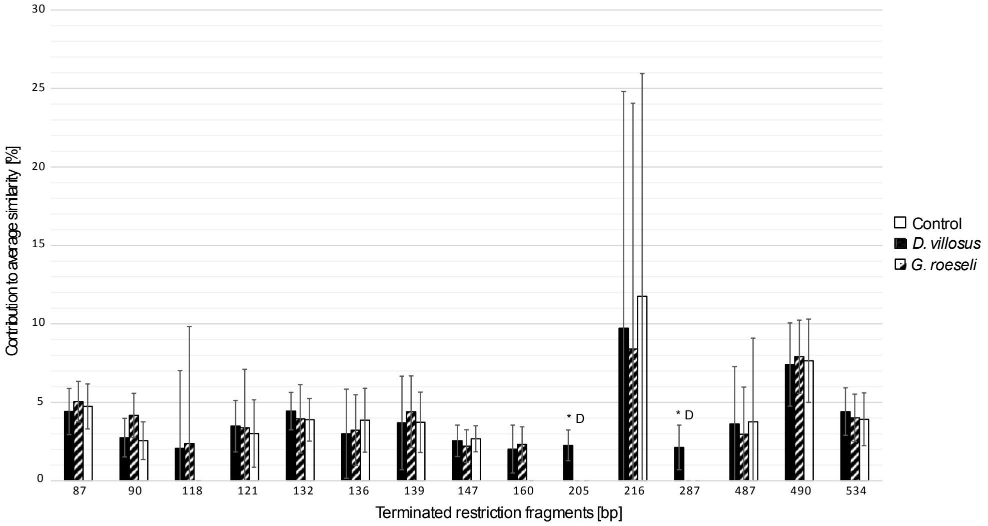


Fig. 5.3: Contribution of individual bacterial 16S rRNA gene T-RFs to the average similarity within treatments in a similarity percentage (SIMPER) analysis based on the abundance of terminated restriction fragments of samples from the substrate surface (Fig 5.3a) and from 5 cm substrate depth (Fig 5.3b). Presented are T-RFs contributing more than 2 % to the average similarity. The error bars represent the standard deviation of contribution to the average similarity between replicates. Different fillings of bars represent different treatments. T-RFs labeled with * G were exclusively present in the *G. roeseli* treatment and T-RFs labeled with * D were exclusively present in the *D. villosus* treatment.

The variance in OTU composition can be partially explained by the electric conductivity at the substrate surface (5.49 %) and at 5 cm substrate depth (3.44 %) (DISTLMs, adjusted R^2, all $p < 0.05$). In the substrate, fluoride (5.1 %) followed by ammonium (3.6 %) and sulfate (2.9 %) influenced the composition of the microbial community (DISTLM, adjusted R^2, all $p < 0.05$).

Based on the MiCa database assignments, aerobic as well as anaerobic bacteria (facultative and fastidious) were present in all treatments. The number of bacteria associated with plant decomposition and decaying matter processes (*Cytophaga* sp., *Caldicellulosiruptor* sp., *Brevibacillus* sp., *Gemella* sp.) was higher in the amphipod treatments compared to the control (*Spirochaeta smaragdine*.).

5.4.3 Alteration of physico-chemical habitat properties by invasive and non-invasive amphipods

Before the amphipods were introduced into the experimental boxes, neither the physico-chemical conditions in open water, nor those in interstitial water differed between treatments (PERMANOVA, treatment nested in day, for day 0 all $p > 0.05$), indicating highly similar starting conditions for the experiment.

Also after the introduction of amphipods, the physico-chemical habitat properties in open and interstitial water were mostly similar between the treatments (pairwise Bonferroni corrected Wilcoxon rank sum tests for pH, O_2 concentration, O_2 saturation, electric conductivity and redox potential, all $p > 0.05$) (Tab. 5.1, Tab. 5.2). Over time, a trend towards a differentiation of abiotic habitat properties in the substrate between sampling time points could be detected, but this development was largely independent of the treatment. Despite of a lack of significance, there was a trend towards lower redox potentials and higher electric conductance in the substrate of those treatments with the highest leaf litter decomposition (i.e. boxes containing *G. roeseli*) compared to boxes containing *D. villosus* and the control. Open water conditions were generally similar throughout all treatments and exposure time.

In line with the physico-chemical measurements in the substrate, the ion composition in the substrate was similar between treatments (pairwise Wilcoxon rank sum tests, Bonferroni corrected, all $p > 0.05$), except for sulfate: in the control, the sulfate concentrations were lower (34.3 ± 7.6 mg/l) compared to the *D. villosus* treatment (41.5

± 8.2 mg/l) and the *G. roeseli* treatment (40.0 ± 5.0 mg/l) (pairwise Wilcoxon rank sum test, Bonferroni corrected, all $p < 0.01$). Over time, ammonium (control: 10-fold decrease), magnesium (control: 1.2-fold decrease) and fluoride concentrations (control: decrease from 0.03 to nearly 0.0 mg/l) decreased (pairwise Wilcoxon rank sum tests, Bonferroni corrected, all $p < 0.05$). The chloride concentrations increased from day 0 to day 20 in all treatments (control: by 7 %) (pairwise Wilcoxon rank sum test, Bonferroni corrected, all $p < 0.01$).

Table 5.1: Abiotic habitat parameters in the open water and common diversity indices based on bacterial OTUs for all sampling time points. Note that ions which were not different between treatments are not presented.

	Day 0			Day 10			Day 20		
Variable	control	*D. villosus*	*G. roeseli*	control	*D. villosus*	*G. roeseli*	control	*D. villosus*	*G. roeseli*
O_2 concentration [mg/l] ± SD	9.7 ± 0.2	9.8 ± 0.3	9.8 ± 0.1	9.6 ± 0.3	9.4 ± 0.1	9.2 ± 0.5	9.2 ± 0.2	8.8 ± 0.8	8.9 ± 0.4
O_2 saturation [%] ± SD	93.2 ± 1.6	93.0 ± 1.6	93.2 ± 1.4	91.3 ± 1.6	89.7 ± 1.6	89.0 ± 3.9	91.1 ± 1.2	86.7 ± 6.9	88.3 ± 3.5
pH ± SD	7.8 ± 0.1	7.8 ± 0.0	7.8 ± 0.1	7.9 ± 0.0	7.9 ± 0.0	7.9 ± 0.0	7.9 ± 0.0	7.9 ± 0.0	7.9 ± 0.0
Redox potential [mV] ± SD	494.9 ± 3.6	490.7 ± 14.8	493.7 ± 6.1	491.6 ± 7.0	493.6 ± 6.3	491.5 ± 8.1	504.3 ± 5.8	503.65 ± 5.0	504.6 ± 5.3
Electric conductance [µS/cm] ± SD	804.6 ± 3.5	809.1 ± 3.0	805.8 ± 4.1	791.8 ± 5.1	794.3 ± 1.2	794.0 ± 5.0	788.2 ± 6.5	790.9 ± 4.2	791.9 ± 3.8
OTU richness	12.4 ± 2.0	10.5 ± 2.1	12.6 ± 2.3	12.4 ± 2.9	11.2 ± 3.1	11.6 ± 3.7	12.7 ± 2.0	11.7 ± 1.9	11.1 ± 1.2
Evenness	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
Shannon	3.7 ± 0.2	3.6 ± 0.2	3.7 ± 0.1	3.2 ± 0.2	3.1 ± 0.3	3.1 ± 0.5	3.7 ± 0.2	3.6 ± 0.1	3.5 ± 0.1
Simpson	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

Table 5.2: Abiotic habitat parameters and common diversity indices based on bacterial OTUs in the substrate for all sampling time points. Note that ions which were not different between treatments are not presented.

	Day 0			Day 10			Day 20		
Variable	control	*D. villosus*	*G. roeseli*	control	*D. villosus*	*G. roeseli*	control	*D. villosus*	*G. roeseli*
O_2 concentration [mg/l] ± SD	2.6 ± 1.5	2.2 ± 0.3	2.0 ± 0.3	2.5 ± 1.8	2.0 ± 0.3	2.0 ± 0.3	2.1 ± 1.4	1.8 ± 0.2	1.7 ± 0.3
O_2 saturation [%] ± SD	26.1 ± 15.4	21.3 ± 3.1	20.2 ± 2.66	24.9 ± 18.7	19.7 ± 3.2	20.0 ± 3.4	21.2 ± 1.4	17.7 ± 1.8	17.5 ± 3.4
pH ± SD	7.6 ± 0.1	7.6 ± 0.1	7.6 ± 0.0	7.6 ± 0.1	7.6 ± 0.0	7.6 ± 0.0	7.6 ± 0.1	7.6 ± 0.1	7.6 ± 0.1
Redox potential [mV] ± SD	-108.3 ± 257.1	-228.8 ± 14.4	-228.1 ± 17.1	-94.0 ± 272.8	-125.0 ± 78.8	-139.6 ± 177.8	-68.6 ± 314.2	-123.9 ± 109.5	-203.3 ± 116.4
Electric conductance [µS/cm] ± SD	949.6 ± 113.2	929.9 ± 63.3	951.7 ± 79.2	900.9 ± 98.5	907.1 ± 59.7	925.9 ± 82.6	891.3 ± 83.2	874.4 ± 67.07	921.6 ± 69.1
Sulfate [mg/l]	36.2 ± 4.8	44.6 ± 7.9	42.9 ± 3.9	32.7 ± 11.9	41.4 ± 8.4	40.1 ± 5.0	34.1 ± 3.8	37.9 ± 7.7	36.6 ± 4.3
OTU richness	14.4 ± 2.7	14.8 ± 2.7	13.6 ± 4.1	13.1 ± 4.1	14.2 ± 2.5	14.6 ± 1.5	13.6 ± 2.8	12.5 ± 3.0	12.9 ± 4.6
Evenness	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Shannon	3.7 ± 0.4	3.6 ± 0.4	3.8 ± 0.3	3.0 ± 0.2	3.1 ± 0.3	3.0 ± 0.4	3.4 ± 0.4	3.4 ± 0.4	3.2 ± 0.5
Simpson	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.1

5.5 Discussion

The results of this study show that the rates of leaf litter decomposition by invasive and indigenous amphipods differ significantly under standardized laboratory conditions, in contrast to highly similar initial conditions among treatments. The present study provides first evidence that the microbial community composition can also be altered if indigenous amphipods are replaced by invasive species. Consequently, the currently occurring replacement of native *G. roeseli* populations by non-native *D. villosus* in many aquatic ecosystems likely results in ecologically relevant functional changes, affecting leaf litter decomposition and related ecosystem services such as water purification and nutrient cycling.

The observed strong differences in the feeding rate of the two amphipod species used in this study can be explained by their different functional roles in the food web, which in turn has an influence on ecosystem services such as leaf litter shredding nutrient turnover. According to the literature, the dietary preferences and the trophic niche of *D. villosus* are controversial. On the one hand, *D. villosus* is often referred to as the carnivorous “killer shrimp” (see citations in MacNeil et al. 2013) which is considered a much more effective predator compared to *G. roeseli* (Dick & Platvoet 2000; Dick & Kelly 2002; Kinzler & Maier 2003; Krisp & Maier 2005). On the other hand, studies examining stable isotopes or fatty acids concluded that the dietary niche of *D. villosus* is not different from that of native amphipods but that *D. villosus* is opportunistic and omnivorous (Maazouzi et al. 2007; Maazouzi et al. 2009; Koester & Gergs 2014). This latter theory is also supported by anatomical studies which suggest that the mouthparts of *D. villosus* do not indicate any specialization for a carnivorous diet (Mayer et al. 2008). For *G. roeseli*, Mayer et al. (2009) concluded that no specialized adaptations of mandibles for feeding on animal prey are present and that the antennulae and antennae are highly suitable for detritus feeding and collecting organic food. This is in line with Kelly et al. (2002) assigning the genus *Gammarus* to the shredder organisms because test animals exhibited leaf shredding in presence of animal prey.

The results from our study suggest that *G. roeseli* is able to solely cover its energy demand by leaf shredding over a time period of at least a few weeks, whereas *D. villosus* exhibited a much lower feeding rate and a higher mortality rate, indicating that the animals starved due to absent animal prey or that the invasive amphipods exhibited cannibalism. Cannibalism might be possible since single dead individuals of *D. villosus* were not fully intact when collected after the 24h intervals. No such observation was made in any of the *G. roeseli* treatments. Differences in size between species cannot explain the observed differences in feeding preferences since only individuals of the same size were used. Instead, the standardized animal size in the experiments might even have underestimated the dietary differences between the two species since *D. villosus* can reach greater sizes than *G. roeseli* and since larger specimens are generally more likely to be carnivorous than smaller ones. Additionally, under natural conditions, *D. villosus* would have been able to feed on animal prey in addition to leaf litter, which was not the case under the experimental conditions in the present study. Taken together, it is likely that *G. roeseli* and *D. villosus* occupy different niches in the food web, which would be expected according to the limiting similarity hypothesis (reviewed in Catford et al. 2009). This is also relevant for the ecosystem services both species provide: if *D. villosus* is a scavenger amongst other nutrition habits, then it probably provides ecosystem services like water purification and the containment of diseases transmitted by carrion, whereas *G. roeseli* is more likely to affect the nutrient cycling and microbial biofilm formation.

As indicated by the results for the physico-chemical parameters, our hypothesis that the amphipods would affect the microbial community by altering physico-chemical habitat properties has to be rejected. Instead, a direct effect of the different leaf shredding activities in the two species on microbial communities is likely, as evident from the observed OTU differences at the substrate surface. It is well established that the gut microflora depends on the dietary composition (Pedrotti et al. 2015 and references therein), and the excretion of different microbiota by the two species as a consequence of the differences in their diets could explain the observed microbial differences between the treatments. Considering the higher feeding rate on alder leaves of *G. roeseli*, their gut microbiota must be highly influenced by the herbivorous diet. On the contrary, *D. villosus* of the same size exhibited an eleven-fold lower feeding rate on alder leaves compared to *G. roeseli* and occasional cannibalism was observed in *D. villosus*. These differences in dietary composition are likely to result in differences in gut microflora between the invasive and indigenous species. Since the gut microflora is also present in feces due to the contact to the colon epithelium (Choy et al. 2014), the observed differences in OTU composition likely represent differences in the gut microflora excreted to the substrate surface. In addition to the bacteria surviving the digestive tract and consequently being present in fecal pellets, the latter are colonized rapidly by external bacteria since the microbes are possibly attracted by leaching DOM (reviewed in Wotton & Malmqvist 2001). After the described colonization with microbes, the pellets are broken down over time and in this colonized and protein enriched state (Shepard & Minshall 1981) they serve as high quality food resource for detritivorous invertebrates (discussed in Wotton & Malmqvist 2001; Joyce et al. 2007; Joyce & Wotton 2008). Thus, native amphipods provide essential ecosystem services in freshwater ecosystems: in addition to the leaf litter degradation, the egestion of fecal pellets is indispensable for primary food web structure.

The explanation that the differences in OTU composition are likely to be induced by differences in nutrition, and therefore gut microbiota, would be also in line with the observation that differences in OTU composition between treatments were only detected at the substrate surface, but not deeper in the substrate. Due to the observed low sediment reworking behavior of both amphipods, no or very low amounts of feces were transferred deeper into the substrate which may be different if other species are present. The consequences of the observed alteration of microbial community composition at the substrate surface may have an impact on grazers feeding on these biofilms and thus on the food web, as well as on the nutrient cycling and the ability for biological degradation of organic contaminants.

Besides the classic effects on ecosystems (e.g., on the food web) there are many more effects which should be considered when assessing the impact of invasive species on supporting ecosystem services. Our standardized experimental conditions did not fully imitate naturally occurring conditions concerning the amounts of organic matter input into freshwater ecosystems and other species which also contribute to oxygen consumption within the substrate were excluded. This may have underestimated the effects on physicochemical conditions. For instance, streams in temperate regions are characterized by a singular input of large amounts of leaves within a short time period during autumn. Based on the observed differences in leaf litter decomposition rates, the replacement of *G. roeseli* by *D. villosus* can have strong effects on the shredded amounts of leaf litter input, and consequently on the timeline of oxygen consumption during this process. The time it takes to degrade all fallen leaves in a freshwater ecosystem would be more than eleven times longer in presence of *D. villosus* compared to *G. roeseli* with possible adverse effects on habitat quality. The decomposition of the naturally occurring large amounts of leaf litter affects the N-cycling, the ion composition and oxygen availability. The longer it takes to shred the large amounts of leaf litter, the longer the time-period in autumn and winter when a potential oxygen-depletion (because of an increased oxygen-demand due to the remineralization of organic matter) can occur, with potential negative consequences for all species depending on certain concentrations of oxygen. This might not be of great importance in the open water of oxygen-rich streams with high current, but the situation can be different upstream of weirs and dams with lake-like hydrological properties (Nykänen et al. 2012). Furthermore, the large amounts of leaves which are not shredded sink down to the substrate surface and can clog the pores of the stream bed, decreasing the exchange between open and interstitial water. If this happens, the reproductive success of lithophilic fish species depending on an oxygen-rich stream bed is adverse affected, as in the case of salmonid eggs which mostly develop in the interstitial zone over winter (Hancock 2002 and references therein; Sternecker et al. 2013a; Sternecker et al. 2013b). Similarly, juvenile stages of highly endangered freshwater mussels (Unionoida) also strongly depend on oxygen-rich substrates with high exchange rates to the open water (Geist & Auerswald 2007; Denic et al. 2014b and references therein). As obvious from the physico-chemical data from different sampling time points, the conditions within the substrate change over time. The results for physico-chemical variables, which were not significantly different between treatments, likely underestimate the complexity of the naturally occurring situation. However, mean values are probably less important than extremes, e.g., in the case of oxygen minima.

In summary, our results indicate that the observed effects of invasive amphipods are greater and more diverse than currently assumed, especially if the invasive species replace keystone fauna which are indispensable for the overall organic matter processing and nutrient turnover in freshwater ecosystems, such as amphipods. In many aquatic ecosystems in Europe, native gammarids have already been largely replaced by non-native ones which is also true in the natural habitat where the test organisms were sampled (Kley et al. 2006). The presented alterations in leaf litter decomposition and microbial community composition can have negative impacts on ecosystem functioning and thus on supporting ecosystem services, which in turn can also affect the human well-being.

6. General discussion

The results of the thesis presented here contribute to a better understanding of interrelated processes between abiotic habitat properties, ecosystem engineers and microbial communities in the stream interstitial zone and an adjacent mesohabitat – the open water. The strong correlations between abiotic habitat properties created by different ecosystem engineers and microbial communities suggest to consider mesohabitats with their different trophic levels of organization and intertwined processes not as single research units, but as a whole ecosystem with countless interactions and severe consequences for ecosystem functioning, if one part of this puzzle is replaced or removed.

6.1 Closing gaps in methodology

To model and predict future alterations and establish conservation plans, it is indispensable to understand ecological processes and interrelationships among abiotic and biotic factors within and between different mesohabitats. Before prediction models can be established in a young field of study, like the interstitial zone research, defined questions regarding single components of a complex system have to be answered. Standardized laboratory experiments as illustrated in chapter two are suitable to keep experimental conditions constant to leave a focus on evaluating defined hypotheses. This thesis added a new dimension to former studies, which considered only effects on single factors (e. g. N-cycling or predation rates) influenced by ecosystem engineers (e.g., 2012; Vaughn & Hakenkamp, 2001; de Haas et al., 2005; Gallon et al., 2008; Marshall & Hall, 2004) or invasive species (Pellan et al., 2015; Médoc et al., 2015). To close the gaps in stream interstitial zone research, a new standardized method was established to study abiotic habitat properties and microbial, invertebrate and vertebrate assemblages at the same time, and to allow direct comparisons between functionally different organisms. Microcosms were constructed with low financial expenses, facilitating a high number of biological replicates. A high number of biological replicates is crucial for significant results and an estimation of variance components (Judd et al., 2017). Additionally, the measurements in the interstitial zone were non-invasive, since the redox electrodes were installed in the measurement depth of five cm before the experiments started. All interstitial water measurements as well as the sample collection for microbial community analyses were undertaken from water samples taken from a valve, which was also fixed in the substratum measurement depth of five cm. This experimental setup allowed an unaltered sample collection from interstitial zone water. The mesocosms and experimental setup developed in this thesis can help to improve a minimal invasive sample collection in future experiments. To control the quality of the setup, measurements were performed at day zero with the result that none of the experimental boxes indicated significant differences in physico-chemical habitat properties or microbial community composition between the treatments (Boeker & Geist, 2015; Boeker & Geist 2016; Boeker et al., 2016) at that time point. Moreover, typical conditions in regard to oxygen availability in the open water and the unturbated stream interstitial zone were detected at day zero (i.e. without ecosystem engineers) (Boeker & Geist, 2015; Boeker & Geist 2016; Boeker et al., 2016). These results were important indicators for a well-functioning methodological approach and crucial for reliable data.

The chosen size of the microcosms used in the presented experiments was suitable to compare many types of treatments including animals of different sizes (from tubificid worms to lamprey larvae). Nevertheless, in case of *E. danica* treatments (chapter 3), the water samples for physico-chemical measurements as well as the substratum samples for microbiological analyses were taken a few centimetres deeper than the test animals´ burrows were, in favour of exactly the same experimental setup as the other treatments. This resulted in a dilution of the effects on physico-chemical and microbial habitat properties for the *E. danica* treatments (Boeker et al., 2016). The observed alterations might therefore even be greater than detected with the experimental setup used in this study, which is not a problem as long as there is an awareness of such effects. The described phenomenon points out that the results found under laboratory experiments (e.g., microcosms) should be further evaluated under field conditions. This is necessary, because laboratory experiments can never fully imitate natural conditions. The microcosms used in chapters three to five for example cannot be used to simulate natural flow conditions of a stream interstitial zone that is influenced by a groundwater aquifer. Therefore, the term ´interstitial zone´ instead of ´hyporheic zone´ is used in this thesis, because it seems more appropriate to describe the conditions in the microcosms.

6.2 Ecosystem engineers in context of contemporary impacts on the interstitial zone and ecosystem services

The burrowing organisms examined in the present thesis can clearly be classified as ecosystem engineers, because they engineered the habitat by their burrowing behaviour and physiological traits and thus altered physico-chemical habitat properties in the interstitial zone (oxygen availability, conductivity, nutrients) and consequently the bacterial community composition (chapters 3, 4 and 5).

As evident from chapter three and four, the impact of burrowing invertebrates and vertebrates on interstitial microbial community composition increases with bioturbation intensity (Boeker et al., 2016). Moreover, the results indicate that the way how organisms burrow (e.g. frequency of position change and density of burrows) is also important. Zieritz et al. (2014) reported on a high burrowing activity of mussels, which could be also observed in the experiment presented in chapter three. The finding of Zieritz et al. (2014) was complemented by elucidating important effects that result from this mode of life such as high influx rates of oxygen-rich surface water, resulting in a high oxygen availability in the interstitial zone and followed by an alteration in microbial community composition. High redox potentials could be observed in the *Eudontomyzon* sp. treatments (chapter 4), as also mentioned in Shirakawa et al. (2013). Above that, the results presented in chapter four added a new dimension of information to that, because the observed differences in physico-chemical parameters compared to the controls without bioturbators also caused a shift in microbial community composition. In contrast to that, in the *T. tubifex* treatments no increased oxygen availability was observed due to the very small burrows of the tubificid worms. In addition to the mode of sediment reworking, the results of the experiment presented in chapters three and four indicate that also physiological characteristics of ecosystem engineers play a key role in the interaction between habitat engineers and their closer environment. The effects of high influx rates of oxygen-rich open water in the *A. anatina* treatment was probably enhanced by their way of respiration and excretion: mussels use their siphon for respiration of oxygen from open water and excrete pseudofeces into open water. This probably caused a low oxygen depletion in the substrate due to respiration on the one hand, and a low biodeposition of feces and thus a low ammonium concentration in the interstitial zone on the other hand. Consequently, little oxygen was consumed in the interstitial zone via nitrification. In the *T. tubifex* treatment, completely different physiological traits (respiration of oxygen available in the burrows, excretion of feces to the sediment surface) may have caused high oxygen depletion in the interstitial zone. This effect was probably further increased through the direct excretion of ammonium by oligochaetes (Gardner et al., 1983) that leads to oxygen consumption via nitrification. The high metabolic activity of bacteria at the burrow walls of *T. tubifex* mobilizes ions to the interstitial water, which might explain the high electric conductance in the *T. tubifex* treatments compared to the other treatments.

Taken together, the ecosystem services provided by habitat engineers investigated in the herein presented studies (gas exchange, stimulation of nutrient cycling) all contribute to the overall ecosystem functioning and are therefore basis for most ecosystem services freshwaters provide (Millenium Ecosystem Assessment, 2005). The knowledge gained from chapters three, four and five suggests that the currently less considered interactions between micro- and macrobiota can have strong effects on ecosystem services and functioning and should be considered in hydraulic engineering plans and conservation management.

In addition to the naturally occurring impacts on the interstitial zone, its location at the interface between stream surface water and groundwater exposes it to a variety of anthropogenic pressures (Hancock, 2002). Both water bodies, surface and groundwater are increasingly exploited by humans. The impacts on the interstitial zone can be divided into two scenarios: the first one alters abiotic habitat properties and consequently affects several biota indirectly; the second affects the biotic community composition directly (e. g. via introduction of invasive species). The outcomes of the experiments undertaken in this study clearly indicate that dense populations of certain ecosystem engineers have the potential to modulate (mussels and lamprey larvae created suitable microhabitats for other species including meiofauna) or even enhance (tubificid worms created almost anoxic microenvironments) anthropogenic pressures. For example, seasonal peak exposure of freshwater unionoid mussels to de-icing salt, which is known to be a serious pollutant to freshwaters (Kaushal et al., 2005) in winter and spring across the northern hemisphere, has been reported to affect behaviour patterns of mussels (*Anodonta anatina*) (Hartmann et al., 2016) and viability of mussel larvae (Beggel & Geist, 2015). Transferred to the results from the *A. anatina* treatments (chapter 3) de-icing salt pollution might cause a decrease in burrowing activity and consequently an oxygen depletion in the interstitial zone with expectable shifts in microbial community composition and consequences for nutrient cycling. Also other pollutants such as high heavy metal concentrations around mines (Ciszewski 1998; Soares et al., 1999) or agricultural chemicals (e.g., atrazine, deethylatrazine, deisopropylatrazine) (Squillace et al., 1993) have the potential to alter interstitial habitat properties and thus biotic communities or at least their feeding and burrowing habits.

A further impact on the interstitial zone is the input of fine sediments into rivers. This can be the case for rivers with catchment areas dominated by forestry and cattle farming (Maridet et al., 1996) as well as agricultural land use (Allan, 2004; Wasson et al., 2010). Increased fine sediment loads in the interstitial zone result in clogging and colmation of the interstices with serious effects on interstitial bioturbators (decrease in number and activity), which in turn alters the porosity of the substratum, because of a declined feeding and burrowing activity (Brunke & Gonser, 1997). As revealed by the herein presented studies, alterations in burrowing activity of variable ecosystem engineers has severe consequences for abiotic habitat properties and microbial community composition, which can influence food web structure and thus ecosystem functioning.

Above all these anthropogenic impacts, Meyer et al. (1999) see the climate change as a potential high impact on the interstitial zone since stream morphology and hydrology, which are highly influenced by climatological variables, determine interstitial processes. Global warming is expected to reduce the permanent water depth due to alterations in precipitation patterns, which could lead to permanent streams becoming temporary streams (Stanley & Valett, 1991) with effects on interstitial communities. Facilitated through climate change is the deliberate or undeliberate introduction of invasive species, which can lead to species replacements by highly competitive invaders (Caravaggi et al., 2016). As evident from chapter five, the invasive *D. villosus* does not fulfil the same ecological functions as the indigenous *G. roeseli*. Pronounced differences could be observed in the feeding rates with an 11-fold increased decomposition rate of alder leaves by the indigenous compared to the invasive species. Moreover, the higher mortality rate in combination of not fully intact dead individuals of *D. villosus* lets assume, that cannibalism might have occurred in the *D. villosus* treatments, whereas in the *G. roeseli* treatments such observations have not been made (Boeker & Geist, 2016). Therefore it is likely that the investigated amphipod species occupy different niches in the food web. This in turn might have influenced bacterial community composition at the substrate surface, because of the excretion of different microbiota by the two species as a consequence of distinct diets (Boeker & Geist, 2015). These findings are highly relevant in context of ecosystem services, the different species provide: if *D. villosus* is a scavenger among other nutrition habits, then it probably provides ecosystem services such as water purification and the containment of diseases transmitted by carrion, whereas *G. roeseli* is more likely to affect the nutrient cycling and microbial biofilm formation. Consequently, the currently occurring replacement of indigenous *G. roeseli* populations by invasive *D. villosus* in many aquatic ecosystems likely results in ecologically relevant functional changes affecting leaf litter decomposition and related ecosystem services such as water purification and nutrient cycling (Boeker & Geist, 2015).

6.3 The stream interstitial zone as a key for conservation planning

Mayfly larvae, mussels and lamprey larvae require a well-oxygenated gravel-bed (Hardisty, 1979; Geist & Auerswald 2007; Krieger et al., 2007; Geist 2010, Lopes-Lima et al., 2017), according to the river continuum concept indicative for largely undisturbed habitats in headwater regions. In contrast, the presence of tubificid worms is more favoured by conditions found in highly altered habitats (high fine-sediment and fine organic matter load, low amounts of oxygen) (Jablonska, 2014). If largely undisturbed habitats are further subject to alterations in flow regime and sediment transport, as caused by the construction of weirs and dams (Kondolf, 1997; Shields et al., 2000) and intensive agricultural land-use in the catchment area (Maridet et al., 1996; Allan, 2004; Wasson et al., 2010), a shift in interstitial habitat properties and benthic species composition has to be expected (Mueller et al., 2011, Caschetto et al., 2014). The construction of dam walls isolates the interstitial fauna, possibly leading to a separation of ecosystem engineers. As reported in chapters three and four, functionally different bioturbators provide diverse ecosystem services (e. g. venting the interstitial zone) that are likely to complement one another when considering the stream interstitial as an entire ecotone. If the correlation of these variable ecosystem services is separated, the functioning of the interstitial zone might be endangered. Moreover, pumping of water for human consumption or irrigation lowers the water table and therefore reduces the hydraulic pressure with which water is pushed into and through the interstitial zone – preventing from flushing out fine sediments. Considering the findings presented in chapters three and four it is likely that the exchange of nutrients (e. g. metabolites from the N-cycling) between the stream intersititial zone and the open water decreases with a reduced hydraulic pressure and a lowered water table. Additionally, a lowered water table decreases the extent of the interstitial zone, especially that of parafluvial zones (Hancock, 2002) resulting in a decreased area that can be used as habitat for different organisms and in particular for microbes – less space for crucial ecosystem services provided by interstitial zone organisms (Boeker & Geist, 2015; Boeker & Geist 2016; Boeker et al., 2016). Therefore, conservation of freshwater ecosystem functioning including ecosystem services must be among the top priorities for future generations.

Different conservation concepts have been established in order to protect single species or local ecosystems, but not all of them are suitable for the preservation of ecosystem services. As discussed in Geist (2010; 2015) there are four main approaches in species conservation: flagship species (prominent and well-known species that are unfortunately not always crucial for ecosystem functioning), indicator species (indicative for good habitat properties at the local scale such as water and substrate quality), umbrella species (species with large home ranges whose protection automatically includes species with smaller home ranges (Geist, 2015)), and, probably the most important: keystone species. The latter are very important for ecosystem functioning (e.g., for habitat quality, food webs, nutrient input) and therefore other species are protected at the same time. The keystone species approach might be useful for the conservation of biodiversity – certainly the crux of establishing resilient systems. Nevertheless, the protection of entire ecosystems and the services they provide demands a more comprehensive approach. Intertwined abiotic and biotic dynamics as revealed by the present thesis, complex and in undisturbed systems perfectly balanced interactions have to be considered at the same time with growing anthropogenic pressures and alterations. Variable stakeholders have to elaborate conservation management plans, which have to be scientifically monitored and can be rapidly modulated if necessary.

6.4 Conclusions and Outlook

The functional interactions in the stream interstitial zone strongly depend on resident biota and processes they influence and regulate. In particular, regulated processes are exchange rates between surface and subsurface water including the transport of important gases and nutrients (oxygen, ammonium, nitrate) as well as the metabolism taking place in the interstitial zone via bacteria. For a correct evaluation of hypotheses that concern intertwined relationships, it is essential to design an appropriate methodology that allows for a simultaneous investigation of abiotic and biotic parameters in a standardized environment.

The next scientific steps should be the investigation of further functional groups, like gravel spawning fish species, and functionally different bioturbators combined in one microcosm. Moreover, flora-fauna interactions should also be adressed in such studies. Once the key drivers and dependent processes are identified in standardized experiments, it is crucial to upscale these observations to the reach and catchment scale in field experiments. Therefore, it is of great importance to include adjacent ecotones, such as groundwater aquifers, the parafluvial zone and the flood plain. Combined data from laboratory experiments and field studies can then lead to a comprehensive understanding of ecological processes and serve as a basis for modelling to predict alterations in ecosystem functioning in case of variable situations (e.g., global warming, invasive species, pollution).

A fundamental and interdisciplinary understanding of ecological interrelations is the key to preserve resilient ecosystems that can provide a variety of ecosystem services humans benefit from. Conservation management plans aimed at the interstitial zone should be combined with effective public ecological education (children and adults) and policy to reach the broader public in order to call attention for this hidden ecotone.

7. Publications and oral presentations

7.1 Publications related to the thesis

C. Boeker and J. Geist (2015) Effects of invasive and indigenous amphipods on physico-chemical and microbial properties in freshwater substrates. Aquatic Ecology 49:467-480.

C. Boeker and J. Geist (2016) Lampreys as ecosystem engineers: burrows of *Eudontomyzon* sp. and their impact on physical, chemical and microbial properties in freshwater substrates. Hydrobiologia 777:171-181.

C. Boeker, T. Lueders, M. Mueller, J. Pander and J. Geist (2016) Alteration of physico-chemical and microbial properties in freshwater substrates by burrowing invertebrates. Limnologica – Ecology and Management of Inland Waters 59:131-139.

7.2 Further Publications

G. Eissner, I. Hartmann, A. Kesikli, E. Holler, S. Haffner, T. Sax, C. Schray, B. Meiser, B. Reichart (2011) CD4+CD25+FoxP3+ regulatory T cells enhance the allogeneic activity of endothelial-specific CD8+/CD28−CTL. *International Immunology* 23:485–592.

M. v. Hauff, F.-T. Gottwald, K. Stöckl, J. Kurz, C. Böker (2016) Towards sustainable economies. In: P. A. Wilderer, M. Grambow (eds.) Global Stability through Decentralization? Strategies for Sustainability. *Springer International Publishing*, Switzerland. DOI 10.1007/978-3-319-24358-0.

I. Studer, C. Boeker, J. Geist (2017) Physicochemical and microbiological indicators of surface water body contamination with different sources of digestates from biogas power plants. *Ecological Indicators* 77:314-322.

A. F. Cerwenka, A. Pagnotta, C. Boeker, J. Brandner, J. Geist, U. K. Schliewen (2017) Little association of biological trait values with environmental variables in invasive alien round goby (*Neogobius melanostomus*). *Ecology and Evolution 7*:4076-4085. DOI:10.1002/ece3.2942.

7.3 Oral presentations

C. Schray, A. F. Cerwenka, J. Brandner, J. Geist, U. K. Schliewen (2012) Abschätzung von Alter und Wachstum anhand von Schuppenmerkmalen bei Neogobius melanostomus. Jahrestagung der Deutschen Gesellschaft für Limnologie, Koblenz.

C. Schray, J. Geist (2014) Effects of burrowing stream invertebrates on physicochemical and microbial properties of the hyporheic zone. Statustreffen im Rahmen des Helmholtz Wasserzentrums, Neuherberg.

C. Boeker (2015) Einfluss grabender Invertebraten auf physikochemische Habitatparameter und mikrobielle Diversität im hyporheischen Interstitial. Zwischenevaluierung des Promotionsvorhabens an der Technischen Universität München, Freising.

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